Local Opiate Withdrawal in Locus Coeruleus Neurons In Vitro

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Ivanov, Alexander and Gary Aston-Jones. Local opiate withdrawal in locus coeruleus neurons in vitro. J Neurophysiol 85: 2388–2397, 2001. Noradrenergic neurons of the brain nucleus locus coeruleus (LC) become hyperactive during opiate withdrawal. It has been uncertain to what extent such hyperactivity reflects changes in intrinsic properties of these cells. The effects of withdrawal from chronic morphine on the activity of LC neurons were studied using intracellular recordings in rat brain slices. LC neurons in slices from chronically morphine-treated rats exhibited more than twice the frequency of spontaneous action potentials after naloxone compared with LC neurons from control rats. However, after naloxone treatment, the resting membrane potential (MP) of LC neurons from dependent rats was not significantly different from that in control rats. Neither resting MP nor spontaneous discharge rate (SDR) was altered by naloxone in LC neurons from control rats. Neither kynurenic acid nor a cocktail of glutamate and GABA antagonists (6-cyano-7-nitroquinoxalene-2,3-dione + 2-amino-5-phosphonopentanoic acid + bicuculline) blocked the hyperactivity of LC neurons precipitated by naloxone in slices from morphine-dependent rats. The effects of ouabain on MP and SDR were similar in LC neurons from control and morphine-dependent rats. These results indicate that an adaptive change in glutamatergic or GABAergic synaptic mechanisms or altered Na/K pump activity does not underlie the withdrawal-induced activation of LC neurons in vitro. Specific inhibitors of protein kinase A [Rp-cAMPS or N-(2-[p-bromocinnamylamino]ethyl)-5-isouquinolinesulfonamide (H-89)] partially suppressed the withdrawal hyperactivity of LC neurons, and activators of cAMP (forskolin) or protein kinase A (Sp-cAMPS) increased the discharge rate of LC neurons from control rats. These results suggest that upregulation of cAMP-dependent protein kinase A during chronic morphine treatment is involved in the withdrawal-induced hyperactivity of LC neurons.

INTRODUCTION

Recent results clearly establish that extrinsic glutamate inputs from the ventrolateral medulla play a prominent role in the hyperactivity of locus coeruleus (LC) neurons after morphine withdrawal (e.g., Akaoka and Aston-Jones 1991; Ennis and Aston-Jones 1988; Rasmussen and Aghajanian 1989). However, a possible additional intrinsic mechanism has been controversial. Some previous studies did not observe an increase in the spontaneous firing rate of LC neurons in brain slices from chronically morphine-treated rats (Andrade et al. 1983; Bell and Grant 1998; Christie et al. 1987a), indicating that an intrinsic mechanism may not exist. In another study, LC neurons recorded extracellularly in slices from chronically morphine-treated rats were reported to have elevated impulse rates (Kogan et al. 1992). Another recent study in vivo (Aston-Jones et al. 1997) found increased activity of LC neurons of dependent rats after local microinfusion of methyl naloxone, consistent with a local withdrawal mechanism. However, the possible involvement of residual synaptic inputs in these studies was not evaluated. Thus it remains uncertain to what degree changes in intrinsic mechanisms within LC neurons participate in their hyperactivity during opiate withdrawal. Demonstration of intrinsic changes with opiate withdrawal would reveal neuronal-level alterations produced by chronic morphine and could be important for understanding the cellular basis of opiate dependence as well as for developing pharmacotherapies for opiate addiction.

The purpose of this study was to characterize the intracellular responses of LC neurons to naloxone in brain slices from drug-naive and chronically morphine-treated rats to evaluate possible intrinsic changes in LC neurons during chronic morphine exposure that contribute to LC hyperactivity during opiate withdrawal.

METHODS

Male Sprague-Dawley rats (n = 55; ~150 g) were used in these experiments. Brain slices were prepared as described previously (Ivanov and Aston-Jones 1996). “Quasihorizontal” brain slices (angled ventrostral to caudodorsal to maximize inclusion of LC dendrites) (Shipley et al. 1996) containing the LC were incubated in artificial cerebrospinal fluid (ACSF) of the following composition (in mM): 126 NaCl, 5 KCl, 2.4 CaCl₂, 1.3 MgSO₄, 1.2 NaH₂PO₄, 24 NaHCO₃, and 11 glucose. The ACSF was pH 7.4 and continuously saturated with 95% O₂-5% CO₂.

Experimental groups

Membrane potential and the frequency of spontaneous discharge of LC neurons were examined in three experimental groups: control group—slices prepared from morphine-naive (untreated) rats; acute morphine group—slices prepared from untreated rats that were continuously maintained in morphine (5 μM) for the duration of experiments; and morphine-dependent group—slices taken from rats treated chronically with morphine and continuously maintained in morphine (5 μM) for the duration of experiments. Two methods were used for chronic morphine treatment: 1) pellets containing 75 mg of morphine base each were implanted subcutaneously on alternate days for 5 days (1, 2, then 2 pellets). In this case, chronically treated rats were used for electrophysiological experiments 2 days after the final implantation. 2) Only two pellets were implanted, and rats were used in experiments 4 days after implantation. The results were identical with these two treatments and were pooled. Each of these treatments

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has been previously shown to induce dependence as indicated by strong withdrawal behaviors following opiate antagonist administration in waking rats (Christie et al. 1987b; Gold et al. 1994; Harris and Aston-Jones 2001). Sham pellets were not used as our previous behavioral and electrophysiological studies showed no difference from untreated controls. Slices in the acute morphine and morphine-dependent groups were continuously superfused with morphine (5 μM) during the electrophysiological experiments. Previous data indicate that this mimics the level of morphine in the brain during chronic morphine treatment (Christie et al. 1987a).

Electrophysiological recording and drugs

Electrodes for intracellular recording were made on a Brown-Flaming puller from Kwik-fil glass micropipettes and were filled with 2 M KCl (resistance = 30–50 MΩ). Intracellular potentials were amplified using an Axoclamp 2A amplifier (Axon Instruments) and monitored on a chart recorder on-line (Gould 2200) or digitized with a CED 1401 interface (CED, Cambridge, UK) and stored on a computer disk. Data analysis was performed on the computer employing Chart 2.0, Spike 2, and Sigma-Plot software. All average values presented in the following text are means ± SE.

The following drugs were applied to the slice perfusion solution: naloxone (1 μM), tetrodotoxin (TTX, 1 μM; Sigma), bicuculline (15 μM; Sigma), kynurenic acid (500 μM; Sigma), 6-cyano-7-nitroquinoxalene-2,3-dione (CNQX, 20 μM; RBI), 2-amino-5-phosphono- pentanoic acid (AP5, 20–50 μM; RBI), forskolin (10–30 μM; RBI), 1,9-dideoxyforskolin (10 μM; RBI), ouabain (0.5–1 μM; RBI), Rp-cAMPS (10–50 μM; RBI), N-(2-[p-bromocinnamylamino]ethyl)-5-isouquinolinesulfonamide (H-89, 10 μM; Calbiochem), and Sp-cAMP (10 μM; RBI). In some experiments, BaCl2 (2 mM) was added to ACSF in which NaH2PO4 was omitted.

RESULTS

Control group

Twenty-five neurons in 20 slices taken from control rats were studied. Only neurons that had a stable frequency of spontaneous discharge and a stable membrane potential close to −60 mV for 1 h were included in the data analysis. The spontaneous discharge rate (SDR) of LC neurons in these slices ranged from 0.5/s to 1.4/s (mean = 1.0 ± 0.1/s; n = 25); the range of resting membrane potential (RMP) recorded was −58 to −65 mV (mean = 60.4 ± 0.3 mV; Fig. 1B). In eight cells from this group, the effect of naloxone was tested. No significant change in RMP (−60.0 ± 0.4 vs. −60.3 ± 0.7 mV) or SDR (1.1 ± 0.2/s vs. 1.1 ± 0.2/s) was observed after 5–10 min.

Acute morphine group

The RMP of 23 neurons (12 slices) from the acute morphine group (1 h superfusion with 5 μM morphine) was significantly lower (−72.9 ± 1.0 mV; n = 23) than in the control group without morphine (−60.4 ± 0.3 mV; n = 25; Fig. 1B). Spontaneous discharge was suppressed in all LC neurons tested in the presence of morphine in this group. All neurons were depolarized to control values (by −13 mV) when naloxone was added (average membrane potential after naloxone = −60.6 ± 0.1 mV; n = 23; Fig. 1, A and B). RMP and SDR recorded in this group of neurons in the presence of naloxone were not significantly different from the values observed in LC neurons in the control group (Fig. 1C; 1.3 ± 0.2/s, n = 23 vs. 1.0 ± 0.1/s; n = 25, P > 0.05; t-test). The preceding data indicate that intrinsic properties reflected in RMP and SDR were not changed by superfusion with morphine for 1–2 h.

Morphine-dependent group

The majority of LC neurons (19 of 24) from this group exhibited spontaneous firing at an average rate of 0.3 ± 0.1/s in the presence of 5 μM morphine. The remaining neurons were silent. This is in contrast to LC neurons in control or acute morphine groups in which a complete suppression of impulse activity occurred with 5 μM morphine (described in the preceding text). The membrane potential of neurons in the morphine-dependent group (23 slices) ranged from −60 to −68 mV (mean = −61.7 ± 0.6 mV; n = 24) in the presence of 5 μM morphine (Fig. 2, A and B). This was significantly hyper-
polarized compared with control slices without morphine (Fig. 2B; \( P = 0.04 \)), t-test). However, morphine hyperpolarized LC neurons significantly less in slices from the morphine-dependent group compared with the acute morphine group (-72.9 ± 1.0 mV). These data indicate that a prominent tolerance to morphine developed in LC neurons during the chronic morphine treatment. Nonetheless, morphine in the bath had a significant effect on LC neurons because the frequency of spontaneous discharge was lower in the morphine-dependent group than observed in the control group.

A clear depolarization was observed in 16 of 24 neurons in the morphine-dependent group after naloxone. The mean depolarization for this group of 16 neurons was 3.5 ± 0.5 mV. The overall average depolarization in LC neurons after naloxone in the morphine-dependent group was -2 mV (from -61.7 ± 0.6 to -59.5 ± 0.6 mV, \( n = 24 \) cells, \( P < 0.05 \); Fig. 2B). The remaining eight cells from this group did not show depolarization after naloxone (-59.6 ± 0.5 vs. 60.1 ± 0.8 mV; \( P > 0.05 \)).

Overall, LC neurons from dependent animals exhibited a higher SDR after naloxone than either the control or acute morphine groups. This increase in SDR occurred similarly in cells of the morphine-dependent group that demonstrated no depolarization after naloxone (2.6 ± 0.3/s; \( n = 8 \)) and cells that were depolarized by naloxone (2.5 ± 0.3/s; \( n = 16 \)). Thus the higher frequency of spontaneous impulses in LC neurons from dependent rats after naloxone was not obviously dependent on a naloxone-induced depolarization.

It is important to note that the average RMP in LC neurons from dependent rats in the presence of naloxone did not differ significantly from the RMP in LC neurons from control rats (Fig. 2B). These results indicate that chronic morphine treatment did not significantly change the RMP in LC neurons from morphine-dependent rats compared with control rats. However, although their RMP postnaloxone was not different from control-naive cells, LC neurons from dependent rats displayed a markedly higher SDR after naloxone compared with that recorded from LC neurons in control rats (2.5 ± 0.3 vs. 1.0 ± 0.1/s, \( P < 0.01 \); Fig. 2C). Thus a comparison of LC neurons after naloxone from control versus dependent rats revealed a more than twofold difference in SDR without a significant difference in the average RMP.

To further examine the possible role of RMP in the elevated discharge of LC neurons after withdrawal, we calculated the rate of spontaneous discharge in LC neurons from control rats as a function of RMP. Cells with an average RMP of -57.2 ± 0.4 mV (\( n = 4 \)) had an average discharge frequency of 1.4 ± 0.1/s; cells whose RMP = -60.0 ± 0.1 mV (\( n = 6 \)) had an average discharge frequency of 1.1 ± 0.1/s; and cells with an RMP of -62.5 ± 0.3 mV (\( n = 6 \)) had a corresponding discharge of 0.8 ± 0.1/s. Thus a difference of 5 mV in membrane potential caused a relatively small change in discharge frequency (from 0.8 to 1.4/s) compared with that observed in neurons from morphine-dependent rats after naloxone.

We also examined the effect of repolarizing the MP to the prenaloxone level in three cells from morphine-dependent rats (average MP = -61.5 ± 0.3 mV; rate of spontaneous spikes, 0.3 ± 0.1/s). Naloxone depolarized these cells 3.4 ± 0.1 mV on average and increased the rate of spontaneous spikes to 2.4 ± 0.5/s. Hyperpolarization of these neurons to the membrane potential before naloxone only partially suppressed the spontaneous discharge rate (1.1 ± 0.3/s). Note that this rate was still significantly higher (\( P < 0.05 \)) than the rate of spontaneous discharge in these cells before naloxone. Together these data support the possibility that depolarization is not the only mechanism responsible for increasing spontaneous LC discharge rate in morphine-dependent rats after naloxone.

**TTX and Ba\(^{2+}\)**

TTX was used to block possible residual synaptic inputs to LC neurons in the slice during naloxone-precipitated withdrawal. We found, as in our previous report (Ivanov and
Aston-Jones 1995), that TTX (1 μM) not only blocked synaptic responses [excitatory and inhibitory postsynaptic potentials (EPSPs and IPSPs)] but also suppressed spontaneous impulse activity in 93% of LC neurons (n = 59). Spontaneous spikes could be easily elicited in all LC neurons tested during TTX superfusion by injection of a small inward current through the microelectrode (Fig. 3A). Spikes evoked by slight depolarization during TTX perfusion were classified as Ca 2+ spikes because they were persistent in the presence of 1 μM TTX, had lower amplitude (~40 mV) and longer duration (>10 ms) than spikes in control solutions, and were blocked by Co 2+ (2 mM) or nifedipine (15 μM). On chart records these spikes exhibited relatively large amplitude because of their long duration (Fig. 3A). TTX did not change the membrane potential in LC neurons from morphine-dependent rats (n = 6).

Although spontaneous impulses were also absent in neurons from dependent slices in the presence of TTX, naloxone superfusion evoked depolarization and tonic impulse activity (calcium spikes; see Fig. 3B). Thus TTX did not prevent the naloxone-precipitated depolarization in LC neurons from dependent rats. The frequency of impulse activity during naloxone and TTX superfusion in LC neurons from dependent rats was significantly lower (1.1 ± 0.1/s, Fig. 3C; n = 6) than without TTX (2.5 ± 0.3/s; n = 24). However, note that the increase in impulse activity due to naloxone was similar with or without TTX.

The naloxone-induced depolarization might arise as a result of decreased outward potassium conductance (e.g., blockade of the residual effect of morphine by naloxone) or as a result of a new inward current caused by adaptive changes during chronic morphine exposure. On average, the RMP of cells that did not demonstrate depolarization after naloxone was significantly smaller (~60.1 ± 0.8; n = 8, P < 0.05) than that of cells that with depolarization after naloxone (~62.9 ± 0.5 mV; n = 16). After naloxone treatment, the MPs of both groups of cells were similar (59.2 ± 0.7 and 59.5 ± 0.4 mV). These data indicate that the naloxone-induced depolarization observed here may primarily reflect blockade a residual effect of morphine.

To further examine this issue, experiments with TTX + Ba 2+ were performed. 1 μM TTX + 2 mM Ba 2+ depolarized LC neurons by ~10 mV (to ~52.1 ± 1.1 mV; n = 6) and increased the number of cells without depolarization following naloxone from ~33% in control ACSF to ~66% (4 of 6 cells). However, a clear depolarization (~3 mV) was still observed after naloxone in the two remaining cells when their membrane potential was artificially repolarized to the level observed before TTX + Ba 2+ (Fig. 4A). Thus neither TTX nor Ba 2+ blocked the naloxone-induced depolarization in some dependent LC neurons.

We also observed subthreshold membrane oscillations in LC cells when spontaneous discharge was suppressed by hyperpolarization to ~65 to ~70 mV in the presence of TTX + Ba 2+, similar to those previously reported (Williams et al. 1984). The frequency of subthreshold membrane oscillations in LC neurons from morphine-dependent rats after naloxone were higher (1.3 to 2.5/s) than that in LC neurons from control rats (0.5–1.1/s). Further studies are necessary to determine the possible role of these oscillations in the response of LC neurons to withdrawal.

In additional experiments, cells were hyperpolarized to ~85 mV (close to E k with 5 mM K+ in the ACSF) (Andrade and Aghajanian 1984a) or external K+ was increased to 10 mM and cells were hyperpolarized to between ~70 and ~80 mV. Under these conditions and in the presence of 1 μM TTX, direct stimulation of neurons (10-ms inward current pulses) revealed no afterhyperpolarization following evoked spikes, confirming that the membrane potential was close to E k (Fig. 4B, inset). In these recordings, a naloxone-precipitated depolarization (4.1 ± 0.5 mV; n = 6) was observed in 6 of 11 cells (Fig. 4B). The remaining cells (n = 5) did not yield a depolarization. These data indicate that the naloxone-induced depolarization in some cells results from elimination of the stim-
Loration of potassium channels by residual morphine. However, the fact that depolarization was observed in several cells despite setting their membrane potential near or at $E_k$ indicates that another current may also be involved.

**Glutamate and GABA antagonists**

Previous studies have shown that naloxone given to morphine-dependent rats causes a large release of glutamate within the LC (Aghajanian et al. 1994; Akaoka and Aston-Jones 1991). To test whether such synaptic release plays a role in the elevated SDR found in LC neurons from dependent rats after naloxone, we studied the effects of a nonselective antagonist of glutamatergic receptors (kynurenic acid), a GABA antagonist (bicuculline), or a cocktail of glutamate and GABA antagonists (CNQX + AP5 + bicuculline), in morphine-dependent rats.

Pretreatment with kynurenic acid (500 μM) for 5 min before naloxone administration slightly (≈4 mV) hyperpolarized membrane potential and suppressed SDR (SDR was decreased on average from 0.3 to 0.1/s; $n = 4$; the remaining 2 neurons became silent). Such kynurenic acid treatment did not prevent a naloxone-precipitated depolarization ($-60.6 \pm 0.7$ vs. $-62.8 \pm 1.5$ mV; $n = 6$). The frequency of spikes during naloxone in LC neurons with kynurenic acid treatment from dependent rats ($1.8 \pm 0.1/s; n = 6$) was significantly higher than that in control rats ($1.0 \pm 0.1/s; P < 0.01$), indicating that kynurenic acid did not block the withdrawal-induced hyperactivity in LC neurons in vitro. The average increase in SDR after naloxone was slightly but not significantly lower in LC neurons from dependent rats with kynurenic acid than without kynurenic acid (Fig. 5B), and impulse frequency increased when kynurenic acid was washed from these slices. Note that kynurenic acid similarly decreased LC impulse activity in slices from dependent rats (see preceding text) and from control rats (from $1.1 \pm 0.2$ to $0.9 \pm 0.2/s; n = 9$; data not shown). This indicates that this agent slightly decreased activity in LC neurons from control and morphine-treated rats in a similar manner.

The effect of more selective and potent antagonists of glutamate receptors (20 μM CNQX +50 μM AP5), in combination with bicuculline (15 μM), was also examined. This cocktail suppressed synaptic responses evoked by stimulation of the slice through a bipolar electrode during 5 min of superfusion in all three cells tested (Fig. 5A). This cocktail did not prevent the naloxone-precipitated elevation in impulse frequency in any of the eight cells tested (activity increased by $1.8 \pm 0.4/s$ after naloxone in the presence of the cocktail; $P < 0.05$). The average elevations in the frequencies of discharge for naloxone, kynurenic acid and naloxone + cocktail are illustrated on Fig. 5B.

In addition, the hyperactivity of LC neurons after naloxone in dependent rats was not prevented by 15 μM bicuculline (activity increased by $1.9 \pm 0.4/s$ after naloxone in the presence of bicuculline, $P < 0.05; n = 7$; not illustrated). Bicuculline had an inconsistent effect on membrane potential in LC neurons from control and dependent rats (most cells were slightly hyperpolarized in the presence of bicuculline).

Thus although kynurenic acid or the antagonist cocktail slightly altered the baseline frequency in control and morphine-dependent rats, these treatments did not prevent the naloxone-precipitated hyperactivity of LC neurons from dependent rats. These results indicate that release of glutamate or GABA from residual synaptic inputs during naloxone-precipitated withdrawal does not produce the hyperactivity observed in LC neurons in slices from dependent rats.

**Ouabain**

A recent study indicated that S neurons of the myenteric plexus in chronically morphine-treated animals were depolarized by $-10$ mV compared with S neurons from naive animals and that this difference was associated with a reduction in
electrogenic Na\(^+\)/K\(^+\) pumping (Kong et al. 1992, 1997). As noted in the preceding text, we did not find a significant difference in RMP of LC neurons from control versus morphine-dependent rats (see Fig. 2). However, it seemed possible that chronic morphine could reduce Na\(^+\)/K\(^+\) pump activity in LC neurons, leading to hyperexcitability, which would become apparent after opiate withdrawal. To test this possibility, we examined the effect of ouabain on the SDR and membrane potential of LC neurons from control and dependent slices. SDR in LC neurons from control and morphine-dependent rats began to increase 1–1.5 min following the onset of superfusion with ouabain (0.5–1 \mu M; Fig. 6A2). We noted that the increase in firing rate of LC neurons from dependent rats evoked by ouabain always developed with a slowly progressing depolarization that was never observed following naloxone. After 4 min of superfusion with ouabain, LC neurons were depolarized by \(\sim 10\) mV on average (53.0 \pm 1.2 vs. 62.0 \pm 1.0 mV, Fig. 6B; \(n = 5\)). The frequency of SDR in LC neurons from both control and dependent rats strongly increased (up to 6 spikes/s, \(\text{mean} = 5.0 \pm 0.6/s\) in control and \(6.0 \pm 1.2/s\) in dependent rats) after 4 min of superfusion with ouabain (Fig. 6C). Notably, there was no significant difference between LC neurons from control versus morphine-dependent rats in the depolarization or increased impulse activity following ouabain. Naloxone added after washing ouabain from slices of dependent rats for 10 min increased the impulse activity of LC neurons without any depolarization (Fig. 6A2). These results indicate that the increased excitability of LC neurons in dependent rats after naloxone is unlikely to be due to an inhibition of an electrogenic Na\(^+\)/K\(^+\) pump as a consequence of chronic morphine treatment.

**Protein kinase inhibitors**

The effect of naloxone before and 10 min after superfusion with protein kinase inhibitors (Rp-cAMPS or H-89) was tested on LC neurons from control and morphine-dependent rats. In slices from control rats, Rp-cAMPS (10 \mu M) did not significantly change LC membrane potential (−64.4 \pm 1.1 vs. −64.5 \pm 0.7 mV; \(n = 3\)) or spontaneous discharge rate after 10–15 min of superfusion. However, with a higher concentration (50 \mu M), Rp-cAMPS significantly increased the impulse activity of LC neurons from control rats (1.9 \pm 0.2 vs. 1.2 \pm 0.2/s; \(P < 0.05\)) without a significant change of membrane potential. It was not clear why Rp-cAMPS increased spontaneous discharge rate in LC neurons from control rats. One possibility is that the excessive concentration of Rp-cAMPS may activate (rather than suppress) protein kinase A (Pedarzani and Storm 1993).

Rp-cAMPS (10 \mu M) was tested on the LC cells from dependent slices. First, naloxone was tested and then after \(\sim 1\) h wash the same cell was perfused for 10–15 min with Rp-cAMPS (10 \mu M), after which naloxone was again added. Pretreatment with Rp-cAMPS (10 \mu M) did not change the membrane potential or spontaneous discharge rate (62.9 \pm 1.3 vs. 63.1 \pm 0.7 mV; \(n = 3\)) but significantly decreased the impulse activity induced by naloxone (Fig. 7, A and B; 1.6 \pm 0.2 vs. 3.2 \pm 0.3/s; \(P < 0.05\)).

Another specific inhibitor of protein kinase A (PKA), H-89 (10 \mu M), did not significantly change the RMP in neurons from dependent rats (−61.2 \pm 1.4 vs. −60.7 \pm 1.1 mV; \(n = 12\), Fig. 7C) but significantly suppressed the postnaloxone SDR (1.5 \pm 0.2 vs. 2.7 \pm 0.3/s; \(P < 0.01\), \(n = 12\); Fig. 7D). Thus although inhibitors of protein kinase A did not change the membrane potential in LC neurons from dependent rats, they significantly suppressed the naloxone-precipitated impulse hyperactivity.

**Activators of cAMP and PKA**

The PKA activator Sp-cAMPS (10 \mu M) slightly but significantly hyperpolarized LC neurons from control rats (Fig. 8A; from −61.3 \pm 0.9 to −63.6 \pm 0.7 mV; \(n = 9\), \(P < 0.05\)) and strongly (1.9 \pm 0.3 vs. 0.9 \pm 0.1/s; \(P < 0.01\)) increased the frequency of spontaneous action potentials and subthreshold membrane oscillations (Fig. 8, A and B). Forskolin (10–15 \mu M) also increased the frequency of spontaneous discharge in four of seven LC neurons (from 0.8 \pm 0.2 to 1.6 \pm 0.2/s; \(P < 0.05\), \(n = 7\), Fig. 8C) without obviously changing membrane potential, in agreement with previous reports (Harris and Williams 1991; Wang and Aghajanian 1987). The inactive forskolin analogue, 1,9-dideoxyforskolin, at the same concentration had no effect on spontaneous discharge (3 cells tested). In contrast, forskolin at a higher concentration (30 \mu M) completely suppressed spontaneous firing and hyperpolarized LC neurons (not illustrated; 3 cells tested), in agreement with
previous reports (Osborne and Williams 1996). Thus these results indicate that PKA or adenylate cyclase activators significantly increased the frequency of spontaneous spikes in LC neurons from naïve rats without membrane depolarization.

DISCUSSION

The main findings of this study are that LC neurons in slices from chronically morphine-treated rats have a higher tonic discharge rate after naloxone compared with control rats but do not exhibit a significant difference in RMP. This indicates that LC neurons in vitro exhibit a local withdrawal response as we previously reported with in vivo experiments (Aston-Jones et al. 1997). Our experiments with neurotransmitter antagonists, and with TTX, indicate that this withdrawal-induced hyperactivity in vitro is not produced by neurotransmitter release and presumably results from adaptations produced by chronic morphine treatment in these neurons. Our studies also indicate that this adaptation likely involves changes in the cAMP-PKA cascade.
that in neurons from naïve control animals. In addition, hyperactivity of LC neurons in vitro during withdrawal occurs without significant depolarization, is independent of presynaptic transmitter release, and may involve a cAMP/PKA mechanism.

Our findings differ in some regards from a previous intracellular study that reported no obvious difference in the mean frequency of LC firing between control tissue and morphine-treated rats (Christie et al. 1987a). The reason for this discrepancy in results is not clear but may result from differences in methods (e.g., lines of rats used or the presence or absence of morphine in the bath of slices from dependent rats). Nonetheless, our study does agree with this previous report in finding no difference in RMP of LC neurons in slices from nonmorphine-treated animals versus dependent slices after naloxone.

That previous study also found that membrane resistance ($R_{in}$) was not different for LC neurons from morphine-dependent versus naïve rats (Christie et al. 1987a). This result is consistent with our previous observations of $R_{in}$ when LC neurons were depolarized by other agents (glutamate and hypocretin), which indicated that reliably detectable changes in $R_{in}$ (~10% or more) were observed only if the change of membrane potential exceeded 5 mV (Ivanov and Aston-Jones 1996, 2000). In the present studies, the average depolarization of LC neurons in morphine-dependent rats after naloxone was ~2.2 mV, and the difference between RMP in LC neurons from control and morphine-dependent rats was not significant. Thus no difference in $R_{in}$ would be expected.

Our data support earlier results that LC neurons in vitro demonstrate a marked tolerance to morphine after chronic morphine treatment (Andrade et al. 1983; Christie et al. 1987a). The signs of tolerance included a diminished effect of morphine on the membrane potential and spontaneous discharge in many LC neurons from dependent rats in spite of morphine in the bath. However, as also previously reported, tolerance appeared to be incomplete as LC neurons from dependent slices exhibited spontaneous firing in the presence of morphine at a rate that was lower than LC neurons from control rats without morphine in the bath. In addition, morphine hyperpolarized dependent cells, albeit to a smaller extent than in slices from control rats. Such apparent incomplete tolerance may reflect tolerance in multiple cellular processes that exhibit different time courses.

It is important to note that the naloxone-precipitated depolarization in individual LC neurons from dependent rats could not be observed in ~50% of cells whose MP was shifted close to $E_k$ (Fig. 4C3). These results indicate that the depolarization observed after naloxone in at least some LC neurons from morphine-dependent rats might be caused by elimination of a residual effect of morphine. However, a possible additional source of depolarization also appears likely in some cells as depolarization was occasionally observed despite a MP near $E_k$. It is notable that the appearance of persistent depolarization after chronic morphine treatment was found in neurons from the periaqueductal gray (Chieng and Christie 1996).

It was reported that chronic morphine treatment could modulate the release of GABA and glutamate from synaptic terminals (Bonci and Williams 1997; Martin et al. 1999; Pinnock 1992). Those results indicated that residual synaptic inputs might influence the excitability of LC neurons in slices from morphine-dependent rats. However, we found that withdrawal-induced hyperactivity of LC neurons was only slightly sup-

Previous data have demonstrated that the majority of the withdrawal-induced activation of LC neurons in vivo is mediated indirectly, via excitatory amino acid inputs from the rostral ventrolateral medulla (Akaoka and Aston-Jones 1991; Ennis and Aston-Jones 1988; Rasmussen and Aghajanian 1989). However, these data did not exclude the possible additional involvement of an intrinsic mechanism in the hyperactivity of LC neurons during withdrawal. In fact, in these prior studies withdrawal-induced hyperactivity was not completely eliminated by antagonizing amino acid inputs or by lesions of LC inputs. Our present findings are in agreement with a previous extracellular study in vitro (Kogan et al. 1992) in showing that LC neurons in brain slices from morphine-dependent rats exhibit elevated impulse activity in response to opiate withdrawal. However, that study did not establish that the withdrawal-induced activation was due to an intrinsic change in LC neurons and not to other factors, e.g., increased synaptic activation of LC cells. In addition, that prior study did not examine the membrane potential of LC neurons during withdrawal as extracellular recording methods were used, and possible mechanisms involved were not examined. The present study extends that report by showing that although withdrawal activates LC cells in vitro above discharge rates in naïve rats, the resting membrane potential of LC neurons from morphine-dependent rats after naloxone did not significantly differ from

FIG. 8. Sp-cAMPS and forskolin increased impulse activity in LC neurons from control rats. $A$: example of change in spontaneous discharge of an LC neuron before and 5 min after superfusion with Sp-cAMPS (10 μM). Sp-cAMPS consistently increased the frequencies of spontaneous discharge and slow membrane oscillations, and simultaneously slightly hyperpolarized LC neurons (9 cells tested in different slices). $B$: average discharge rate was significantly increased during superfusion of Sp-cAMPS ($P < 0.01$; $n = 9$). $C$: average discharge rate was significantly increased during superfusion of forskolin ($P < 0.05$; $n = 8$).
pressed by antagonists of glutamate and GABA_A receptors. Moreover, these antagonists had a similar effect on LC neurons from dependent and control slices, and there was no tendency of these antagonists to selectively suppress withdrawal-induced activity. Therefore little if any of the withdrawal hyperactivity in LC neurons in vitro appears to be involve amino acid inputs. This conclusion is consistent with the results of our experiments using TTX to block synaptic transmission. TTX completely blocked spontaneous (pacemaker) discharge in LC neurons from control rats (as we have previously reported) (Horvath et al. 1999; Ivanov and Aston-Jones 1995) but only partially suppressed it in LC neurons from dependent rats after naloxone. We also demonstrated in these previous studies that depolarization of TTX-treated LC neurons restored spontaneous impulse activity in the form of Ca^{2+} spikes. These results indicate that the lower rate of discharge of LC neurons from dependent slices treated with TTX compared with control cells probably is not due to blockade of a withdrawal-specific current. Rather this lower rate is due to an overall suppressive effect of TTX on LC impulse activity, and impulses are restored by naloxone-induced withdrawal despite TTX. Indeed a similar increase in activity occurs after naloxone in dependent slices regardless of whether TTX is present.

Previous results indicated that the inhibition of a Na^+/K^+ pump after chronic morphine treatment caused depolarization and increased the excitability of S neurons from myenteric plexus (Kong et al. 1997). The absence of a significant difference in the RMP between LC neurons from control and morphine-dependent rats, and a similar effect of ouabain on the RMP and SDR in LC neurons from both groups of animals, make such a mechanism unlikely in the withdrawal-induced hyperactivity of LC neurons.

Chronic morphine administration has previously been shown to upregulate the cAMP second-messenger and protein phosphorylation pathways in LC neurons (Nestler 1992; Nestler and Tallman 1988). It was hypothesized that the elevated cAMP activates cAMP-dependent TTX-insensitive sodium channels, which depolarize LC neurons and increases their excitability (Alreja and Aghajanian 1991; Wang and Aghajanian 1987). However, this suggestion was not consistent with other results (Travalgi et al. 1995). The present findings also do not support this hypothesis. First, LC neurons from dependent rats were not significantly depolarized compared with LC neurons from control rats, as would be expected for cells with a persistently increased Na^+ conductance. Moreover, in ~30% of tested cells, the withdrawal-induced increase in impulse activity of LC neurons developed without a detectable depolarization, and a few cells even exhibited a small hyperpolarization after naloxone. Second, the selective activator of PKA, Sp-cAMPS, increased impulse activity without depolarizing LC neurons from control rats, and, selective inhibitors of PKA (Rp-cAMPS and H-89) suppressed the withdrawal-induced hyperactivity of LC neurons without significantly hyperpolarizing their membrane potential. Moreover, the cAMP stimulator forskolin had no consistent effect on the membrane potential of LC neurons as was also reported in a previous study (Harris and Williams 1991). Increased impulse activity following forskolin could reflect attenuation of the AHP in LC neurons (Shiekhattar and Aston-Jones 1994).

Together, the present results indicate that elevated impulse activity in LC neurons during opiate withdrawal and activation of the cAMP cascade was not caused simply by depolarization of these cells subsequent to an increased Na conductance. However, it is possible that distal dendrites become depolarized during withdrawal without being detectable with recordings in the soma. Such an effect could lead to elevated spike rates, particularly in neurons whose axons originate from proximal dendrites, as in the LC (Groves and Wilson 1980). This possibility requires further analysis.

In addition, the participation of an unidentified inward current that slightly depolarizes LC neurons in dependent rats cannot be excluded by the present results. It is logical to suggest that potassium channels activated by morphine during chronic treatment are the primary targets for adaptive changes. The present findings (no changes in membrane potential and increase in spontaneous discharge rate after chronic morphine treatment) lead us to propose that a downregulation of potassium channels that control spontaneous discharge rate resulting from activation of PKA causes the naloxone-precipitated hyperexcitability in LC neurons. Indeed, the most prominent effect after naloxone observed in this study was increased frequency of spontaneous action potentials. Previous work indicated that two types of potassium currents are primarily involved in the regulation of spike frequency in central neurons: the A current and the SK current (Rudy 1988). These currents exist in LC neurons (Osmanovic and Shefner 1993; Williams et al. 1984) and participate in the regulation of SDR (Andrade and Aghajanian 1984b). Furthermore Rp-cAMPS selectively prevented the inhibitory effect of cAMP on the SK current in hippocampal neurons (Pedarzani and Storm 1993), and activation of either PKA or PKC downregulated the transient A-type K^+ channels in dendrites of hippocampal CA1 pyramidal neurons (Hoffman and Johnston 1998). These findings are consistent with our hypothesis that chronic morphine administration (and the associated increase in cAMP) lead to decreased K^+ channel activity in LC neurons, and that this change underlies the locally induced withdrawal-precipitated hyperactivity in LC cells.

Thus our results demonstrate that the withdrawal-induced hyperactivity in LC neurons in vitro does not involve release of neurotransmitters but presumably results from adaptations produced by chronic morphine treatment. Our studies also indicate that this adaptation may involve downregulation of conductances in the family of potassium channels. Further studies using voltage-clamp methods and a preparation with truncated LC dendrites are needed to test this hypothesis, and to elucidate the specific channel(s) that are altered during chronic morphine exposure.

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