LOCUS COERULEUS ACTIVITY *IN VITRO*: INTRINSIC REGULATION BY A CALCIUM-DEPENDENT POTASSIUM CONDUCTANCE BUT NOT α_2 -ADRENOCEPTORS¹

RODRIGO ANDRADE² AND GEORGE K. AGHAJANIAN

Departments of Pharmacology and Psychiatry, Yale University School of Medicine and the Connecticut Mental Health Center, New Haven, Connecticut 06508

Received May 12, 1983; Revised August 4, 1983; Accepted August 20, 1983

Abstract

Locus coeruleus neurons recorded intracellularly in rat brainstem slices exhibited spontaneous activity and a marked afterhyperpolarization following a burst of spikes. This afterhyperpolarization was associated with an increase in membrane conductance and resulted in a marked postactivation inhibition of spontaneous activity. Since the reversal potential of the afterhyperpolarization was found to be virtually identical when recorded with KCl or potassium acetate-filled electrodes and shifted in the hyperpolarizing and depolarizing direction with decreases and increases in extracellular potassium concentrations, respectively, the afterhyperpolarization seen following a burst of spikes in this cell group appears to be mediated by an increase in potassium conductance.

The afterhyperpolarization and postactivation inhibition were markedly attenuated by reducing calcium influx by either omitting extracellular calcium in the bathing medium or blocking calcium channels with manganese or cadmium. Thus, the afterhyperpolarization and the resulting postactivation inhibition appear to be largely mediated by the activation of a calcium-dependent potassium conductance.

Previous reports *in vivo* have suggested that activation of α_2 -adrenoceptors by norepinephrine release from recurrent axons or dendrites may mediate self-inhibition in the locus coeruleus. In this study, we examined the effect of blocking α_2 -adrenoceptors on the afterhyperpolarization and postactivation inhibition. Administration of the α_2 -adrenoceptor antagonist piperoxane failed to produce any changes in either of these parameters, suggesting that at least *in vitro* the afterhyperpolarization and postactivation inhibition seen in locus coeruleus neurons do not result from the activation of α_2 -adrenoceptors.

The locus coeruleus (LC) consists of a tightly packed noradrenergic cell group (Dahlström and Fuxe, 1965; Swanson and Hartman, 1975) containing approximately half of the noradrenergic neurons in the rat brain (Nygren and Olson, 1977). Previous *in vivo* studies have shown these cells to be spontaneously active (Graham and Aghajanian, 1971; Faiers and Mogenson, 1976) and to exhibit a marked postactivation inhibition following orthodromic (Cedarbaum and Aghajanian, 1978a; Watabe and Satoh, 1979; Foote et al., 1980) or antidromic

(Aghajanian et al., 1977; Watabe and Satoh, 1979) stimulation. Although the presence of such a postactivation inhibition in the LC is well established, the mechanisms that underlie it are not completely understood. Recently, intracellular studies *in vivo* have shown that this postactivation inhibition is associated with a long-lasting afterhyperpolarization (AHP) (Aghajanian and VanderMaelen, 1982). Since LC neurons are under the tonic regulation of α_2 -adrenoceptors (Cedarbaum and Aghajanian, 1976), one possible mechanism mediating the AHP could involve activation of α_2 -adrenergic somatodendritic "autoreceptors" by the release of norepinephrine from recurrent autoinhibitory axonal branches or possibly from dendrites. This hypothesis has derived some support from the observation that, *in vivo*, the α_2 -adrenoceptor antagonist piperoxane appears to reduce the AHP following a burst of spikes (Aghajanian and VanderMaelen, 1982). This interpretation, however, must be tempered by the possibility that this treatment

¹ We thank Dr. C. P. VanderMaelen for helpful discussions, Ms. Nancy Margiotta and Ms. Annette Zimnewicz for their technical assistance, and Ms. Leslie Fields for typing the manuscript. This research was supported by United States Public Health Service Grants GM-07527, MH-17871, MH-14276, and the State of Connecticut.

² To whom correspondence should be addressed, at Department of Pharmacology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06508.

could also block extrinsic noradrenergic or adrenergic influences onto the LC. Such a blockade would remove a tonic hyperpolarizing influence onto LC neurons, thereby reducing the AHP and increasing spontaneous activity. More recent studies using *in vivo* intracellular injections of the calcium chelator EGTA (Aghajanian et al., 1983) have suggested a second possible mechanism which could mediate the postactivation inhibition, namely the activation of a calcium-dependent potassium conductance. Activation of this current by calcium influx during a burst of spikes would result in a marked increase in potassium conductance and the generation of a long-lasting AHP. The present study was undertaken to characterize further the ionic mechanisms underlying the AHP in the LC and to examine the role of calcium and α_2 -adrenoceptors in its generation.

Materials and Methods

A total of 38 male albino rats (150 to 250 gm) was used in these experiments. They were anesthetized with chloral hydrate (400 mg/kg, i.p.) and given 100% oxygen to breathe for at least 5 min. They were then decapitated and their brains were removed and cooled in ice-cold artificial cerebrospinal fluid (ACSF). One or two pontine brainstem slices (200 to 400 μ m actual thickness) containing the LC were prepared using a Sorval Tissue Sectioner as previously described (Andrade et al., 1983). The brain slices were then transferred to a chamber modified from the design of Haas et al. (1979), where they rested on a stage consisting of a Plexiglas frame covered on top with a fine epoxy-coated Dacron mesh and lined with lens paper. Oxygenated ACSF flowed over the stage at a rate of 0.4 to 1.0 ml/min. Pooling of fluid was reduced by the use of Kimwipe strips placed on the outflow side of the stage. A thorough perfusion of both sides of the slice was ensured by covering the slice with a single layer of surgical gauze. Humidified 95% O_2 and 5% CO_2 flowed continuously over the slice. The chamber and perfusion fluid were maintained at a constant temperature throughout each experiment; this temperature was set between 32 and 37°C.

Extracellular recordings were as previously described (Andrade et al., 1983). Intracellular recordings were obtained with Microstar (Radnoti) or Kwik-fil (W-P Instruments) micropipettes filled with either 3 M KCl or 2 to 4 M potassium acetate. These exhibited impedances ranging from 35 to 65 megohms. Intracellular potentials were amplified using an M-707 Amplifier (W-P Instruments) and were displayed on an oscilloscope. DC drift was prevented by the use of silver/silver chloride leads for the electrode and the chamber ground connection. Intracellular stimulation was accomplished using square pulses delivered by a digital pulse generator/stimulus isolater (W-P Instrument series 1800) through the bridge circuit of the intracellular amplifier. The bridge circuit was balanced inside each cell. Recordings were stored on FM tape and the data analyzed from oscilloscope photographs obtained either on-line or retrieved from tape.

All recordings were obtained from the visually identified LC. This cell group appeared, when illuminated obliquely with an overhead light, as a distinct dark, granular area lateral to the central grey and immediately

anterior to the descending VIIth nerve. We have previously ascertained that this area corresponds to the histologically identified LC (Andrade et al., 1983). Cells were impaled by ringing the capacity compensation. Upon impalement, cells were hyperpolarized by current injection (up to 2 nA) for variable periods of time. This procedure appeared to promote sealing. Recordings were considered acceptable if the action potential amplitude exceeded 60 mV, input resistance was over 50 megohms, and spontaneous activity was regular and of less than 3 spikes/sec. All cells were impaled between 1 and 10 hr postdecapitation. Stable intracellular impalements were maintained for up to 9 hr. Absolute membrane potential was determined at the end of the experiment with respect to extracellular zero.

ACSF of standard composition (in millimoles: NaCl, 130; KCl, 5; $NaH_2PO_4 \cdot H_2O$, 1.25; $NaHCO_3$, 24; $CaCl_2$, 2.5; $MgSO_4$, 1.5; D-glucose, 10) and equilibrated with 95% O_2 and 5% CO_2 was used to collect the slices and throughout most of the experiments. Drugs were administered at known concentrations dissolved in this media. Changes in potassium concentration were accomplished by equimolar replacement of potassium by sodium. When a calcium-free solution was needed, calcium was substituted by varying amounts of magnesium (up to 2.6 mM magnesium per multiple of calcium replaced). Since phosphate and sulfate anions caused cadmium and manganese salts to precipitate, a modified ACSF without phosphate or sulfate was used when these divalent cations were administered. This modified ACSF was identical in most respects to the standard ACSF except phosphate was omitted, $MgSO_4$ was replaced by $MgCl_2$, and the $NaHCO_3$ concentration was lowered to 12 mM to maintain a pH of 7.4 (Wong and Prince, 1981). The modified ACSF was administered for 10 to 30 min before and after administration of the divalent cations, and any changes seen following cadmium or manganese administration were compared to this base line and/or to the recovery in modified ACSF. This procedure was established because the modified medium by itself produced a variable hyperpolarization and decreased or abolished spontaneous activity. In a few experiments the ACSF was supplemented with L-tyrosine (Sigma, 20 μ M), the reduced tyrosine hydroxylase pteridine cofactor DL-6-methyl-5,6,7,8-tetrahydropterine (Calbiochem, 1 μ M), and ascorbic acid (Calbiochem, 5.7 μ M), as an antioxidant, in an attempt to increase norepinephrine synthesis in the slice. Exogenously administered L-tyrosine is known to be converted to norepinephrine in *in vitro* brain slices (Salzman and Roth, 1980). This enriched ACSF was administered for variable periods of time ranging from 17 min to several hours before testing the effect of piperoxane. Ouabain was obtained from Sigma, clonidine from Boehringer Ingelheim, and piperoxane from Rhône Poulenc.

Results

As previously reported for rat brain slices (Williams et al., 1982a; Andrade et al., 1983) most LC neurons recorded either extracellularly or with stable intracellular impalements were found to fire regularly with rates of up to 3 spikes/sec. Intracellular recordings revealed that

this pattern of spontaneous activity was associated with a stereotyped cycling of the membrane potential (Fig. 1A). A spontaneous action potential was followed by an AHP which decayed slowly and led, in slow-firing cells, to a "plateau" phase in which there was little change in membrane potential. This "plateau" phase, which was of variable duration and occurred at about -55 mV, was

terminated by a short depolarization which triggered the next action potential. When a single spike was elicited by current injection 400 to 600 msec following a spontaneous spike, the stimulus-elicited action potential simply replaced a spontaneous spike (Fig. 1B). However, when two or more action potentials were triggered by increasing the duration of the depolarizing pulse, the burst of

Figure 1. Relationship between number of spikes in a burst, AHP amplitude and duration, and postactivation inhibition. *A*, Intracellular record showing the characteristic cycling of the membrane potential associated with spontaneous activity in an LC neuron. Each spontaneous action potential is followed by an AHP which decays slowly, leading to a "plateau" phase of little change in membrane potential. The next spontaneous spike can be seen to arise from a brief depolarization originating from this "plateau" phase. *B* to *D*, A single spike triggered by intracellular current injection simply replaces a spontaneous spike (*A*). However, increasing the number of spikes in the current induced burst (*C* = 4 spikes; *D* = 6 spikes) results in a larger AHP and longer lasting postactivation inhibition. *E* to *G*, While the amplitude of the AHP appeared to begin to saturate after a few spikes (*G*), its duration (50% AHP recovery time, *E*) and the latency to the first spike following the burst (a measure of the duration of the postactivation inhibition, *F*) increase linearly with increasing number of spikes. *E*, $r = 0.92$, $p < 0.001$. *F*, $r = 0.94$, $p < 0.001$. *G*, curve fitted by hand.

spikes was followed by an AHP which increased both in amplitude and duration with the number of spikes in the burst, and it resulted in a marked postactivation inhibition (Fig. 1, *C* and *D*). Interestingly, the duration of the AHP (time to 50% recovery) and the length of the postactivation inhibition (latency to first spike after the burst) increased linearly with increasing number of spikes in the range tested (Fig. 1, *E* and *F*), while the amplitude of the AHP began to saturate after a few spikes (Fig. 1*G*).

When the membrane input resistance was tested by a brief (100 msec) hyperpolarizing current pulse immediately before a burst of spikes and during the resulting AHP (Fig. 2*A*), the second hyperpolarizing pulse caused a substantially smaller voltage deflection (Fig. 2*A*), suggesting that a decrease in membrane resistance (increase in conductance) had occurred during the AHP. However, because these cells also show an apparent decrease in slope resistance with increasing hyperpolarization (anomalous rectification) (cf. Fig. 3), a phenomenon

which would result in an apparent decrease in membrane resistance during the AHP, we re-examined changes in membrane input resistance following a burst of spikes at the reversal potential of the AHP. This manipulation allowed changes in membrane conductance to be measured in the absence of changes in membrane potential. As seen in Figure 2*B*, when this experiment was conducted while hyperpolarizing the cell to the reversal potential of the AHP, a burst of spikes was followed by a marked decrease in membrane input resistance even in the absence of changes in membrane potential. This change in membrane resistance exhibited a time course consistent with that of the AHP (Fig. 2, *A* and *B*).

The reversal potential for the AHP was found to be consistently 20 to 35 mV more negative than the "plateau" phase of the firing cycle (Fig. 3, *A* and *B*). Such a reversal potential indicated that the AHP could result from an increase in conductance to either potassium or chloride. To distinguish between these possibilities, we compared intracellular recordings obtained with electrodes filled with KCl or potassium acetate. Electrodes containing chloride would be expected to release this anion into the cell and change its equilibrium potential. If the AHP were mediated by an increase in chloride conductance, such changes would then decrease or reverse the AHP and displace the reversal potential for the

Figure 2. Changes in input resistance during the AHP. *A*, The voltage deflection produced by a short (100 msec) hyperpolarizing pulse (-0.2 nA) was found to be substantially diminished during the AHP following a burst of spikes when compared to an identical pulse delivered just before the burst. This decrease suggested that the AHP is associated with an increase in conductance. *B*, This decrease in slope resistance was unlikely to result from the presence of anomalous rectification in these cells since it was seen even when the experiment was repeated at the reversal potential of the AHP where there is no change in membrane potential following the burst of spikes. This figure is a composite of 10 superimposed sweeps with hyperpolarizing or depolarizing pulses (± 0.4 nA) delivered just before or at varying times following the burst of spikes.

Figure 3. Comparison of the reversal potentials calculated with KCl or potassium acetate-filled electrodes. *A* and *C*, Two sets of five superimposed sweeps each, illustrating the voltage deflections produced by series of hyperpolarizing pulses (0.0, -0.2 , -0.4 , -0.6 , -0.8 nA) delivered just before and after a burst of spikes in two cells, one being recorded with KCl (*A*) and the other with a potassium acetate (*C*)-filled electrode. *B* and *D*, I-V plots ($\bullet$, preburst; $\circ$, postburst) for the data shown in *A* and *C*. The i.v. curves for these cells give an apparent reversal potential for the KCl electrode of -82 mV and for the potassium acetate electrode of -81.5 mV. The marked deviation from linearity of the i.v. relationship denotes the presence of substantial anomalous rectification (Kandel and Tauc, 1966) in these cells.

AHP in the depolarizing direction. Electrodes filled with acetate, an anion much larger than chloride and, therefore, thought not to permeate efficiently through chloride channels (Araki et al., 1961), would not be expected to affect the AHP. When the reversal potential for the AHP calculated by using KCl electrodes ($84.0 \text{ mV} \pm 3.1 \text{ mV}$, mean $\pm$ SD; $n = 13$ cells; e.g., Fig. 3, *A* and *B*) was compared to that found by using potassium acetate electrodes ($84.4 \text{ mV} \pm 2.7 \text{ mV}$, mean $\pm$ SD, $n = 5$ cells; e.g., Fig. 3, *C* and *D*), they were not found to be significantly different. Moreover, intracellular recordings for up to 9 hr with KCl (3 M)-filled electrodes resulted in no noticeable change in either the amplitude or the duration of the AHP. This lack of effect was in strong contrast to the ready reversal of chloride-mediated potentials seen in other systems when recording with KCl-filled electrodes (e.g., Coombs et al., 1955; Alger and Nicoll, 1980; VanderMaelen and Aghajanian, 1982). These results suggested the AHP was mediated by an increase in potassium rather than chloride conductance. However, to test more directly the possibility that a change in potassium conductance indeed mediates the AHP, we examined the dependence of the reversal potential of the AHP on the extracellular concentration of potassium. If the AHP were mediated by a pure increase in potassium conductance, changing the external concentration of this ion, which would alter the equilibrium potential for potassium, should shift the reversal potential of the AHP in accordance with the Nernst equation (ideally $\pm 60 \text{ mV}$ for a 10-fold increase or decrease in potassium concentration at 32°C). Consistent with such a prediction, increasing the potassium concentration from 5 to 10 mM or reducing it from 5 to 2.5 mM produced shifts in the reversal potential of the AHP in the depolarizing and hyperpolarizing direction, respectively, with a slope of 41 mV for a 10-fold change in external potassium concentration (Fig. 4, $n = 5$ cells).

Previous experiments *in vivo* using intracellular injections of the calcium chelator EGTA have shown that intracellular calcium is necessary for the generation of the AHP following a burst of spikes in the LC (Aghajanian et al., 1983). This calcium dependency suggests that the AHP is mediated by a calcium-dependent potassium conductance activated by calcium influx during the burst of action potentials. In order to test this hypothesis more fully, we examined the amplitude and duration of the AHP and the duration of the postactivation inhibition after a burst of spikes under conditions which would alter calcium entry into the cell. Two different approaches were used to manipulate calcium influx. In a first group of cells, the normal ACSF was replaced with ACSF in which all calcium had been replaced with varying concentrations of magnesium. This procedure resulted in a marked decrease in the AHP following a burst of action potentials (Fig. 5*A*) and a pronounced increase in spontaneous activity. The magnitude of these effects depended on the length of time the slice was exposed to calcium-free ACSF. In 10 cells exposed to calcium-free medium for 8 to 38 min, the mean amplitude of the AHP following a burst of spikes decreased by $42\% \pm 14.6\%$ (mean $\pm$ SD, $p < 0.001$), and its duration was reduced by $53\% \pm 18.9\%$ (mean $\pm$ SD, $p < 0.001$). The postacti-

Figure 4. Effect of changing the extracellular concentrations of potassium on the reversal potential of the AHP. *Right*, the reversal potential of the AHP was $-84.0 \pm 3.6 \text{ mV}$ (mean $\pm$ SD) in ACSF containing 5 mM potassium. Increasing the potassium concentration to 10 mM resulted in a depolarizing shift in the reversal potential for the AHP to $-71 \pm 3.6 \text{ mV}$ (mean $\pm$ SD). Decreasing the potassium concentration to 2.5 mM shifted the reversal potential for the AHP in the hyperpolarizing direction to $96 \pm 1.0 \text{ mV}$ (mean $\pm$ SD). *Left*, three sets of five superimposed sweeps each showing the series of current pulses (+0.2, 0.0, -0.2, -0.4, -0.6, -0.8 nA) used to calculate the reversal potential of the AHP at each of the three potassium concentrations examined. Notice how the amplitude of the AHP increased and decreased with decreases and increases in potassium concentrations, respectively.

vation inhibition was also significantly reduced in seven spontaneously active cells by an average of $82\% \pm 11.0\%$ (mean $\pm$ SD, $p < 0.001$). In the remaining three cells, which were silent at the onset of the experiment, spontaneous activity was initiated during the perfusion with calcium-free ACSF. The AHP following single spontaneous spikes was also reduced in the presence of the calcium-free medium (Fig. 5*A*). These changes were seen even when the concentration of magnesium was increased up to 8 mM. In a second group of slices, calcium influx into the cell was blocked by administration of the calcium channel blockers manganese or cadmium (Kostyuk, 1980; Hagiwara and Byerly, 1981). Administration of manganese (2 to 4 mM, $n = 3$ cells) for 12 to 36 min reduced the mean amplitude of the AHP following a burst of spikes by $63\% \pm 3.4\%$ (mean $\pm$ SD, $p < 0.001$) and its duration by $73\% \pm 14.8\%$ (mean $\pm$ SD, $p < 0.02$; Fig. 5*B*). The spontaneous activity, suppressed by the modified ACSF, was not restored by the administration of manganese. Administration of cadmium (0.5 to 2 mM) resulted in a marked depolarization and a dramatic increase in spontaneous activity (Fig. 5*C*, $n = 3$ cells). This effect was progressive and resulted, after a few minutes, in a decrease in the action potential amplitude and eventually lead to depolarization block. However, shortly after the administration of cadmium, the AHP following a burst of spikes could be seen to be markedly reduced (Fig. 5*C*). This reduction was most evident when the

Figure 5. Effect of removing calcium from the ACSF (A) or blocking calcium channels with manganese (B) or cadmium (C) on the AHP following a burst of spikes. **A, Top trace:** Two superimposed sweeps, one showing the AHP following a burst of spikes and a second one showing a 2-sec sampling of spontaneous activity containing one spontaneous action potential. The dotted line marks the threshold for the generation of action potentials. Notice that in this cell in standard ACSF, the AHP following the burst did not fully recover by the end of the sweep and the membrane potential remained well below threshold. **Middle trace:** Following 23 min in calcium-free ACSF the AHP amplitude is markedly diminished and its duration reduced. This resulted in a faster depolarization to threshold and consequently a marked reduction in the postactivation inhibition. The AHP following single spikes is also reduced. Spontaneous activity is markedly increased in the calcium-free ACSF. **Lower trace:** Two superimposed sweeps, similar to those shown in the top trace, following recovery from the calcium-free ACSF. **B, Top trace:** A single sweep showing the AHP following a burst of spikes in modified (no phosphate or sulfate) ACSF. The dotted line demarks this cell's resting potential. **Middle trace:** Burst and AHP following 12 min in 4 mM manganese. The AHP following the burst is almost totally abolished under these conditions. **Lower trace:** The AHP has largely recovered after a 20-min wash in modified ACSF. **C, Top trace:** Single sweep showing the AHP following a burst of spikes in modified ACSF. **Middle trace:** Single sweep showing repetitive firing induced by the injection of a steady depolarizing current in modified ACSF. **Lower trace:** Single sweeps showing the AHP following a burst of spikes 4 min after the administration of cadmium (2 mM). Notice that even when firing rates were matched, the AHPs following single spikes or even a burst of spikes were markedly reduced in amplitude by cadmium. The dotted line marks the threshold for generation of action potentials.

AHP following a burst of spikes in the presence of cadmium was compared to that seen in standard ACSF while depolarizing the cell by current injection to elicit comparable repetitive firing (Fig. 5C, $n = 2$ cells). When the AHP amplitude of a single spontaneous spike following cadmium was compared with that after single spikes triggered by steady depolarizing current at a matched firing rate, these were also found to be markedly reduced (Fig. 5C, $n = 2$ cells).

Previous reports dealing with certain other systems have implicated the activation of a Na^+/K^+ electrogenic metabolic pump in the generation of postactivation hyperpolarizations (Nakajima and Takahashi, 1966; Rang and Ritchie, 1968). We have examined the possible involvement of such a pump in the generation of the AHP in the LC. Administration of the Na^+/K^+ ATPase inhibitor ouabain in concentrations up to those needed to block the Na^+/K^+ ATPase in rat brain by 70 to 80% (Sweadner, 1979) was without effect on the amplitude and duration of the AHP (250 nM to 10 μM , $n = 4$ cells, Fig. 6). Higher concentrations of ouabain depolarized the

cell into inactivation, presumably because at those concentrations the remaining pump cannot maintain the sodium and potassium gradients across the membrane.

The possibility that the postactivation inhibition in the LC *in vitro* could, at least partially, be mediated by activation of α_2 -adrenoceptors was also examined. Administration of high concentrations of the α_2 -antagonist piperoxane (10 to 30 μM) failed to reduce the amplitude or duration of the AHP (not shown) or alter spontaneous activity (Fig. 7A). These results were in striking contrast to previous observations *in vivo* where piperoxane reduced the AHP, shortened the postactivation inhibition, and increased spontaneous activity (Aghajanian et al., 1983). However, since it is possible that in the *in vitro* brain slice norepinephrine synthesis is reduced due to the lack of the precursor amino acid L-tyrosine, a situation which could effectively block any norepinephrine-mediated autoinhibition, we repeated this experiment after administering an ACSF enriched by the addition of tyrosine (20 μM) plus the reduced pteridine cofactor of the synthetic enzyme tyrosine hydroxylase (DL-6-methyl-5,6,7,8,

Figure 6. Effect of ouabain on the AHP. *A*, Two superimposed traces showing the AHP following a burst of spikes before the administration of ouabain. *B*, Similar superimposed trace but taken 10 min after the beginning of perfusion with standard ACSF containing 10 μ M ouabain. The broken line was drawn on both figures at the membrane potential just previous to the burst.

tetrahydropterine; 1 μ M). However, even under these conditions, piperoxane (6 μ M) failed to produce any consistent changes in either the amplitude or duration of the AHP (Fig. 7, *B* and *C*, $n = 4$ cells) or alter spontaneous activity (not shown). *In vivo*, LC neurons are thought to receive a dense catecholaminergic innervation (Hökfelt, 1967; Descarries and Droz, 1968). It is possible then that under *in vivo* conditions LC neurons are under a continuous noradrenergic or adrenergic tone. Such a tone, if present, could perhaps explain the differences between the effects of α_2 -antagonists *in vivo* and *in vitro*. In an attempt to test this hypothesis, a tonic activation of α_2 -receptors was induced by the administration of low concentrations of clonidine (5 to 15 nM, $n = 4$ cells) which would reduce or barely suppress spontaneous activity in this preparation (Andrade et al., 1983). Administration of piperoxane (6 μ M) under these circumstances produced a marked increase in spontaneous activity, reduced the duration of the AHP by $65\% \pm 7.7\%$ (mean $\pm$ SD, $p < 0.001$), and reduced the postactivation inhibition by $60\% \pm 21.3\%$ (mean $\pm$ SD, $p < 0.05$) (Fig. 7, *D* and *E*).

Discussion

The present results indicate that in the rat brainstem slice, activation of LC neurons by intracellular current

Figure 7. Effect of piperoxane on spontaneous activity and on the AHP following a burst of spikes. *A*, Frequency histogram obtained while recording extracellularly from an LC neuron. Administration of piperoxane for 15 min to this cell failed to produce any consistent changes in spontaneous activity. *B*, AHP following perfusion of the slice for several hours with standard ACSF enriched by the addition of tyrosine and the pteridine cofactor of the synthetic enzyme tyrosine hydroxylase. *C*, Same as *B* but 20 min following the addition of 6 μ M piperoxane to the enriched ACSF. The AHP appears identical to that seen before piperoxane. The broken line was drawn at the membrane potential just before the burst for comparison purposes. *D*, Two superimposed traces taken while continuously perfusing with 15 nM clonidine. One trace shows the AHP following a burst of spikes and the second a 2-sec sample of spontaneous activity. Under these conditions the AHP recovers slowly, and by the end of the sweep the membrane potential is still well below threshold. *E*, Same as *D* except following the administration of 3 μ M piperoxane. Following piperoxane the AHP decays substantially faster, and now by the end of the sweep this cell's membrane potential almost reached threshold. The spontaneous activity was also increased by piperoxane.

pulses results in a marked AHP which is mediated primarily by the activation of a calcium-dependent potassium conductance; no evidence was found for the involvement of α_2 -adrenoceptors in this response. This AHP appears to be largely responsible for the pronounced postactivation inhibition of spontaneous activity following the burst of spikes. The evidence presented here for the mediation of the AHP by an increase in potassium conductance is 3-fold. First, the AHP is accompanied by a decrease in membrane input resistance, and its reversal potential is strongly in the hyperpolarizing direction as expected for a potassium-mediated AHP. Second, the reversal potential for the AHP was virtually identical regardless of whether it was estimated with KCl or

potassium acetate-filled electrodes. Third, changing the extracellular concentration of potassium resulted in shifts in the reversal potential of the AHP qualitatively consistent with its mediation by a change in potassium permeability. Although these shifts approximated the quantitative predictions for a potassium-mediated AHP, they fell somewhat short of the theoretical value for a pure change in potassium conductance. Such small deviations could result from incomplete exchange of the extracellular potassium concentration in the slice. However, they might also result from the activation of other ionic currents following the burst (e.g., a calcium-activated nonselective cation channel (Yellen, 1982)) or to a less than perfect selectivity filter at the potassium channels (cf. Kostyuk et al., 1981). Interestingly, the shift in the reversal potential for the calcium-activated outward current underlying the AHP in hippocampal pyramidal cells has also been shown to fall short of the theoretical prediction (41 mV/10-fold shift in potassium concentration (Brown and Griffith, 1983)). These results suggest that the AHP is mediated largely but probably not exclusively by an increase in potassium conductance. The increase in potassium conductance following a burst of spikes appeared to be critically dependent on calcium influx. Thus, the AHP could be greatly attenuated by reducing calcium entry into the cells by either removing calcium from the extracellular fluid or by blocking calcium channels with manganese or cadmium. Thus, the present results, in addition to the previous observation that intracellular injections of EGTA *in vivo* abolish the AHP, indicate that LC neurons possess a calcium-dependent potassium conductance which plays an important role in regulating their activity.

The possibility that the release of norepinephrine by recurrent axonal branches or dendrites could also participate in the production of the AHP *in vitro* was also tested in this study. LC neurons receive numerous synapses from putative catecholaminergic presynaptic elements (Hököfelt, 1967; Descarries and Droz, 1968), some of which could emanate from LC neurons themselves (Shimizu and Imamoto, 1970; Swanson, 1976). If these elements were to synapse onto their cells of origin, they could conceivably contribute to the AHP and the postactivation inhibition. The possibility that such a mechanism could play a large role in the generation of the AHP in the LC *in vitro* was excluded in this study by showing that blockade of α_2 -adrenoceptors did not reduce the AHP even when norepinephrine synthesis was promoted by administration of L-tyrosine in the ACSF. Thus, *in vitro* it appears that α_2 -adrenoceptors do not participate in the generation of the AHP. Interestingly, spontaneous activity was not increased following the blockade of α_2 -adrenoceptors. This observation suggests that in the brain slice there is little if any recurrent inhibition by neighboring LC neurons by release of norepinephrine from either axonal collaterals or dendrites. Since the observation that piperoxane was without noticeable effect on LC activity in the *in vitro* brain slice was at odds with a previous report that α_2 -adrenoceptor blockade reduced the AHP and increased spontaneous activity *in vivo*, the possibility was considered that this discrepancy

could result from the presence *in vivo* of a tonic activation of α_2 -adrenoceptors in the LC by extrinsic catecholaminergic inputs or recurrent collaterals. Of course, these extrinsic catecholaminergic inputs would be absent in the slice and axon collaterals would be partially removed due to the slicing procedure, possibly accounting, at least partially, for differences between the *in vivo* and *in vitro* conditions. In support of this hypothesis, we have shown that in the presence of a sustained activation of α_2 -adrenoceptors, produced by the administration of low concentrations of clonidine, LC neurons *in vitro* could closely mimic the behavior of LC neurons *in vivo*. Thus, it is possible that *in vivo* LC neurons are under a continuous adrenergic or noradrenergic tone from either recurrent collaterals (Shimizu and Imamoto, 1970; Swanson, 1976) or from extrinsic adrenergic (Hököfelt et al., 1974) or possibly noradrenergic (Cedarbaum and Aghajanian, 1978) inputs, and that upon administration of piperoxane this steady hyperpolarizing influence is reduced and thus allows for a faster recovery from the AHP and an increase in spontaneous activity. In this regard, it is interesting that field stimulation of LC slices which would result in neurotransmitter release from presynaptic elements results in a poststimulation hyperpolarization which appears to be mediated by the activation of α_2 -adrenoceptors (Williams et al., 1982b).

Previous studies have shown that activation of a metabolic electrogenic pump underlies the marked postactivation hyperpolarization seen in some other systems. That this was not the case in the LC was shown by several observations. First, a metabolic pump would not be expected to produce an AHP with a reversal potential (Nakajima and Takahashi, 1966) such as the one seen in these experiments. Second, increasing and decreasing extracellular potassium concentrations should increase and decrease, respectively, the AHP if it were mediated by a metabolic pump. In these experiments, changes in the extracellular concentration of potassium produced exactly the opposite results. Third, partial blockade of the pump with ouabain at concentrations which inhibit the Na^+/K^+ ATPase by 70 to 80% failed to alter either the amplitude or duration of the AHP. Taken together, these results strongly argue against a major involvement of an electrogenic metabolic pump in the generation of the AHP in the LC.

The present results indicate that the AHP following a burst of spikes in the LC results from the summation of individual AHPs following each action potential in a burst. Thus, there is a strong correlation between the number of spikes in a burst and the duration and amplitude of the AHP, although the latter effect tended to saturate with increasing number of spikes. Interestingly, the duration of the AHP and postactivation inhibition both increased linearly with increasing number of spikes, thus re-emphasizing the importance of the AHP in the control of the postactivation inhibition. The presence of an AHP mediated by a calcium-dependent potassium conductance following each individual action potential raises the question of a possible involvement of this current in the regulation of spontaneous activity, particularly since this current has been shown to be responsible

for sustained rhythmic firing at low frequencies (Partridge, 1982). In the present experiments, reducing calcium influx into LC neurons by either removing calcium or blocking calcium channels with cadmium both resulted in a decrease in the AHP following individual spikes and an increase in spontaneous activity. This increase in spontaneous activity was unlikely to result from the membrane-destabilizing effects of the calcium-free ACSF since it was seen even at concentrations of magnesium which should have compensated for the removal of calcium (Frankenhaeuser and Hodgkin, 1957) and a similar increase was seen after adding cadmium, a divalent cation with calcium channel-blocking properties. Thus, these observations, taken together with our previous observations that LC neurons intracellularly injected with EGTA fired significantly faster than control impaled cells (Aghajanian et al., 1983), lend support to a major role for the calcium-dependent potassium conductance in the control of pacemaker activity in the LC.

References

- Aghajanian, G. K., and C. P. VanderMaelen (1982) α_2 -Adrenoceptor-mediated hyperpolarization of locus coeruleus neurons: Intracellular studies *in vivo*. *Science* 215: 1394–1396.
- Aghajanian, G. K., J. M. Cedarbaum, and R. Y. Wang (1977) Evidence for norepinephrine-mediated collateral inhibition of locus coeruleus neurons. *Brain Res.* 136: 570–577.
- Aghajanian, G. K., C. P. VanderMaelen, and R. Andrade (1983) Intracellular studies on the role of calcium in regulating the activity and reactivity of locus coeruleus neurons *in vivo*. *Brain Res.* 273: 237–243.
- Alger, B. E., and R. A. Nicoll (1980) Spontaneous inhibitory post-synaptic potentials in hippocampus: Mechanism for tonic inhibition. *Brain Res.* 200: 195–200.
- Andrade, R., C. P. VanderMaelen, and G. K. Aghajanian (1983) Morphine tolerance and dependence in the locus coeruleus: Single cell studies in brain slices. *Eur. J. Pharmacol.* 91: 161–169.
- Araki, T., M. Ito, and O. Oscarsson (1961) Anion permeability of the synaptic and non-synaptic motoneurone membrane. *J. Physiol. (Lond.)* 159: 410–435.
- Brown, D. A., and W. H. Griffith (1983) Calcium-activated outward current in voltage-clamped hippocampal neurones of the guinea-pig. *J. Physiol. (Lond.)* 337: 287–301.
- Cedarbaum, J. M., and G. K. Aghajanian (1976) Noradrenergic neurons of the locus coeruleus: Inhibition by epinephrine and activation by the α -antagonist piperoxane. *Brain Res.* 112: 413–419.
- Cedarbaum, J. M., and G. K. Aghajanian (1978a) Activation of locus coeruleus neurons by peripheral stimuli: Modulation by a collateral inhibitory mechanism. *Life Sci.* 23: 1383–1392.
- Cedarbaum, J. M., and G. K. Aghajanian (1978b) Afferent projections to the rat locus coeruleus as determined by a retrograde tracing technique. *J. Comp. Neurol.* 178: 1–16.
- Coombs, J. S., J. C. Eccles, and P. Fatt (1955) The specific ionic conductances and the ionic movements across the motoneuronal membrane that produce the inhibitory post-synaptic potential. *J. Physiol. (Lond.)* 130: 326–373.
- Dahlström, A., and K. Fuxe (1965) Evidence for the existence of monoamine-containing neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brain stem neurons. *Acta Physiol. Scand.* 62: Suppl. 232, 1–55.
- Descarries, M., and B. Droz (1968) Incorporation de noradrenaline- ^3H (NA- ^3H) dans le système nerveux central du rat adulte. Etudes radio-autographique en microscopie électronique. *C. R. Acad. Sci. (Paris)* 266: 2480–2482.
- Fairst, A. A., and G. J. Mogenson (1976) Electrophysiological identification of neurons in locus coeruleus. *Exp. Neurol.* 53: 254–266.
- Foot, S. L., G. Aston-Jones, and F. E. Bloom (1980) Impulse activity of locus coeruleus neurons in awake rats and monkeys is a function of sensory stimulation and arousal. *Proc. Natl. Acad. Sci. U. S. A.* 77: 3033–3037.
- Frankenhaeuser, B., and A. L. Hodgkin (1957) The action of calcium on the electrical properties of squid axons. *J. Physiol. (Lond.)* 137: 218–244.
- Graham, A. W., and G. K. Aghajanian (1971) Effects of amphetamine on single cell activity in a catecholamine nucleus, the locus coeruleus. *Nature* 234: 100–102.
- Haas, H. L., B. Schaerer, and V. Vosmansky (1979) A simple perfusion chamber for the study of nervous tissue slices *in vitro*. *J. Neurosci. Methods* 1: 323–325.
- Hagiwara, S., and L. Byerly (1981) Calcium channel. *Annu. Rev. Neurosci.* 4: 69–125.
- Hökfelt, T. (1967) On the ultrastructural localization of noradrenaline in the central nervous system of the rat. *Z. Zellforsch.* 79: 110–117.
- Hökfelt, T., K. Fuxe, M. Goldstein, and O. Johansson (1974) Immunohistochemical evidence for the existence of adrenaline neurons in the rat brain. *Brain Res.* 66: 235–251.
- Kandel, E. R., and L. Tauc (1966) Anomalous rectification in the metacerebral giant cells and its consequences for synaptic transmission. *J. Physiol. (Lond.)* 183: 287–304.
- Kostyuk, P. G. (1980) Calcium ionic channels in electrically excitable membrane. *Neuroscience* 5: 945–959.
- Kostyuk, P. G., N. S. Veselovsky, S. A. Fedulova, and A. Y. Tsyndrenko (1981) Ionic currents in the somatic membrane of rat dorsal root ganglion neurons. III. Potassium currents. *Neuroscience* 6: 2439–2444.
- Nakajima, S., and K. Takahashi (1966) Post-tetanic hyperpolarization and electrogenic Na pump in stretch receptor neurone of crayfish. *J. Physiol. (Lond.)* 187: 105–127.
- Nygren, L. G., and L. Olson (1977) Intracisternal neurotoxins and monoamine neurons innervating the spinal cord: Acute and chronic effects on cell and axon counts and nerve terminals densities. *Histochemistry* 52: 281–306.
- Partridge, L. D. (1982) A mechanism for the control of low-frequency repetitive firing. *Cell. Mol. Neurobiol.* 2: 33–45.
- Rang, H. P., and J. M. Ritchie (1968) On the electrogenic sodium pump in mammalian non-myelinated nerve fibres and its activation by various external cations. *J. Physiol. (Lond.)* 196: 183–221.
- Salzman, P. M., and R. H. Roth (1980) Poststimulation catecholamine synthesis and tyrosine hydroxylase activation in central noradrenergic neurons. II. Depolarized hippocampal slices. *J. Pharmacol. Exp. Ther.* 212: 74–84.
- Shimizu, N., and K. Imamoto (1970) Fine structure of the locus coeruleus in the rat. *Arch. Histol. Jpn.* 31: 229–246.
- Swanson, L. W. (1976) The locus coeruleus: A cytoarchitectonic, Golgi and immunohistochemical study in the albino rat. *Brain Res.* 110: 39–56.
- Swanson, L. W., and B. K. Hartman (1975) The central adrenergic system. An immunofluorescence study of the location of cell bodies and their efferent connections in the rat utilizing dopamine- β -hydroxylase as a marker. *J. Comp. Neurol.* 163: 467–506.
- Sweadner, K. J. (1979) Two molecular forms of ($\text{Na}^+ + \text{K}^+$)-stimulated ATPase in brain. *J. Biol. Chem.* 254: 6060–6067.
- VanderMaelen, C. P., and G. K. Aghajanian (1982) Serotonin-induced depolarization of rat facial motoneurons *in vivo*: Comparison with amino acid transmitters. *Brain Res.* 239:

- 139-152.
- Watabe, K., and T. Satoh (1979) Mechanism underlying prolonged inhibition of rat locus coeruleus neurons following anti- and orthodromic activation. *Brain Res.* 5: 343-347.
- Williams, J. T., T. M. Egan, and R. A. North (1982a) Enkephalin opens potassium channels on mammalian central neurones. *Nature* 299: 74-77.
- Williams, J. T., T. M. Egan, R. A. North, and G. Henderson (1982b) Noradrenaline mediated inhibitory postsynaptic potentials in locus coeruleus neurons. *Soc. Neurosci. Abstr.* 8: 374.
- Wong, R. K. S., and D. A. Prince (1981) Afterpotential generation in hippocampal pyramidal cells. *J. Neurophysiol.* 45: 86-97.
- Yellen, G. (1982) Single Ca^{2+} -activated nonselective cation channels in neuroblastoma. *Nature* 296: 357-359.