Voltage-Dependent Calcium Currents in Bulbospinal Neurons of Neonatal Rat Rostral Ventrolateral Medulla: Modulation by α2-Adrenergic Receptors

YU-WEN LI, PATRICE G. GUYENET, AND DOUGLAS A. BAYLISS

Department of Pharmacology, University of Virginia, Charlottesville, Virginia 22908

Voltage-dependent calcium currents in bulbospinal neurons of neonatal rat rostral ventrolateral medulla: modulation by α2-adrenergic receptors. J. Neurophysiol. 79: 583–594, 1998. The properties and modulation by norepinephrine (NE) of voltage-dependent calcium currents were studied in bulbospinal neurons (n = 116) of the rostral ventrolateral medulla (RVLM) using whole cell patch-clamp techniques in neonatal rat brain stem slices. RVLM bulbospinal neurons were identified visually by their location in slices and by the presence of fluorescein isothiocyanate-tagged microbeads, which were injected into the spinal cord before the experiment; RVLM neurons were filled with Lucifer yellow during recordings, and the slice was processed for detection of tyrosine hydroxylase immunoreactivity (TH-IR). Thirty-four of 42 recovered cells (81%) were positive for TH-IR, indicating that most recorded cells were C1 neurons. Bulbospinal RVLM neurons expressed a prominent high-voltage-activated (HVA) calcium current, which began to activate at ~30 to ~40 mV (from a holding potential of ~60 or ~70 mV) and peaked at ~0 mV (0.8 ± 0.1 nA; mean ± SE). HVA current comprised predominantly ω-conotoxin GVIA-sensitive, N-type, and ω-agatoxin IVA-sensitive, P/Q-type components, with smaller dihydropyridine-sensitive, L-type, and residual current components. Most RVLM bulbospinal neurons (n = 44/52, including 12/14 histologically identified C1 cells) also expressed low-voltage-activated (LVA) calcium current. LVA current began to activate at ~60 mV (from a holding potential of ~100 mV) and was nearly completely inactivated at ~50 mV with a half-inactivation potential of ~70 ± 2 mV. The amplitude of LVA current at ~50 mV was 78 ± 24 pA with Ba2+ and 156 ± 38 pA with Ca2+ as a charge carrier. NE inhibited HVA current in most bulbospinal RVLM neurons (n = 70/77) with an EC50 of 1.2 μM; NE had no effect on LVA current. Current inhibition by NE was mediated by α2-adrenergic receptors (α2-ARs) as the effect was mimicked by the selective α2-AR agonist, UK-14,304, and blocked by idazoxan, an α2-AR antagonist, but unaffected by prazosin and propranolol (α1- and β-AR antagonists, respectively). Most of the NE-sensitive calcium current was N- and P/Q-type. NE-induced inhibition of calcium current evoked by action potential waveforms (APWs) was significantly larger than that evoked by depolarizing steps (34 ± 2.5 vs. 23 ± 2.7%; P < 0.05). Although inhibition of calcium current was voltage dependent and partially relieved by strong depolarizations, when calcium currents were evoked with a 10-Hz train of APWs as a voltage command, the inhibitory effect of NE was maintained throughout the train. In conclusion, bulbospinal RVLM neurons, including C1 cells, express multiple types of calcium currents. Inhibition of HVA calcium current by NE may modulate input-output relationships and release of transmitters from C1 cells.

INTRODUCTION

Central neurons possess multiple types of voltage-gated calcium channels (Bean 1989; Dunlap et al. 1995; Huguenard 1996; Llinas 1988). Based on differences in voltage-dependent activation and inactivation properties, calcium currents have been subdivided into low-voltage-activated (LVA) and high-voltage-activated (HVA) components (Carbone and Swandulla 1993). LVA current activates at relatively hyperpolarized potentials and inactivates more rapidly than HVA currents (Huguenard 1996). HVA current has been further subdivided into: ω-conotoxin GVIA (CgTx)-sensitive, N-type component; ω-agatoxin IVA (AgaTx)-sensitive P/Q-type component; dihydropyridine-sensitive L-type component; and a residual component (R type) that is insensitive to calcium channel antagonists (Dunlap et al. 1995). Each calcium channel subtype may have a specialized physiological role. For example, N- and P/Q-type HVA currents are essential for neurotransmitter release from presynaptic terminals (Bean 1989; Dunlap et al. 1995; Takahashi and Momiyama 1993) and contribute to activation of Ca2+-dependent K+ channels (Umemiyai and Berger 1994; Viana et al. 1993). L-type HVA channels may be important for mediating effects of Ca2+ on gene expression (Bading et al. 1993; Murphy et al. 1991). Finally, because they operate at subthreshold levels of membrane potential, LVA currents are uniquely poised to contribute to intrinsic neuronal excitability and rhythmic activity (Huguenard 1996; Llinas 1988).

The rostral ventrolateral medulla (RVLM) contains neurons that project monosynaptically to sympathetic preganglionic neurons in the spinal cord. The majority of RVLM bulbospinal neurons are C1 cells, which contain all the enzymes necessary for synthesis of adrenaline. Studies in anesthetized animals suggest that RVLM bulbospinal cells are tonically active and provide a major excitatory drive to sympathetic preganglionic neurons. Therefore the activity of these medullary neurons is apparently critical for maintaining vasomotor tone and blood pressure (Dampney 1994; Guyenet et al. 1996). Although the mechanisms responsible for the generation of basal sympathetic tone are not fully understood, a contributing factor may be the intrinsic pacemaker activity of RVLM bulbospinal neurons (Guyenet et al. 1996). In neonatal rat brain slices, for example, the spontaneous discharge of C1 neurons appears to be due to intrinsic properties rather than excitatory synaptic inputs (Li et al. 1996). Currently, relatively little is known of the intrinsic properties of C1 and other RVLM bulbospinal neurons. In the neonatal rat, C1 neurons were found to express various voltage-dependent K+ currents and a hyperpolarization-activated cationic current (Kangrga and Loewy 1995; Li et al.
1996), but nothing is known regarding voltage-dependent calcium currents in this cell group. A description of the properties of these currents is, therefore, a major objective of the present report.

RVLM C1 neurons express \( \alpha_{2A} \)-adrenergic receptors (Guyenet et al. 1994), and many bulbospinal RVLM vasomotor neurons in vivo are inhibited by activation of \( \alpha_{2A} \)-adrenergic receptors (\( \alpha_{2} \)-ARs) (Allen and Guyenet 1993). We have shown previously that activation of \( \alpha_{2A} \)-ARs hyperpolarizes bulbospinal C1 adrenergic neurons of neonatal rats in vitro by activating an inwardly rectifying K\(^+\) conductance (Li et al. 1996). In other neurons, norepinephrine (NE) also inhibits voltage-activated calcium channels via \( \alpha_{2A} \)-ARs (Chen and Schofield 1993; Ishibashi and Akaike 1995; Li and Bayliss 1997; Trombley 1992). Accordingly, in the present study, we tested effects of NE on calcium currents in RVLM bulbospinal neurons.

In brief, the results reveal that RVLM bulbospinal neurons, including C1 cells, expressed both HVA and LVA currents. HVA current comprised predominantly \( \alpha_{2} \)- and \( P/Q \)-type components with smaller \( \alpha \)- and \( \delta \)-type components. Activation of \( \alpha_{2} \)-ARs inhibited primarily \( \alpha_{2} \)- and \( P/Q \)-components of HVA current but had no effect on LVA current. NE-induced inhibition of calcium current was greater when calcium current was evoked by action potential waveforms instead of depolarizing steps. Thus, RVLM bulbospinal C1 neurons express multiple calcium channel types; calcium current inhibition by NE may contribute to autoregulation of these neurons by \( \alpha_{2} \)-AR agonists.

**Methods**

**Retrograde labeling of bulbospinal neurons in the RVLM**

The procedures for retrogradely labeling bulbospinal neurons in the RVLM and for preparing brain slices were as described previously (Bayliss et al. 1997a; Li et al. 1996). Neonatal Sprague-Dawley rats (1–3 days postnatal) were anesthetized by hypothermia. The thoracic spinal cord was exposed using aseptic methods. A suspension of microspheres labeled with fluorescein isothiocyanate (FITC-tagged microbeads, Lumafour) was injected bilaterally into the upper thoracic spinal cord (0.5–0.8 \( \mu l \) total volume) using a calibrated glass pipette. The skin was closed with cyanoacrylate glue and the pups were returned to a lactating female.

**Preparation of brain stem slices**

Two to five days after the spinal injections, the rats (3–8 day postnatal) were anesthetized deeply by hypothermia and decapitated. The lower brain stem was removed quickly and immersed in an ice-cold Ringer solution (see following section for composition of solutions) and sectioned into transverse slices (150 \( \mu m \)) using a microslicer (DSK-1500E, Dosaka). Slices were incubated for 1 h at 37\(^\circ\)C and then maintained at room temperature (22–25\(^\circ\)C) in the Ringer solution.

**Solutions and drugs**

The solution used for preparing slices contained (in mM) 130 NaCl, 3 KCl, 5 MgCl\(_2\), 1 CaCl\(_2\), 1.25 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), and 10 glucose (equilibrated with 95%O\(_2\)-5% CO\(_2\)). For incubation of slices, the solution was the same except that CaCl\(_2\) and MgCl\(_2\) were both at 2 mM and lactic acid was added (4 mM). The external solution used for recording barium currents through calcium channels contained (in mM) 140 NaCl, 3 KCl, 10 N-[2-hydroxyethyl-piperazine]-N’-[2-ethanesulfonic acid] (HEPES), 2 BaCl\(_2\), 2 MgCl\(_2\), 10 glucose, and 0.001 tetrodotoxin (TTX; pH = 7.3 using NaOH). In some experiments, BaCl\(_2\) was replaced with equimolar CaCl\(_2\). The internal solution contained (in mM) 30 Na-ethyleneglycol-bis-(\(\beta\)-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 4 NaCl, 1 MgCl\(_2\), 0.5 CaCl\(_2\), 3 ATP-Mg, and 0.3 GTP-tris (hydroxymethyl)-aminoethanemethane (GTP-Tris; pH = 7.2 using CsOH or TEAOH).

All drugs were prepared as stock solutions and kept at –20\(^\circ\)C; they were thawed and diluted to final working concentrations on the experimental day. NE (0.1–30 \( \mu M \)) was obtained from Sigma. UK-14,304 (1 \( \mu M \)), propranolol (1 \( \mu M \)), prazosin (1 \( \mu M \)), and idazoxan (1 \( \mu M \)) were obtained from Research Biochemicals International. TTX (1 \( \mu M \)) was from Calbiochem. Stock solutions of CgTx (Bachem) and AgaTx (generous gift from Pfizer, Groton, CT) were prepared and added to perfuse containing 0.1% cytochrome C. Toxins were used at concentrations previously shown to completely block nonoverlapping components of HVA calcium current (2 \( \mu M \) for CgTx and 0.2 \( \mu M \) for AgaTx) (Bayliss et al. 1997b; Mintz et al. 1992). This was verified in the present experiments as we found that the percentage of current sensitive to each toxin was independent of the order of toxin application.

**Recording and data analysis**

Slices were submerged in a chamber mounted on a fixed-stage fluorescence microscope (Axioskop FS, Zeiss) equipped with Nomarski optics and a \( \times40 \) water-immersion objective. Neurons selected for recording were located in the rostral ventrolateral medulla at the level of the rostral tip of the inferior olive. Bulbospinal neurons in the RVLM were identified by the presence of FITC-tagged beads in the cell bodies. Patch electrodes were fabricated from borosilicate glass (Clark Electromedical, UK) on a horizontal puller (Sutter P-97) to a DC resistance of 4–7 M\(\Omega\) and connected to the headstage of an Axopatch-200A patch-clamp amplifier (Axon Instruments). Signals were filtered at 2–5 kHz and digitized at 5–10 kHz. Series resistance was usually \(<20\) M\(\Omega\) and compensated by \(\sim70\)%. Whole cell currents were recorded and analyzed using pClamp software (Axon Instruments). Electrical recordings were performed at room temperature. All recordings of calcium channel currents were leak-subtracted using a P/4 protocol. Because of the potential for voltage clamp errors when recording from intact neurons with extended processes, we were vigilant for cases when currents were obviously uncontrolled; we excluded from further analysis any currents that were not smooth and continuous functions of voltage and time.

To generate calcium channel current-voltage (\(I-V\)) curves under whole cell voltage clamp, currents were elicited by applying voltage step commands (50 ms) to varying potentials from a holding potential of \(-70\) mV. Difference currents obtained by digital subtraction of currents elicited during depolarizing voltage steps from \(-60\) and \(-100\) mV were used to define LVA currents. To test effects of NE or calcium channel toxins, HVA currents were elicited during 20-ms voltage steps to 0 mV from \(-70\) mV at 12- to 20-s intervals. The peak current during the voltage step was measured under control conditions and then isochronally throughout the experiment. The percent current inhibition attributed to NE or calcium channel toxins was determined by comparing the current in NE or toxin to the initial control current. In all cases in which run-down was evident, the data were fitted with a linear function, and the current inhibition was calculated by comparing the current in the presence of NE or toxin to a point estimated based on the linear interpolation of the run-down. Kinetic slowing induced by NE was evaluated by comparing the percent inhibition early in the pulse, the time of the control peak current, to that obtained at the end of the voltage step. To test whether current inhibition by NE was relieved by strong depolarizations, we used a protocol in which a test pulse to 0 mV was generated before and then after a
step to +70 mV. To record currents evoked by action potential waveforms (APWs), action potentials were recorded from bulbospinal RVLM neurons in current-clamp configuration and used to create a voltage command template.

Data were expressed as means ± SE and analyzed statistically using paired t-tests or one-factor analysis of variance (ANOVA). To evaluate prior hypothesized differences among group means, ANOVAs were followed by the Bonferroni t-test. In all cases significance was accepted if \( P < 0.05 \).

**Immunohistochemical detection of tyrosine hydroxylase**

Recorded bulbospinal neurons were marked during the experiment by including Lucifer yellow (0.02%) in the pipette solution. Slices containing recorded cells were immersed in 4% paraformaldehyde solution for 1–4 days before they were processed by immunohistochemical staining for tyrosine hydroxylase (TH, the rate-limiting catecholamine-synthesizing enzyme), as described (Li et al. 1996). After rinses in 0.1 M phosphate buffer (PB) and 1% sodium borohydride in PB, slices were incubated in mouse monoclonal antibody to TH (1:500) in Tris solution (50 mM Tris, 150 mM NaCl, pH 7.4) containing 5% normal goat serum and 0.5% Triton-100 (TS/NGS/TX) for 12–48 h at 4°C. After rinses in TS/NGS/TX, sections were incubated for 1 h at room temperature with biotinylated rabbit anti-mouse antisera (1:100; Vector) in TS/NGS/TX and then incubated for 1 h at room temperature with avidin-Texas red conjugate (1:200; Molecular Probes) in TS. After rinses in TS solution, sections were mounted on gelatin-coated slides and coverslipped in Krystalon (EM Science) before they were examined and photographed with a Zeiss fluorescence microscope.

**RESULTS**

The present report is based on whole cell recordings from 116 RVLM neurons; all recorded neurons were bulbospinal (i.e., retrogradely labeled with FITC-tagged microbeads from the thoracic spinal cord). We recovered a subset of recorded bulbospinal neurons (n = 42) after histological processing for TH-immunoreactivity (TH-IR); of those 42 neurons, the majority was TH-IR (n = 34, 81%). An example of a bulbospinal TH-IR neuron is shown in Fig. 1.

**HVA calcium currents**

Whole cell calcium channel currents were recorded using Ba\(^{2+}\) as a charge carrier under conditions that minimize K\(^{+}\) and Na\(^{+}\) currents. As shown in Fig. 2A, depolarizing steps from a holding potential of −70 mV evoked relatively sustained inward currents in a RVLM bulbospinal neuron. The corresponding current-voltage (I-V) relationship (Fig. 2B, diamonds) reveals that the current began to activate between −40 and −30 mV and peaked at 0 mV with a maximal amplitude of ~1.2 nA. The current was blocked completely by 100 \( \mu M \) Cd\(^{2+}\), a calcium channel blocker (Fig. 2B, squares). HVA current was observed in all bulbospinal RVLM neurons tested; on average, it activated at −40 to −30 mV (from holding potentials of −60 or −70 mV) and had a peak amplitude of 0.8 ± 0.1 nA at 0 mV (n = 26). Of the 26 bulbospinal neurons in this group, 14 were recovered after histological processing; 12 of those 14 were positive for TH-IR, suggesting that they were C1 neurons.

**Pharmacology of HVA current**

HVA current can be subdivided into several components based on sensitivity to selective calcium channel antagonists (Dunlap et al. 1995). To determine which subtypes of HVA calcium channels are expressed in RVLM bulbospinal neurons, we examined the effects of several calcium channel antagonists, as shown in Fig. 2C. HVA current was evoked at 0 mV from −70 mV, and the peak amplitude of the current was plotted as a function of time. In this cell, bath application of 2 \( \mu M \) CgTx, an N-type channel antagonist, inhibited 38% of HVA current. Subsequent application of 0.2 \( \mu M \) AgaTx, a P/Q-type channel antagonist, produced a further 30% decrease in the current. The inhibition was maintained after wash of both toxins, consistent with an irreversible block of N- and P/Q-type channels by these toxins. After CgTx and AgaTx, application of the L-type channel antagonist nimodipine (10 \( \mu M \)) caused an additional 8% inhibition of the current. The residual current (24% in this case) was abol-

![Fig. 1. Bulbospinal C1 neuron recorded in the rostral ventrolateral medulla (RVLM). Bulbospinal neurons in the RVLM were identified by the presence of fluorescein isothiocyanate (FITC)-tagged microbeads that were injected into the spinal cord 2–5 days before the experiment. Recorded neurons were filled with Lucifer yellow through the recording pipettes and immunostained for tyrosine hydroxylase, a catecholamine-synthesizing enzyme. A: fluorescence photomicrograph of a recorded RVLM bulbospinal neuron marked by Lucifer yellow (→). Note the presence of microbeads in the cell body and dendrites. B: fluorescence photomicrograph of Texas red-stained tyrosine hydroxylase immunoreactivity (TH-IR) neurons. Recorded bulbospinal neuron (→) was TH-IR, indicating that it was a C1 neuron. An adjacent TH-IR neuron that was not recorded also was labeled by FITC-tagged microbeads (←). Scale bar, 20 \( \mu M \).](http://jn.physiology.org/)

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Y.-W. Li, P. G. Guyenet, and D. A. BayliSS

FIG. 2. High-voltage ± activated (HVA) calcium current. A: HVA calcium channel currents were recorded from a RVLM bulbospinal neuron during depolarizing steps (10-mV increments) from a holding potential of −70 mV. B: current-voltage (I-V) relationship for calcium channel currents recorded in the same cell in control and in the presence of 100 μM Cd²⁺. C: effects of calcium channel antagonists. Peak currents (evoked at 0 mV from a holding potential of −70 mV) were measured and plotted as a function of time. ω-conotoxin GVIA (CgTx, 2 μM), ω-agatoxin IVA (AgaTx, 0.2 μM), nimodipine (nimo, 10 μM) and Cd²⁺ (100 μM) were applied at the times indicated. D: I-V relationships for calcium channel currents recorded in a different bulbospinal neuron in response to bath application of AgaTx (0.2 μM) and CgTx (2 μM).

lished completely by 100 μM Cd²⁺. Figure 2D shows the effects of AgaTx and CgTx on the I-V relationship for HVA current in a different RVLM bulbospinal neuron. In this cell, AgaTx and CgTx inhibited 28 and 31%, respectively, of HVA current evoked at 0 mV from −70 mV. The current inhibition induced by these toxins was not associated with any apparent shift in the I-V relationship. In 10 bulbospinal neurons tested, the CgTx-sensitive N-type and the AgaTx-sensitive P/Q-type components accounted for 45 ± 4.4% and 33 ± 3.7% of HVA current, respectively. The L-type component contributed 10 ± 1.2% (n = 10), and a residual component resistant to all three antagonists represented 12 ± 2.1% of the total HVA current (n = 5).

LVA calcium current

To characterize LVA current in bulbospinal RVLM cells, we used the two-pulse voltage protocol shown in Fig. 3A in which currents were evoked at potentials between −50 and −30 mV after a 1-s prepulse to −100 mV and then from a holding potential of −60 mV. Currents evoked from −100 mV were of moderate amplitude (40–150 pA) and inactivated rapidly. By contrast, from a holding potential of −60 mV, essentially no current was evoked at −40 or −50 mV and only a small sustained current was evident at −30 mV. Digital subtraction of currents evoked from −60 mV from those evoked after the prepulse to −100 mV resulted in the prepulse-sensitive currents (Fig. 3A, inset). The amplitude and kinetics of the prepulse-sensitive currents were virtually identical to the transient components of currents evoked from −100 mV, suggesting that the transient components of the currents evoked at −50 to −30 mV from −100 mV were predominantly LVA current. The mean amplitude of LVA current evoked at −60, −50, and −40 mV from −100 mV in 12 RVLM bulbospinal neurons was 31 ± 6 pA, 51 ± 9 pA, and 70 ± 14 pA, respectively.

To study steady state inactivation of LVA current in relative isolation, currents were evoked at −50 mV after a 1-s prepulse that varied from −100 mV to −40 mV (Fig. 3B). The data points were fitted with a Boltzmann equation: \( y = \frac{1}{1 + \exp\left(\frac{(V - V_{1/2})}{k}\right)} \), where \( V \) is the prepulse potential, \( V_{1/2} \) is the half-inactivation potential, and \( k \) is a slope factor. Inactivation of the LVA current was steeply voltage dependent between −80 and −60 mV, with a half-inactivation potential about −70 mV and a slope factor of 6 mV. LVA currents were inactivated almost completely at potentials greater than or equal to −50 mV. In four RVLM bulbospinal cells tested, the half-inacti-
CALCIUM CURRENTS IN RVLM NEURONS; MODULATION BY NE

FIG. 4. Norepinephrine (NE) inhibits HVA current without affecting LVA current. A: NE (10 μM) inhibited HVA current (21%) but was without effect on LVA current in a bulbospinal neuron. LVA and HVA currents were evoked using the voltage protocol (top). B: averaged data (n = 10) showing that NE significantly inhibited HVA current but had no significant effect on LVA current. *P < 0.05; NS, P > 0.9.

The LVA calcium current we have described was recorded with Ba²⁺ as the charge carrier. The amplitude of LVA current depends on the permeating divalent cation in some neurons (Huguenard 1996). For example, with Ca²⁺ instead of Ba²⁺ as the charge carrier, LVA currents are larger in thalamic reticular neurons but not different in lateral habenula neurons (Huguenard et al. 1993). Because Ca²⁺ is the physiologically relevant ion, we decided to examine this issue in RVLM bulbospinal neurons. As illustrated in Fig. 3C, a transient LVA current with a peak amplitude of ~40 pA was evoked at −50 mV from −100 mV with external solution containing 2 mM Ba²⁺. After replacing Ba²⁺ with 2 mM Ca²⁺, the peak amplitude of the LVA current increased to ~120 pA with no apparent change in the kinetics (see Fig. 3C, inset). Similar results were seen in four additional neurons. On average, LVA current (measured at −50 mV from V₀ = −100 mV) in 2 mM Ca²⁺ was approximately twice the size of that in 2 mM Ba²⁺ (156 ± 38 pA vs. 78 ± 24 pA, P < 0.05).

We looked for evidence of LVA current in 52 RVLM bulbospinal neurons; 44 (85%) of these neurons had LVA currents (defined as >10 pA transient current evoked at −50 mV from a holding potential of −100 mV). Fourteen of these 44 neurons were recovered after histological processing and 12 were TH-IR. Therefore the data from these experiments indicate that most RVLM bulbospinal neurons, including C1 cells, express LVA calcium currents.
NE inhibits HVA, but not LVA, currents

We have demonstrated previously that RVLM bulbospinal cells, and particularly C1 neurons, express functional $\alpha_2$-ARs; activation of $\alpha_2$-ARs hyperpolarized RVLM neurons by increasing an inwardly rectifying $K^+$ conductance (Li et al. 1996). In a variety of central and peripheral neurons, $\alpha_2$-ARs also mediate inhibition of voltage-activated calcium currents (Chen and Schofield 1993; Ishibashi and Akaike 1995; Li and Bayliss 1998; Trombley 1992). Therefore we tested if the $\alpha_2$-ARs in RVLM bulbospinal neurons are also coupled to calcium channels.

We first determined the effects of NE on both HVA and LVA currents. NE was applied in the perfusate either alone or in the presence of prazosin and propranolol ($\alpha_1$- and $\beta$-AR antagonists, respectively) to eliminate potential effects mediated by those receptors. Because the effects of NE were the same with or without the antagonists present (see further for details on receptor pharmacology), the data were pooled for further analysis. A representative example of the effects of NE on HVA and LVA currents is shown in Fig. 4A. LVA and HVA currents were evoked using a double-pulse protocol (Fig. 4A, top). In the presence of 10 $\mu$M NE, LVA current was not noticeably affected, but HVA current (measured isochronally at the control peak current) was inhibited by 21% (Fig. 4A, bottom). We tested effects of NE on both HVA and LVA current in 10 bulbospinal neurons (including 4 TH-IR cells). As illustrated in Fig. 4B, NE (10 $\mu$M) inhibited 23 $\pm$ 2% of HVA current ($P < 0.01$) but had no significant effect on LVA current recorded in the same 10 neurons.

We tested effects of 10 $\mu$M NE on HVA current in 77 RVLM bulbospinal neurons; 70 (91%) were inhibited by NE (>5% inhibition) and the remaining 7 cells were not affected. Twenty-eight of the 77 neurons were recovered after histological processing and 24 were TH-IR. Of these 24 TH-IR neurons, 23 (96%) were inhibited by NE. The averaged inhibition of HVA currents in TH-IR neurons was 26 $\pm$ 1.6% ($n = 23$), which was not significantly different from those that were not recovered from histology (27 $\pm$ 1.3%, $n = 45$, $P > 0.1$). Of the four neurons that were not TH-IR, two were inhibited by 10 $\mu$M NE (28 and 34% inhibition, respectively). Data from these experiments indicate that NE inhibits HVA current in the majority of bulbospinal RVLM neurons, especially C1 cells; NE was without effect on LVA current in any bulbospinal RVLM neuron.

The inhibitory effect of NE on HVA current was concentration dependent. As shown in Fig. 5A, increasing concentrations of NE produced progressively greater inhibition of peak HVA current in a bulbospinal neuron. The averaged concentration-response of HVA currents to NE is illustrated in Fig. 5B. The data points (averaged from 4 to 6 cells) were fitted to a logistic equation of the form: $y = (a - c)/[1 + ((a/b)/EC_{50})^d] + c$, where $a$ and $c$ are the theoretical minimum and maximum, respectively, and $b$ is a slope function. The calculated EC$_{50}$ of the inhibition was 1.2 $\mu$M.

Calcium current inhibition by NE usually was associated with a slowing in the activation kinetics (e.g., see Fig. 5C, inset). The kinetic slowing induced by 10 $\mu$M NE was analyzed in a subset of neurons ($n = 42$) by comparing the percent inhibition at time points early (i.e., at the control peak current) and late in the voltage step; the inhibition was greatest early in the pulse in all but one cell and was 35 $\pm$ 5% greater at that time than when measured at the end of the voltage step. This measure of kinetic slowing was not correlated with either dose of NE or the percentage of current inhibition (not shown). As illustrated in the $I$-$V$ relationship of Fig. 5C, the inhibition by NE of HVA current was also
CALCIUM CURRENTS IN RVLM NEURONS; MODULATION BY NE

voltage dependent with the largest inhibition in the voltage range between −10 and 0 mV. To explore further the voltage dependence of NE-evoked inhibition, we used a standard double-pulse voltage protocol in which currents were evoked during test pulses to 0 mV from −70 mV, before and after a strong depolarizing prepulse to +70 mV. As seen in Fig. 5D (bottom), under control conditions, calcium current induced by the first and second test pulses were similar in amplitude. However, in the presence of NE, the current evoked during the first test pulse was reduced to a greater extent than during the second test pulse that followed the strong depolarization, and the ratio of those currents increased from 1.0 to 1.4. In six bulbospinal neurons tested, 10 μM NE increased the ratio from 1.1 ± 0.03 to 1.3 ± 0.05 (P < 0.01). These results suggest that the strong depolarizing steps partially relieved calcium current inhibition induced by NE.

α2-ARs mediate calcium current inhibition induced by NE

We performed experiments to determine the adrenergic receptor subtype that mediates calcium current inhibition by NE in bulbospinal RVLM neurons. First, we compared the effect of 10 μM NE in the absence and presence of prazosin and propranolol (α1- and β-AR antagonists, respectively). In the experiment shown in Fig. 6A, NE alone inhibited 34% of peak current; in the presence of prazosin and propranolol (1 μM for both), NE inhibited 39% of the peak currents. There was no significant difference in inhibition induced by NE alone (24 ± 2.1%, n = 18) or in the presence of prazosin and propranolol (27 ± 2.2%, n = 23, P > 0.2, Fig. 6D). Second, we examined the effect of UK 14,304, a selective α2-AR agonist, on calcium current. As exemplified in Fig. 6B, UK 14,304 (1 μM) inhibited 25% of the current in this neuron; on average, 1 μM UK 14,304 inhibited 24 ± 3% of calcium current (n = 6, Fig. 6D). Finally, we tested the effect of the α2-AR antagonist, idazoxan, on calcium current inhibition by NE. In the example shown in Fig. 6C, 10 μM NE inhibited 30% of peak current under control conditions; in the presence of idazoxan (1 μM), however, NE caused only 3% inhibition. After washout of idazoxan, the inhibition of calcium channel current by NE was recovered partially. Grouped data are presented in Fig. 6D. In the presence of 1 μM idazoxan, the NE-induced current inhibition was reduced significantly (from 29 ± 2.1% to 7 ± 1.8%, n = 6; P < 0.01). Results from these experiments indicate that inhibitory effect of NE on calcium current in RVLM bulbospinal neurons is mediated by α2-ARs.

NE inhibits N- and P/Q-types of HVA calcium current

We demonstrated above that RVLM bulbospinal neurons express N- and P/Q-type calcium currents. Therefore, we wished to determine the sensitivity of these two major components of calcium current to NE. As shown in the representative experiment of Fig. 7A, NE (10 μM) caused 37% inhibition of calcium channel current under control conditions. After blocking N-type channels with 1 μM CgTx, the NE-induced inhibition was reduced to
B. cells are shown in Fig. 7.

Therefore 75% of NE-sensitive current in this neuron was N-type and 21% was P/Q-type. Averaged data from six cells are shown in Fig. 7B. Of ~28% of HVA current that was sensitive to NE, 51 ± 9% was N-type and 41 ± 5% was P/Q-type; NE inhibited 36 ± 4% of N-type current compared with 30 ± 6% of P/Q-type. Very little NE-sensitive current remained after blocking N- and P/Q-type currents, suggesting that L-type and residual components do not contribute substantially to calcium current inhibition by NE.

Effect of NE on calcium currents evoked by action potential waveforms

We found that inhibition of HVA calcium current by NE is voltage and time dependent. This suggests that transmitter-induced modulation of calcium currents in RVLM cells may be enhanced when calcium currents are evoked by APWs instead of depolarizing pulses (Pennington et al. 1992; Toth and Miller 1995). Therefore, we used APWs recorded from RVLM bulbospinal neurons as a voltage command to evoke calcium currents and determined the effects of NE on these APW-induced currents. APW voltage commands were applied at a holding potential of −60 mV, and the peak of APWs reached +20 mV. Figure 8A shows a representative example from one of these experiments. The peak amplitude of leak-subtracted, calcium channel currents evoked by APWs was 1.1 nA. In the presence of 10 μM NE, the peak current was reduced to 0.5 nA (i.e., inhibited by 46%). By contrast, 10 μM NE inhibited only 30% of the peak current evoked by rectangular depolarizing pulses in the same neuron (Fig. 8A, inset). On average, NE inhibited 34 ± 2.5% of peak current evoked by APWs but only 23 ± 2.7% of the current evoked by rectangular depolarizing pulses in the same cells (n = 5; P < 0.05).

We demonstrated in an earlier section that strong depolarizations could relieve NE-induced inhibition of calcium channels. Therefore we wished to determine if a similar relief of inhibition might occur during depolarizations associated with repetitive firing. To test this, a train of eight APWs at 10 Hz was used as a voltage command to evoke calcium channel currents (Fig. 8B). Under control conditions, the peak currents evoked by the APWs were the same magnitude throughout the train (Fig. 8B, middle). During application of 10 μM NE, the current associated with the first APW in the train was inhibited by 44%. The relative inhibition of current associated with subsequent APW depolarizations was not reduced (and actually increased slightly; 47−51%). Similar results were obtained from four other RVLM bulbospinal neurons. Thus repetitive APW depolarizations delivered near the maximal steady state firing frequency of these neurons did not relieve the inhibition of calcium channels by NE.

FIG. 7. NE inhibits predominantly N- and P/Q-type calcium currents. A: peak calcium channel currents were plotted as a function of time. NE (10 μM), CgTx (2 μM), AgaTx (0.2 μM), and Cd2+ (100 μM) were applied at the times indicated (solid bars). NE inhibited ~57% of the peak current in control, ~9% after application of CgTx, and ~1.5% after application of AgaTx. Remaining current was abolished during application of Cd2+. B: averaged percentage of NE-sensitive current that was N-type (CgTx-sensitive) and P/Q-type (AgaTx-sensitive) is plotted (left) as is the averaged percentage of N-type (CgTx-sensitive) and P/Q-type (AgaTx-sensitive) current that was inhibited by NE (right). N- and P/Q-type channels contributed most of NE-sensitive current.

FIG. 8. NE-induced calcium current evoked by action potential waveforms. A: calcium channel currents were elicited by action potential waveforms (APWs) in control and in the presence of 10 μM NE and leak-subtracted using a P/4 protocol. APWs previously recorded from an RVLM bulbospinal neuron were applied as a voltage command from a holding potential of −60 mV. Inset: currents evoked by rectangular depolarizing steps from a holding potential of −60 mV in the same cell. NE (10 μM) inhibited 46% of peak current evoked by APWs but only 30% of the current evoked by depolarizing steps. B: NE-induced inhibition of currents evoked by a 10-Hz train of APWs (top). Current inhibition induced by NE (10 μM) was not relieved by the spike train.
The present study provides the first description of calcium currents in bulbospinal RVLM neurons, including C1 cells, and their modulation by NE. RVLM neurons express a relatively sustained HVA current, comprising prominent N- and P/Q-type components and smaller L-type and residual components. A transient LVA current, which began to activate at −60 mV with a half-inactivation potential of approximately −70 mV, was evident in the majority of bulbospinal RVLM cells. NE inhibited primarily N- and P/Q-type components of HVA current but had no effect on LVA current. NE-induced inhibition was mediated by α₂-ARs and was greater when calcium currents were evoked by APWs instead of rectangular depolarizing voltage steps.

Methodological considerations

Some methodological considerations are noteworthy. First, by necessity, brain stem slices were derived from neonatal rats. With current technology, targeting retrogradely labeled neurons under direct vision in the heavily reticulated RVLM region of adult animals is extremely difficult, if not impossible. So, although this is the first description of calcium channels in bulbospinal RVLM neurons, it is not certain that the complement of channels we have described and/or the mechanism of their modulation would be identical in these same neurons from adult rat.

Second, we recorded calcium currents from relatively intact neurons with extended processes in a slice preparation. Under these conditions, it is not known to what extent the membrane potential of distant processes is controlled, especially when large inward currents are evoked. Although it might be possible to mitigate such concerns by recording bulbospinal neurons in a dissociated neuronal preparation, the absence of anatomic landmarks would make it difficult to target specifically the small group of bulbospinal RVLM neurons, and it is likely that other populations of reticulospinal neurons would be sampled (e.g., bulbospinal serotonergic neurons). The approach employed here, based on both retrograde labeling and anatomic localization, assured a high probability of successfully recording RVLM neurons. Nevertheless, although we were careful to exclude data in which currents were clearly uncontrolled, the potential for less obvious space clamp errors with this approach cannot be discounted and caution should be exercised when interpreting the quantitative data presented.

Finally, we used an immunohistochemical method to identify C1 adrenergic neurons that was based on TH- rather than phenylethanolamine N-methyl transferase (PNMT)-immunoreactivity. This approach was chosen because our TH antibody consistently provides a more reliable and intense staining than is possible with PNMT antibodies currently available. Although this leaves open the possibility that noradrenergic (i.e., TH-IR but not PNMT-IR) as well as adrenergic (i.e., both TH- and PNMT-IR) neurons were immunostained, we consider that unlikely in this case. In double-labeling studies of the RVLM region, in which our recordings were made, nearly all TH-IR cells were also PNMT-IR (Tucker et al. 1987). Moreover, A1 noradrenergic neurons that overlap with the caudal pole of the C1 group do not project to the thoracic spinal cord (Westlund et al. 1983). Therefore, by targeting specifically bulbospinal neurons based on their uptake and retrograde transport of FITC-labeled microbeads injected into the thoracic spinal cord, we avoided those A1 noradrenergic cells. Thus it seems reasonable to conclude that most, if not all, bulbospinal RVLM neurons identified positively as TH-IR indeed represent C1 cells.

HVA calcium currents

HVA calcium currents recorded in bulbospinal RVLM neurons, including C1 cells, have properties similar to those found in many other neurons. HVA current activated with a threshold at approximately −30 to −40 mV and a peak amplitude of ~0.8 nA near 0 mV. HVA calcium current was separated into several components according to their sensitivity to calcium channel antagonists. Together, CgTx and AgaTx blocked ~80% of HVA current, indicating that N- and P/Q-type channels accounted for the majority of current. RVLM bulbospinal neurons also possessed a small amount of dihydropyridine-sensitive L-type calcium current and a small residual current. The residual HVA current may have included some LVA current that was not completely inactivated at the holding potential of −70 mV used in most experiments.

Multiple calcium channel types have been observed in other central monoaminergic neurons, although the relative contribution of each subtype appears to vary considerably. For example, dopaminergic neurons in the midbrain also express prominent N- and P/Q-type components but have a more substantial L-type calcium current (28%) (Cardozo and Bean 1995). Serotonergic neurons in the caudal raphe possess primarily N- and P/Q-type currents with essentially no L-type current component (Bayli ss et al. 1997b). Similarly, serotonergic dorsal raphe neurons have no L-type current and a large N-type current but, in contrast to other monoaminergic neurons, they have no P/Q-type current component and express a particularly large toxin-insensitive residual current (Penington and Fox 1995).

LVA calcium currents

The majority of RVLM bulbospinal neurons tested (44/52), including all 12 verified C1 neurons, expressed a transient calcium current that displayed kinetic properties similar to those of LVA calcium currents described in other neurons (Huguenard 1996). LVA calcium currents in RVLM cells began to activate at potentials around −60 mV and had rapid inactivation kinetics. Half-inactivation occurred at −70 mV, and the current was inactivated completely at potentials positive to −50 mV. Similar to results from other central neurons (Huguenard and Prince 1992; Takahashi et al. 1991), the replacement of Ba²⁺ with equimolar Ca²⁺ increased the peak amplitude of LVA currents in RVLM bulbospinal neurons (by ~100%). The pronounced increase in relative current amplitude at −50 mV with Ba²⁺ or Ca²⁺ probably represents differences in permeation of the divalent cations through T-type channels (Huguenard and Prince 1992); it cannot be explained primarily by charge screening effects because Ca²⁺ substitution would be expected to shift the activation threshold to more depolarized potentials, actually providing less current at the same test potential (Lorenzon and Foehring 1995).
In general, LVA currents are implicated in two main physiological functions. First, LVA current can underlie depolarizing afterpotentials that contribute to rhythmic activity of neurons (Burris and Aghajanian 1987; Llinas 1988). Second, LVA current can influence the overall excitability of neurons because it provides a depolarizing influence at sub-threshold membrane potentials (Llinas and Yarom 1981). LVA currents could play a role in the pacemaker activity of bulbospinal RVLM neurons. Although most LVA channels are inactivated at the resting membrane potential (−50 to −55 mV), the high-input resistance of RVLM neurons (−1 GΩ) would allow a small standing inward current to generate a substantial membrane depolarization (Kangraga and Loewy 1995; Li et al. 1996). Moreover, it is conceivable that the afterhyperpolarization (which reaches −70 mV) in RVLM bulbospinal neurons (Kangraga and Loewy 1995; Li et al. 1996) could partially remove inactivation of the LVA current, and, in concert with other active properties (e.g., TTX-sensitive persistent sodium conductance) (Kangraga and Loewy 1995), the LVA current could contribute to the ramp-like depolarization and regular pacemaker-like behavior in these neurons.

**NE inhibits HVA current by activation of α2-ARs**

We found that NE inhibited HVA current but had no apparent effect on LVA current. The selective inhibition of HVA calcium current by NE is in good agreement with observations in other neurons (Allen and Brown 1993; Bayliss et al. 1995; Ishibashi and Akaikie 1995; Viana and Hille 1996). The inhibitory effect of NE on HVA currents was concentration-dependent with an EC50 of 1.2 μM and ~25% inhibition at 10 μM NE. Current inhibition was mediated by α2-ARs because the effect was mimicked by the selective α2-AR agonist, UK 14,304, and blocked by the selective α2-AR antagonist, idazoxan, but not affected by prazosin or propranolol (α1- and β-AR antagonists, respectively). The observation that the majority of RVLM bulbospinal neurons (81%) and virtually all C1 neurons (92%) were inhibited by NE and α2-AR agonists is consistent with the results from our previous study, which suggested that most C1 adrenergic neurons express functional α2-ARs (Li et al. 1996).

We previously demonstrated that α2-ARs activate inwardly rectifying K+ channels in C1 adrenergic bulbospinal neurons, causing a membrane hyperpolarization (Li et al. 1996). Joint modulation of inwardly rectifying K+ channels and voltage-activated calcium channels by G protein-coupled receptors has been demonstrated in other CNS neurons. For example, dopamine (D2) receptors in midbrain dopaminergic neurons (Cardozo and Bean 1995), γ-aminobutyric acid-B receptors in hippocampal neurons (Swartz and Bean 1992) and serotonin-1A (5-HT1A) receptors in caudal raphe serotonergic neurons (Bayliss et al. 1997a,b) can modulate both inwardly rectifying K+ and voltage-activated calcium currents. However, there are examples where G-protein coupled receptors do not modulate both ion channel types. For example, in serotonergic caudal raphe neurons, α2-ARs couple only to voltage-activated calcium channels and not to inwardly rectifying K+ channels even though 5-HT1A receptors modulate both channel types in the same neurons (Bayliss et al. 1997a,b; Li and Bayliss 1998). Thus the differential coupling between α2-ARs and these distinct channel types suggests a functional diversity of signaling mechanisms mediated by α2-ARs in different neurons.

We found that NE inhibited both N- and P/Q-type calcium channels. The inhibition of multiple calcium channel types by NE has been observed in other central neurons. For example, NE inhibited N-, P/Q- and L-type calcium channels in neurons dissociated from the nucleus tractus solitarius (Ishibashi and Akaikie 1995), and it inhibited N- and P/Q-type channels in serotonergic caudal raphe neurons (Li and Bayliss 1997). By contrast, NE inhibited only N-type currents in adrenergic sympathetic postganglionic neurons (Chen and Schofield 1993).

**Effects of NE on calcium currents evoked by APWs**

Calcium currents and their neuromodulation traditionally have been studied by applying rectangular depolarizing pulses and recording the resultant currents. However, several groups recently have used APWs as a voltage command to more closely mimic the “physiological” situation (Brody et al. 1997; Penington et al. 1992; Toth and Miller 1995). For example, Penington et al. (1992) found in dorsal raphe neurons that the inhibitory effect of 5-HT on calcium current was greater when currents were evoked using APWs instead of depolarizing steps. Similarly, Toth and Miller (1995) showed that NE and neuropeptide Y produced a larger inhibition when calcium currents were evoked by APWs in sympathetic neurons rather than by depolarizing steps. In agreement with these reports, we found that a larger percentage of the calcium current was inhibited by NE when currents were evoked by APWs instead of depolarizing rectangular step commands in RVLM bulbospinal neurons. The enhanced inhibition of calcium current evoked by APWs is probably due to the kinetic slowing that usually accompanies NE-induced inhibition because the shorter duration of the APW limits the voltage- and time-dependent relief of current inhibition that can be seen even during a single voltage step (Penington et al. 1992).

Inhibition of calcium current by NE often was associated with a slowing of activation kinetics and was relieved partially after strong depolarizing prepulses (see Fig. 5D). These characteristics of calcium inhibition have been attributed to a transmitter-induced shift in the activation properties of calcium channels (from “willing” to “reluctant” modes) (see Bean 1989). This phenomenon may have physiological relevance if, for example, voltage-dependent relief of transmitter inhibition was to occur during a high-frequency burst of spikes. For this reason, we examined the inhibition by NE of calcium currents evoked by a train of APWs (10 Hz, 0.8-s duration) to determine if any relief occurred during the APW train. We chose a frequency of 10 spikes/s because it is close to the maximal spike frequency that neonatal C1 adrenergic neurons attained after application of neurotransmitters such as angiotensin II and substance P (Li and Guyenet 1996, 1997). We found that NE-evoked inhibition of calcium currents was not relieved during the course of the APW train. These results are in accord with the observations of Toth and Miller (1995) and Penington et al. (1991), who showed that neither trains of short rectangular pulses designed to mimic APWs nor a long train of APWs (even at frequencies as high as 75 Hz) changed the degree of transmitter-induced inhibition in dor-
sal raphe and sympathetic neurons. On the other hand, results from two recent papers suggest that relief of transmitter-induced inhibition can occur during simulated spike firing but that the degree of relief varies considerably depending on the characteristics of the discharge (i.e., firing rate and pattern, action potential duration) (Brody et al. 1997; Williams et al. 1997). However, the discharge rates that supported significant relief of transmitter-induced inhibition in those studies were outside the range of steady state firing frequency of bulbospinal RVLM neurons in vitro (Li et al. 1996).

Functional implications of calcium current inhibition by $\alpha_2$-ARs

The coupling of $\alpha_2$-ARs to inwardly rectifying K$^+$ channels may represent a mechanism by which C1 neurons, which synthesize and probably release catecholamines, can regulate their discharge rate—a so-called “autoinhibition” (Li et al. 1996). The inhibition of voltage-activated calcium currents by $\alpha_2$-ARs that we report here may represent an additional autoinhibitory mechanism. Ultrastructural studies demonstrate that PNMT-IR terminals form occasional synaptic contacts with PNMT-IR cell bodies in the RVLM, suggesting that C1 adrenergic neurons send collateral projections onto themselves or neighboring cells (Miller 1994). Electrochemical detection of catecholamine metabolites in vivo suggests that release of catecholamines in the RVLM correlates with the activity of C1 neurons and may originate from those cells (Mermet and Quintin 1991; Rentero et al. 1993). In addition, clonidine, an $\alpha_2$-AR agonist that is known to inhibit activity of C1 neurons, also inhibited release of catecholamines in RVLM. Although clonidine could inhibit spike-dependent catecholamine release from C1 neurons via $\alpha_2$-AR-mediated inhibition of firing activity, it also might act by inhibiting the calcium currents that support release. In addition, inhibition of calcium current by $\alpha_2$-ARs also could reduce transmitter release from bulbospinal RVLM cells in the spinal cord if $\alpha_2$-ARs, as well as N- and P/Q-type calcium channels, are present on terminals of C1 neurons.

Finally, calcium current inhibition mediated by $\alpha_2$-ARs could alter the firing behavior of the bulbospinal RVLM neurons. In serotonergic caudal raphe neurons and in somatic motoneurons, inhibition of calcium currents by 5-HT decreases the amplitude of the spike afterhyperpolarization and increases the firing frequency response to current inputs (Bayliss et al. 1995, 1997b). As suggested for 5-HT effects on serotonergic raphe neurons, if NE hyperpolarizes C1 neurons (via $\alpha_2$-AR-mediated activation of inwardly rectifying K$^+$ conductance) and simultaneously enhances spike firing responses to current inputs (via $\alpha_2$-AR-mediated inhibition of calcium current and afterhyperpolarization), the functional consequence would be to increase the signal-to-noise ratio of synaptic inputs (i.e., effects of strong, suprathreshold inputs would be enhanced by NE, whereas effects of weak inputs would be attenuated).

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Present address of Y.-W. Li: Bio-Control Systems Group, Dupont Experimental Station, E328/B29, Wilmington, DE 19880-0328.

Address for reprint requests: D. A. Bayliss, Dept. of Pharmacology, Box 448, 5017 Jordan Hall, University of Virginia, Charlottesville, VA 22908.

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