Prototypical Imidazoline-1 Receptor Ligand Moxonidine Activates Alpha2-Adrenoceptors in Bulbospinal Neurons of the RVL

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Hayar, Abdallah and Patrice G. Guyenet. Prototypical imidazoline-1 receptor ligand moxonidine activates alpha2-adrenoceptors in bulbospinal neurons of the RVL. *J. Neurophysiol.* 83: 766–776, 2000. Moxonidine is an antihypertensive drug that lowers sympathetic vasomotor tone by stimulating either alpha2-adrenergic (α2-AR) or imidazoline I1 receptors within the rostral ventrolateral medulla (RVL). In this study, we investigated the effects of moxonidine (10 μM) on RVL neurons in brain stem slices of neonatal rats. We recorded mainly from retrogradely labeled RVL bulbospinal neurons (putative presympathetic neurons) except for some extracellular recordings. Prazosin was used to block alpha1-adrenoceptors. Moxonidine inhibited the extracellularly recorded discharges of all spontaneously active RVL neurons tested (bulbospinal and unidentified). This effect was reversed or blocked by the selective α2-AR antagonist SKF 86466 (10 μM). In contrast, the I1 imidazoline ligand AGN 192403 (10 μM) had no effect on the spontaneous activity. In whole cell recordings (holding potential −70 mV), moxonidine produced a small and variable outward current (mean 7 pA). This current was observed in both tyrosine hydroxylase–immunoreactive and other bulbospinal neurons and was blocked by SKF 86466. Excitatory postsynaptic currents (EPSCs) evoked by focal electrical stimulation were isolated by incubation with gabazine and strychnine, and inhibitory postsynaptic currents (IPSCs) were isolated with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Moxonidine reduced the amplitude of the evoked EPSCs (EC_{50} = 1 μM; 53% inhibition at 10 μM) but not their decay time constant (5.6 ms). The effect of moxonidine on EPSCs persisted in barium (300 μM) and was reduced ~80% by SKF 86466. Moxonidine also reduced the amplitude of evoked IPSCs by 63%. In conclusion, moxonidine inhibits putative RVL presympathetic neurons both presynaptically and postsynaptically. All observed effects in the present study are consistent with an α2-AR agonist activity of moxonidine.

INTRODUCTION

Centrally acting antihypertensive drugs with an imidazoline structure, like clonidine and moxonidine, are effective in treating moderate to severe forms of hypertension (Armah et al. 1988; Prichard et al. 1997; Schafer et al. 1995; Ziegler et al. 1996). There is general agreement that a major site of action of these drugs is within the rostral ventrolateral medulla (RVL) (Punnen et al. 1987; reviewed by Reis 1996), but their specific neuronal targets are unknown. The RVL is critical for sympathetic tone generation and blood pressure control because it contains bulbospinal neurons (presympathetic neurons) that contribute the major excitatory drive to sympathetic vasoconstrictor and cardiac preganglionic neurons (McAllen et al. 1997; Sun 1995). The presympathetic neurons include a catecholaminergic cell group (C1 cells) that express the A subtype of α2-ARs (Guyenet et al. 1994). Activation of α2-ARs by norepinephrine (NE) in RVL bulbospinal neurons in vitro (C1 cells and others) activates a small barium-sensitive inwardly rectifying potassium current (Hayar and Guyenet 1999; Li et al. 1995) and inhibits high-voltage–activated calcium current (Li et al. 1998). However, in the RVL, most immunohistochemically identified α2A-ARs are heteroreceptors located on axon terminals (Milner et al. 1999), suggesting that the effects of α2-AR agonists on presympathetic neurons could largely derive from presynaptic mechanisms. This interpretation is also supported by electrophysiological recordings (Hayar and Guyenet 1999) that showed that NE produces a combination of pre- and postsynaptic inhibition of the RVL presympathetic neurons by stimulating α2-ARs. The first objective of the present study was therefore to determine how closely the action of moxonidine mimics the various pre- and postsynaptic effects of NE previously attributed to α2-AR stimulation in the RVL in vitro.

The molecular mechanism of action of moxonidine and other imidazoline derivatives in RVL is controversial. Specifically, it is unclear whether the sympathoinhibition evoked by moxonidine is mediated via α2-ARs (reviewed by Guyenet 1997) or via I1-imidazoline receptors (Ernsberger et al. 1990; reviewed by Ernsberger and Haxhiu 1997). Accordingly, the second objective of the present study was to determine whether some of the effects of moxonidine in RVL can be attributed to a mechanism other than the activation of α2-ARs. To test this hypothesis, we determined whether moxonidine exerts residual effects after blockade of α2-ARs by the selective agonist SKF 86466 (Ernsberger et al. 1990; Hieble et al. 1986) and whether the selective I1-imidazoline ligand AGN 192403 has any effect.

Whole cell and extracellular recordings were performed in thin coronal slices from neonate rat brain [*postnatal day 4–21* (*P4–P21*)] cut at the level of the RVL (Hayar and Guyenet 1999). Bulbospinal RVL neurons were prelabeled with a retrograde marker injected into the thoracic spinal cord a few days before recording (Li et al. 1995). This method allowed us to restrict our recordings to a population of RVL neurons that consists predominantly of presympathetic neurons (Li et al. 1995) and is therefore of special relevance to understanding the central sympatholytic effects of moxonidine. In some experiments, we further characterized whether the recorded neurons could be classified as C1 neurons using immunostaining for tyrosine hydroxylase (Kangrga and Loewy 1995; Li et al. 1995).

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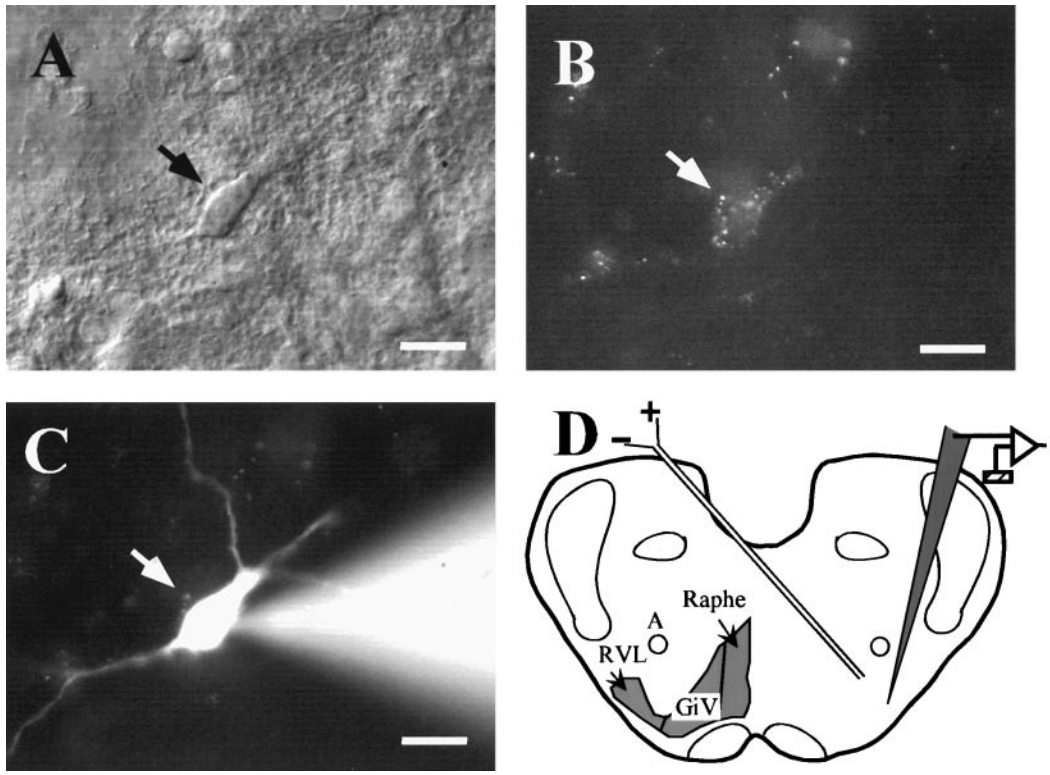

FIG. 1. Identification of rostral ventrolateral medulla (RVL) and RVL bulbospinal neurons. *A*: photomicrograph of an RVL neuron visualized using Infrared Differential Interference Contrast optics. *B*: the neuron was identified as bulbospinal because of the presence of FITC-tagged microbeads that were retrogradely transported from the thoracic spinal cord. *C*: the neuron was filled with Lucifer yellow during whole cell recording. *D*: distribution of retrogradely labeled neurons (gray area) in a typical coronal section cut at the level of the rostral medulla immediately caudal to the facial motor nucleus. The positions of the recording pipette and the stimulating electrode are also shown. Retrograde labeling was symmetrical but is only shown on left side for clarity. Recordings were made exclusively from the lateral island of retrogradely labeled neurons identified as RVL in the figure. GiV, gigantocellular reticular nucleus pars ventralis; A, ambiguus nucleus. Scale bar in *A–C* is 20 μm.

METHODS

Slice preparation

A previously described method was used with minor modifications (Hayar and Guyenet 1999; Li et al. 1995). Briefly, Sprague-Dawley rat pups (2–3 days old) were anesthetized by hypothermia and a suspension of fluorescein isothiocyanate (FITC)-tagged microbeads (0.3–0.5 μl; Lumafluor) was injected bilaterally into the upper thoracic spinal cord to retrogradely label bulbospinal RVL neurons. One to 18 days later, the rats (4–21 days old) were deeply anesthetized either by hypothermia (<7 days old) or by halothane (>7 days old). The rats were decapitated, and their brain stems were blocked and immersed in sucrose-artificial cerebrospinal fluid (sucrose-ACSF) equilibrated with 95% O_2-5% CO_2 (pH 7.38). The sucrose-ACSF had the following composition (in mM): 26 $NaHCO_3$, 1 NaH_2PO_4, 3 KCl, 5 $MgSO_4$, 0.5 $CaCl_2$, 10 glucose, and 248 sucrose.

Coronal slices (200 μm thick) were cut with a Microslicer (Ted Pella, Redding, CA). The slices were then incubated until used at room temperature (22°C) in lactic acid-ACSF equilibrated with 95% O_2-5% CO_2 (composition in mM: 124 NaCl, 26 $NaHCO_3$, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 10 glucose, and 4.5 lactic acid). For recording, a single slice containing the RVL was placed in a recording chamber on an upright epifluorescent microscope (Olympus BH-2). The slices (1 or at most 2 per brain) were selected by examination under a ×10 objective by a set of anatomic characteristics (no facial nucleus visible, inferior olive very small) and a characteristic pattern of retrograde labeling that is found neither rostral nor caudal to that level (Li et al. 1995) (Fig. 1*D*). In this chamber, the slice was continuously superfused at the rate of 1.5 ml/min with normal ACSF equilibrated with 95% O_2-5% CO_2. Recordings were made at 30–31°C, except for a subset of experiments in which we investigated concentration-dependent response of moxonidine at room temperature to minimize rundown of evoked excitatory postsynaptic currents (EPSCs). To prevent precipitation of salts, the ACSF was altered in experiments using barium by eliminating phosphate and sulfate ions (2 mM $MgSO_4$ was replaced with 2 mM $MgCl_2$, and 1.25 mM NaH_2PO_4 was replaced with 1.25 mM NaCl). To eliminate possible interaction with alpha1-adrenoceptors (α1-ARs), moxonidine was always applied in the presence of prazosin (1 μM; see DISCUSSION).

Electrophysiological recordings

Neurons containing microbeads were identified under epifluorescence illumination and viewed with a water-immersion ×40 objective using a closed-circuit television camera (Fig. 1, *A* and *B*). Extracellular recordings from RVL bulbospinal neurons were done under direct visualization by placing the pipette adjacent to a neuron containing fluorescent microbeads. Extracellular recordings from unidentified RVL neurons were done blindly; however, the pipette was directed toward the region with the highest density of neurons containing microbeads within the anatomic boundaries of the RVL (Fig. 1*D*). These extracellular recordings were made with patch electrodes (same as those used for whole cell recordings) in the voltage-clamp track mode of the Axopatch-200B amplifier. Once extracellular electrical events were detected, a small negative pressure was applied to the pipette to clear the neuropil and improve access to the cell. Using this procedure, we were able to improve the signal-to-noise ratio and record action currents (amplitude of 75–200 pA with a noise signal of

~5 pA) for up to 2 h without causing a significant change in the baseline discharge frequency of the cells. To ascertain that some of the extracellular recordings were indeed made from RVL bulbospinal neurons, we applied further suction to the pipette and then stimulated the cell with high-intensity oscillatory current while applying continuous negative current. We could then verify by direct microscopic observation whether the cells filled with Lucifer yellow contained microbeads.

Patch pipettes were pulled from borosilicate glass capillaries with an inner filament (1.5 mm OD, Clark, UK) on a pipette puller (Sutter P87) and were filled with a solution of the following composition (in mM): 114 K-gluconate, 17.5 KCl, 4 NaCl, 4 $MgCl_2$, 10 HEPES, 0.2 EGTA, 3 Mg_2ATP, 0.3 Na_2GTP, and 0.02% Lucifer yellow (Molecular Probes, Eugene, OR). Osmolarity was adjusted to 270 mOsm and pH to 7.3. The pipette tips were coated with silicone elastomer (Sylgard), and their resistance was 4–7 MΩ. Whole cell voltage-clamp recordings were made with the Axopatch-200B amplifier as described before (Hayar and Guyenet 1999). Liquid junction potential was 9–10 mV and all reported voltage measurements have been corrected for these potentials. Only neurons that had access resistance of <30 MΩ were included in this study. No series resistance compensation was performed.

Electrical stimulation was performed using two tungsten wires, teflon coated except at their tips (50 μm diam, A-M Systems, Everett, WA). They were positioned 100 μm apart and were placed on the surface of the slice ~250 μm dorsomedial to the recorded neurons (Fig. 1*D*). The stimulation voltage was set at the minimum necessary to induce a maximal evoked postsynaptic current (potential: 30–50 V; duration: 100–200 μs; frequency: 0.2–0.5 Hz).

Tyrosine-hydroxylase (TH) immunostaining

After recording, images of the recorded neurons (labeled with Lucifer yellow) were digitized using a video card (Snappy video snapshot, Play, Rancho Cordova, CA) and were stored using JPEG format. This procedure was useful to confirm the identity of the recorded neurons after histological processing, in particular when recordings were performed from more than one neuron in a given slice. The slices were fixed for 24–48 h in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and stored in a cryoprotectant solution (Watson et al. 1986) at −20°C for 2–4 wk. Immunostaining for TH was done using an avidin-biotin based reaction (mouse anti-TH monoclonal antiserum; 1:750; Chemicon, Temecula, CA), biotinylated goat anti-mouse antiserum (1:150; Vector Laboratories, Burlingame, CA), and streptavidin-conjugated Cy-3 (1:1000; Jackson Immunoresearch Laboratories, Westgrove, PA). The neurons that displayed detectable TH immunoreactivity were considered catecholaminergic. They were assumed to be C1 adrenergic cells because in double-labeling studies of the RVL region, nearly all bulbospinal TH-immunoreactive cells were also phenylethanolamine *N*-methyl transferase (PNMT)-immunoreactive (Tucker et al. 1987). We preferred to use TH rather than PNMT-immunostaining to identify C1 cells because our TH antibody provided a more reliable and intense staining than was possible with PNMT antibodies available at the time of the study.

Reagents

Drugs and solutions of different ionic content were applied to the slice by switching the perfusion with a three-way electronic valve system. Strychnine HCl, norepinephrine bitartrate, prazosin, and 2-methoxy-idazoxan were obtained from Sigma (St. Louis, MO). Gabazine (SR95531), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), tetrodotoxin (TTX), (2-endo, 3-exo)-3-(methylethyl)-bicyclo[2.2.1]heptan-2-amine hydrochloride (AGN 192403 hydrochloride), and (±)-baclofen were obtained from Research Biochemicals International (Natick, MA). 6-Chloro-*N*-methyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF 86466) was offered by Smith Kline Beecham Pharmaceuticals (Philadelphia, PA).

Data analysis and statistics

During the experiment, analogue signals were low-pass filtered at 2 kHz (Axopatch 200B), digitized at 48 kHz (Vetter, A. R. Vetter, Rebersburg, PA), and stored on a videotape for later analysis. They were also collected through a Digidata-1200A Interface and were digitized at 10–20 kHz. The peak detection of events [postsynaptic currents (PSCs) evoked by focal electrical stimulation and action currents] was performed using the new features available in the Windows version of pClamp7 software (Axon Instruments, Foster City, CA). In extracellular recordings, events were detected on-line by triggering sweeps on the analogue input signal and by setting an appropriate threshold for detection of action currents. The time of occurrence of action currents along with their amplitude and half-width were stored in ASCII files. The data were then imported into Origin 4.1 program (Microcal Software, Northampton, MA) and analyzed using a binning algorithm. The number of action currents was binned at 1 s and two moving averages (10 and 60 points) of the firing frequency were generated (Fig. 2, *A* and *D*). The amplitude of the action currents was inspected for a sudden change that might have been caused by movement of the electrode away or closer to the recorded cell and the data were discarded if such a change had affected the baseline frequency.

In whole cell recordings, peak detection and measurement of the evoked PSC amplitude along with its area and its time-to-peak were also done on-line (pClamp7) and stored in ASCII files. A moving average (10–30 points) of the data was then generated (Origin). The results were discarded if they revealed a rapid rundown in the amplitude of the evoked PSC (>30% per 10 min) or a sudden change in the time-to-peak of the PSCs, which was found to indicate a change in the electrode access resistance. To determine the drug effect on the evoked PSCs, we averaged 8–20 consecutive evoked PSCs 1 min before and 4 min after drug application. The decay time constant of the evoked PSCs was determined by fitting a single exponential from peak to baseline on averaged traces of the evoked PSCs (Origin).

Data are expressed as means ± SE. A one-way repeated measure ANOVA was performed to determine the effects of sequential drug applications on the same neuron (Sigmastat, v. 1.03, Jandel Scientific). In case of significant ANOVA F value, pair-wise multiple comparisons were done using the Student-Newman-Keuls method. Comparisons involving only two groups were made with the paired or unpaired t-test as appropriate (Origin or Sigmastat). In all cases, significance was accepted if $P < 0.05$.

RESULTS

Extracellular recordings were obtained from bulbospinal and unidentified RVL neurons that were spontaneously active. All whole cell recordings were made from RVL bulbospinal neurons identified in the slice by the presence of FITC microbeads in their soma (Fig. 1, *A* and *B*). The membrane properties of RVL bulbospinal neurons have been studied in detail elsewhere (Hayar and Guyenet 1998; Kangrga and Loewy 1995; Li et al. 1995). The recorded neurons were labeled with Lucifer yellow (Fig. 1*C*) for phenotypic identification after immunocytochemical processing for TH immunoreactivity.

Effect of moxonidine on the spontaneous discharges of RVL neurons recorded extracellularly

Moxonidine (10 μM) was tested on spontaneously active RVL neurons using extracellular recordings. Using this method, we avoided altering the ionic gradients or dialyzing

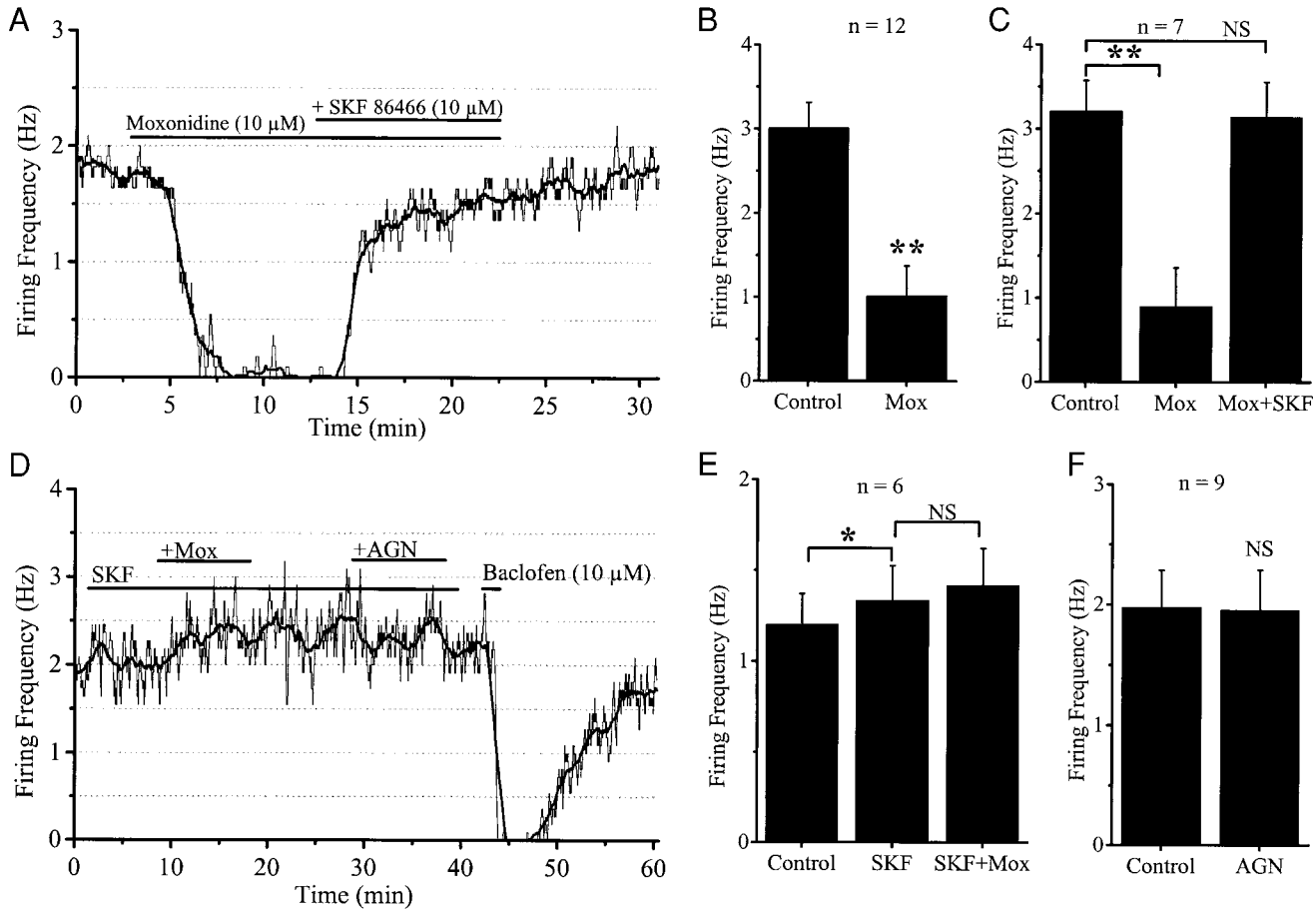

FIG. 2. Inhibitory effect of moxonidine on the discharge rate of RVL neurons and antagonism by SKF 86466 (SKF). All neurons were recorded extracellularly. *A*: moxonidine (Mox) inhibited the firing of an RVL bulbospinal neuron, and this effect was completely reversed by addition of the α2-AR antagonist SKF 86466. *B*: average effect of moxonidine in 12 neurons (8 unidentified and 4 bulbospinal, $^{**}P < 0.0001$, paired *t*-test). *C*: reversal of the inhibitory effect of moxonidine by SKF in 7 neurons (Protocol as in *A*; $^{**}P < 0.0001$; NS, nonsignificant; ANOVA). *D*: SKF 86466 alone did not change the frequency of this unidentified RVL neuron, but it prevented the inhibitory effect of moxonidine. The I1-imidazoline ligand AGN 192403 (10 μM) had no effect, whereas baclofen completely inhibited the cell. *E*: group data: moxonidine produced no significant change in firing frequency (n = 6) in the presence of SKF 86466, which itself produced a small effect ($^{*}P < 0.05$; NS, nonsignificant; Student-Newman-Keuls method). *F*: group data: AGN 192403 had no significant effect in 9 cells tested (NS, nonsignificant; paired *t*-test). In *A* and *D*, thin lines and thick lines represent moving averages (10 and 60 points, respectively) of the number of action currents originally binned at 1 s. Prazosin (1 μM) was present in the bath in all experiments.

intracellular messengers of the recorded cells, which can occur with invasive techniques like patch and intracellular recordings. Moreover, any potential effects of the drug on voltage-dependent conductances that are activated during action potential generation would likely be revealed by a change in the firing frequency. All 12 neurons exposed to moxonidine (8 unidentified and 4 bulbospinal neurons) were inhibited, either partially (n = 6) or completely (n = 6, Fig. 2*A*). Overall, moxonidine reduced the firing frequency of these cells from 3.0 ± 0.3 Hz to 1.0 ± 0.3 Hz (mean ± SE, n = 12, $P <$ 0.0001, paired *t*-test, Fig. 2*B*). There was no correlation between the control firing frequency of the neurons and the decrease in the firing frequency induced by moxonidine (R = 0.14, P = 0.66). The effect of moxonidine had a notably slow onset and required up to 5 min to reach a plateau (Fig. 2*A*).

To compare the kinetics of the response to moxonidine and NE we bath applied a saturating concentration of NE (30 μM) to 18 other neurons (11 unidentified and 7 bulbospinal). Application of NE caused a reduction in the firing frequency of these cells from 2.1 ± 0.3 Hz to 0.5 ± 0.3 Hz ($P <$ 0.0001, paired *t*-test, not illustrated). In all cases, the effect of NE reached its maximum in <2 min. The half-maximum response time, defined as the delay from the start of the application to half-maximal inhibition, was 53 ± 4 s for NE (n = 18) and 171 ± 13 s for moxonidine (n = 12; $P <$ 0.0001, unpaired *t*-test).

The inhibitory effect of moxonidine persisted with no apparent desensitization during prolonged application in all cells tested (up to 10 min, n = 12). Addition of the selective α2-AR antagonist SKF 86466 (10 μM) to the bath completely reversed the inhibition produced by moxonidine in all cells tested (n = 7; 3.2 ± 0.4 Hz in control, 0.9 ± 0.5 Hz in moxonidine, and 3.1 ± 0.4 Hz in moxonidine + SKF 86466; Fig. 2, *A* and *C*). In six other cells, SKF 86466 applied alone (10 μM for 10 min) produced a small change in firing frequency (1.2 ± 0.2 Hz in control and 1.3 ± 0.2 Hz in SKF 86466, n = 6, $P <$ 0.05, ANOVA) indicating that α2-ARs were minimally or not activated under our recording conditions. The subsequent applica-

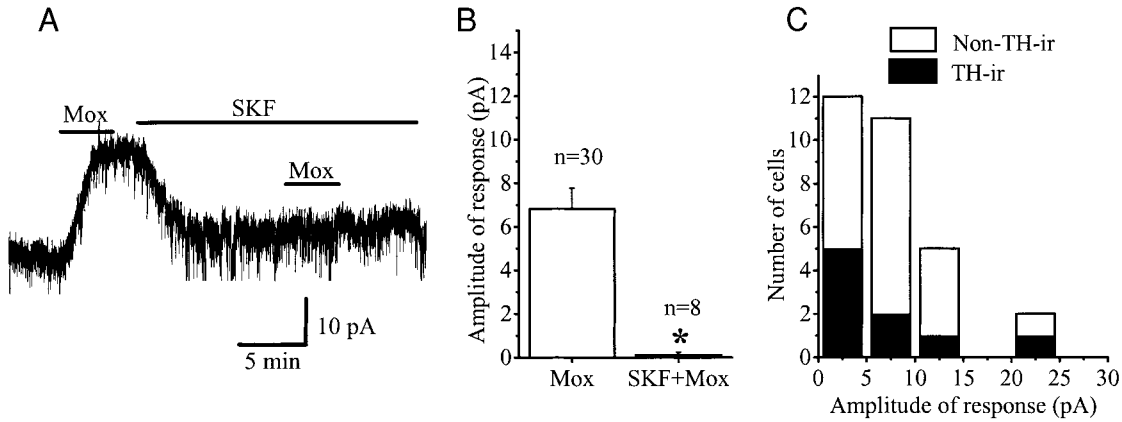

FIG. 3. Postsynaptic effect of moxonidine in RVL neurons. *A*: typical moxonidine (Mox) response in an RVL bulbospinal neuron voltage clamped at −70 mV. The specific α2-AR antagonist SKF 86466 (SKF) blocked the effect of a subsequent application of moxonidine. *B*: mean amplitude of current produced by moxonidine in control and in the presence of SKF 86466 (*$P < 0.001$, paired *t*-test). *C*: distribution histogram of the amplitude of the response to moxonidine in all RVL bulbospinal neurons tested ($n = 30$) including the cells identified as TH-immunoreactive (TH-ir).

tion of moxonidine (10 min, 1st exposure to the slice) in the continued presence of SKF 86466, produced no effect on the firing frequency in any of the six cells tested (1.3 ± 0.2 Hz in SKF 86466, 1.4 ± 0.2 Hz in SKF 86466 plus moxonidine; Fig. 2, *D* and *E*). Four of these six unresponsive cells were later exposed to the $GABA_B$ agonist baclofen (10 μM, 2 min), which inhibited their discharge completely (Fig. 2*D*).

Finally, we also tested the effects of the I1-imidazoline ligand AGN 192403 (Munk et al. 1996) in nine RVL neurons. AGN 192403 (10 μM, 10-min bath application) did not change the firing frequency of any of the nine neurons tested (control: 2.0 ± 0.3 Hz; AGN 192403: 2.0 ± 0.3 Hz, $P = 0.6$, paired *t*-test, Fig. 2, *D* and *F*). In five cases, AGN 192403 was applied in the presence of 1 μM prazosin plus 10 μM SKF 86466, and in the remaining five cases AGN 192403 was applied in the absence of the antagonists. Therefore the results so far have not provided any evidence for the presence of functional imidazoline receptors in our preparation.

Effect of moxonidine on the holding current in RVL bulbospinal neurons

We examined the effect of bath applications of moxonidine (10 μM) on the holding current in 30 RVL bulbospinal neurons clamped at a holding potential of −70 mV (Fig. 3*A*). Moxonidine produced a modest outward current (6.8 ± 0.9 pA, range 0–23, $n = 30$, Fig. 3*B*). Application of a higher concentration (30 μM) produced no additional effect ($n = 2$) and the concentration of 10 μM was considered to be saturating. The magnitude of the current produced by moxonidine was too small to produce a concentration-response curve. Eight of the 30 neurons that responded with >5 pA of outward current to moxonidine (mean 12 ± 2.4 pA) were exposed to the specific α2-AR antagonist SKF 86466 (10 μM) for 8–12 min. Then, still in the presence of the antagonist, the cells were reexposed to a second application of moxonidine (Fig. 3*A*). The second exposure to moxonidine produced no detectable outward current in any of the eight cells tested (0.1 ± 0.1 pA, $P < 0.001$, paired *t*-test, Fig. 3, *A* and *B*). These results suggest that the outward current produced by the moxonidine resulted from the activation of α2-ARs.

The 30 neurons exposed to moxonidine in this series of experiments were processed for immunocytochemical detection of the catecholamine synthesizing enzyme tyrosine hydroxylase (TH). Immunoreactivity for TH could be detected in 9 out of 30 cells. The response of TH-immunoreactive neurons to moxonidine was as variable as that of the general population of bulbospinal neurons (Fig. 3*C*). However, some of the non–TH-immunoreactive neurons may be false negatives because we found that prolonged intracellular dialysis with whole cell recordings reduces the detectability of TH immunoreactivity (Hayar and Guyenet 1998).

Effect of moxonidine on EPSCs and inhibitory postsynaptic currents (IPSCs) evoked by focal stimulation in RVL bulbospinal neurons

The following experiments were designed to determine the effect of moxonidine (10 μM, except where indicated) on the EPSCs and IPSCs evoked in RVL bulbospinal neurons by focal stimulation. A second objective was to assess whether this effect was sensitive to the α2-AR blocker SKF 86466. The stimulation electrode was positioned ~250 μm dorsomedial to the recorded neurons (see Fig. 1*D*).

Evoked EPSCs were isolated in the combined presence of the glycine receptor antagonist strychnine (10 μM) and the $GABA_A$ receptor antagonist gabazine (SR 95531, 3 μM) (Mienville and Vicini 1987). We showed previously that the remaining EPSCs are sensitive to 10 μM CNQX and thus are presumably due to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors (Hayar and Guyenet 1998, 1999). Individual EPSCs displayed their customary variability and a modest degree of time-dependent rundown. This rundown is illustrated in Fig. 4 by a dotted line, henceforth called the rundown line. Figure 4 also illustrates the moving average method used to determine the mean effect of the drug on EPSC amplitude. The percent inhibition of the EPSC was calculated at the end of the drug application by dividing the magnitude of the EPSC (from the moving average curve) by the theoretical control value (from the rundown line at the same time point). Moxonidine reduced the amplitude of the evoked EPSCs in all cells tested irrespective of the mag-

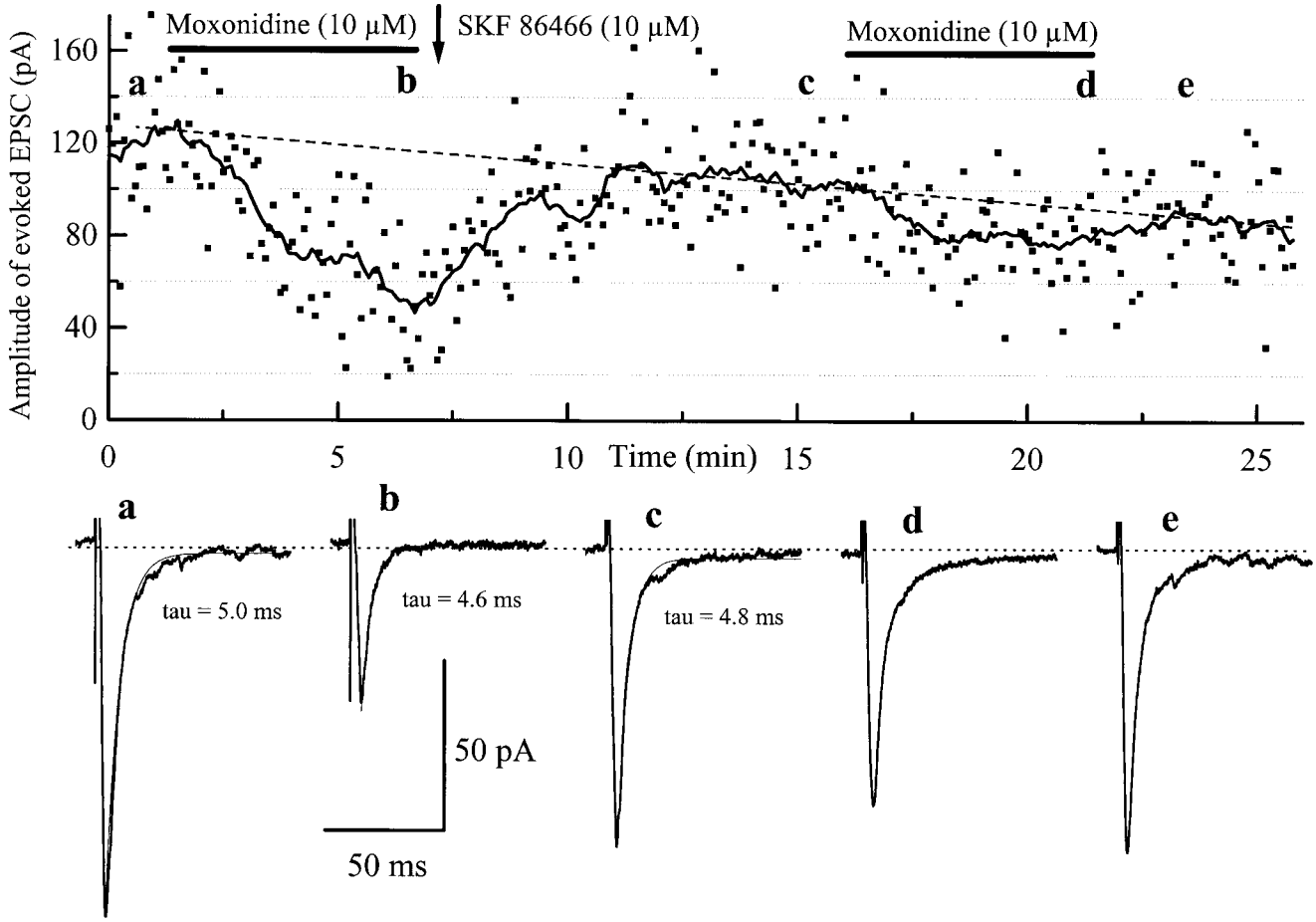

FIG. 4. Inhibition by moxonidine of the evoked excitatory postsynaptic currents (EPSCs) in a bulbospinal RVL neuron and antagonism by SKF 86466. *Top*: amplitude of individual EPSCs (■, stimulation at intervals of 3 s) and moving average (line) of 12 consecutive points. Moxonidine decreased the amplitude of the evoked EPSCs. Application of the α2-AR antagonist SKF 86466 accelerated recovery from moxonidine on wash and attenuated the reduction in the amplitude of the evoked EPSCs caused by a 2nd application of moxonidine. Dashed line is an extrapolation of the baseline amplitude of evoked EPSCs, which decreased during the experiment due to a time-dependent rundown of evoked EPSCs. *Bottom traces* are representative averages of 12 consecutive evoked EPSCs. Letters (*a*–*e*) refer to the sampling periods shown in the *top plot*. The decay time constant (tau) for the traces (*a*–*c*) was calculated after fitting each trace with a single exponential decay function (thin lines). Note that in this neuron, moxonidine did not induce a detectable change in the holding current ($\cdots$).

nitude of the presumed postsynaptic response (outward current) by a mean of 57 ± 4.6% (66 ± 10 pA in control and 28 ± 6 pA in moxonidine, $n = 15$, $P < 0.0001$, paired *t*-test, Fig. 5*A*). Moxonidine did not change the decay time constant of the evoked EPSCs (5.6 ± 0.4 ms in control, 5.6 ± 0.5 ms in moxonidine, $n = 15$, $P = 0.9$, paired *t*-test; Fig. 4).

The effect of moxonidine on the amplitude of the evoked EPSCs was greatly attenuated by SKF 86466 (10 μM). In five cells, the first application of moxonidine produced a reduction in the amplitude of the evoked EPSCs (43 ± 7%). A second application of moxonidine after incubation with SKF 86466 for 10–15 min, reduced the amplitude of the evoked EPSC by only 9 ± 1.8%, an 80% attenuation of the inhibitory effect of moxonidine (Fig. 4). To exclude the possibility that a second application of moxonidine could have produced a smaller response because of receptor desensitization or EPSC rundown, five slices were preincubated with 10 μM SKF 86466 for 5 min and the cells ($n = 5$) were exposed only once to moxonidine. In the continued presence of this antagonist, the first application of moxonidine to the slice produced only a small reduction in the amplitude of evoked EPSCs (9 ± 1.4%, from 82 ± 16 pA to 75 ± 17 pA, $n = 5$, $P < 0.05$, ANOVA; Fig. 5*A*). SKF 86466 alone did not change the amplitude of evoked EPSCs (Fig. 5*A*). On average, SKF 86466 attenuated the inhibitory effect of a first application of moxonidine by 84% ($P < 0.0001$, unpaired *t*-test, Fig. 5*B*).

Additional experiments were done in the presence of barium (300 μM). Barium eliminates the outward current evoked by NE in RVL bulbospinal neurons due to its ability to block the inwardly rectifying potassium current triggered by activation of α2-ARs (Hayar and Guyenet 1999). The reduction in the amplitude of evoked EPSCs by moxonidine persisted in the presence of barium (69 ± 7% reduction, $n = 3$; results not illustrated). Moxonidine did not produce outward current in any of these cells. Thus the reduction in excitatory synaptic transmission by moxonidine is not mediated by the opening of a presynaptic barium-sensitive potassium current.

The inhibition of the EPSC by moxonidine was a sufficiently reliable and robust response to allow examination of its concentration dependence ($n = 4$). The effect of moxonidine was

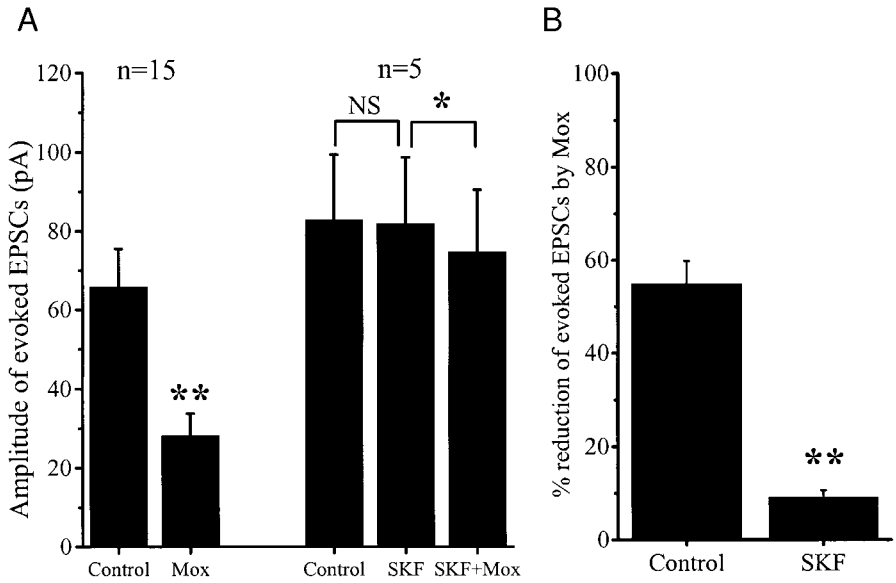

FIG. 5. Antagonism of the presynaptic effect of moxonidine (10 μM) by SKF 86466 (10 μM). *A*: *left 2 columns* show the effect of a 1st application of moxonidine on the amplitude of evoked EPSCs (**$P < 0.0001$, paired *t*-test). *Right 3 columns* show the effect of SKF 86466 and that of moxonidine in the continued presence of SKF 86466. SKF 86466 by itself produced no effect. Additional application of moxonidine produced only a small although statistically significant reduction in the amplitude of evoked EPSCs (NS, nonsignificant; *$P < 0.05$, ANOVA). *B*: replot of the data shown in *A* indicates that SKF 86466 significantly attenuated the inhibitory effect of moxonidine on the evoked EPSCs (**$P < 0.0001$, unpaired *t*-test). Control, effect of moxonidine alone (from *A, left 2 columns*); SKF, effect of moxonidine in the presence of SKF 86466 (from *A, right 2 columns*). Statistical comparisons were made with respect to control except where indicated.

clearly concentration-dependent with a threshold of 100 nM, saturation of 10 μM, and an EC_{50} of 1 μM (Fig. 6).

Evoked IPSCs were isolated in the presence of the non–*N*-methyl-D-aspartate (non-NMDA) receptor antagonist CNQX (10 μM). At the holding potential of −70 mV, the IPSCs were recorded as inward currents because the calculated equilibrium potential for the chloride ions was −38 mV in our recording conditions. Under our experimental conditions a relatively small fraction of RVL bulbospinal neurons (~15%) had a predominant inhibitory component in their evoked monosynaptic postsynaptic current (Hayar and Guyenet 1998; 1999). Therefore we were able to test the effect of moxonidine on IPSCs only in three neurons. IPSC amplitude varied greatly and their average magnitude was determined using a moving average as illustrated in Fig. 7. Also, a small rundown of the amplitude of the evoked IPSC occurred during the course of the experiment (dotted line in Fig. 7). When calculating the percent inhibition of the EPSC by moxonidine, this rundown was taken into consideration in the manner described above for the EPSC. In each cell tested, moxonidine (10 μM) produced a large decrease in the amplitude of the evoked IPSC (60 ± 16%, Fig. 7). In two of these three cells, moxonidine was reapplied after 12 min exposure of the slice to 10 μM SKF 86466 and in the continued presence of this antagonist. In both

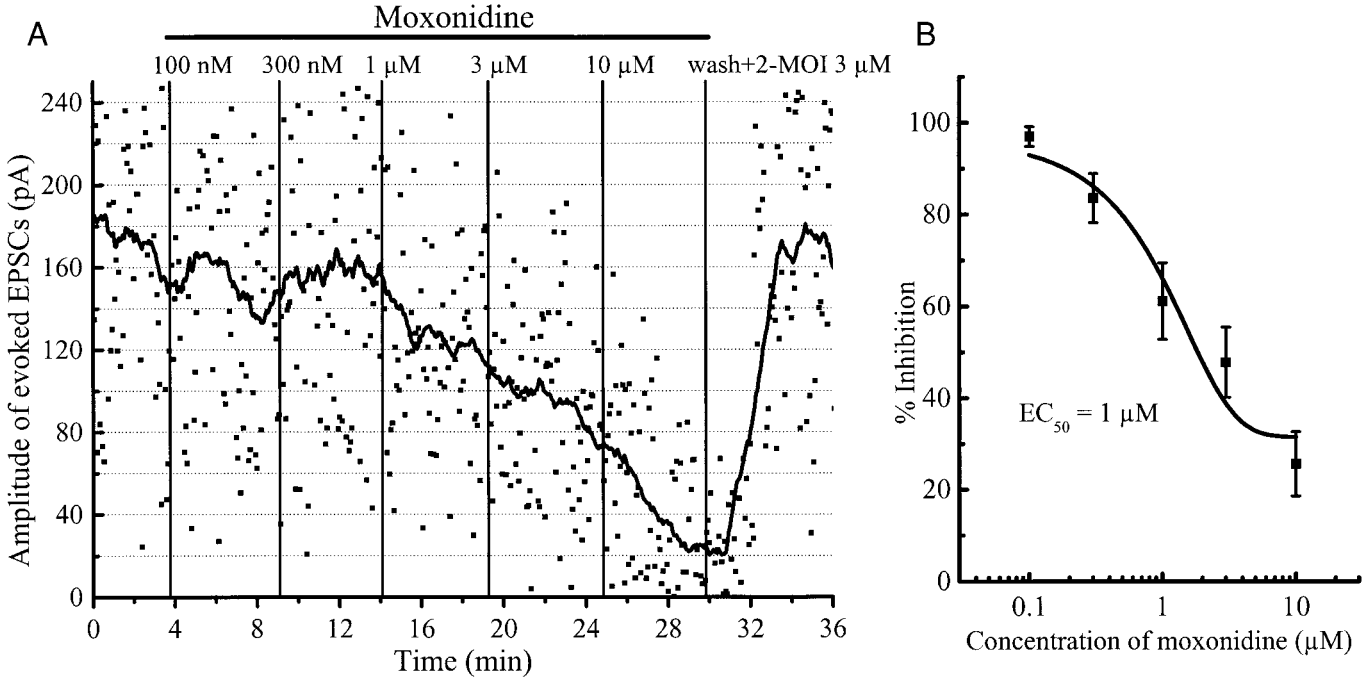

FIG. 6. Concentration-dependent inhibition of the evoked EPSCs by moxonidine. Evoked EPSCs were isolated in the presence of gabazine (3 μM) and strychnine (10 μM). *A*: scatter plot of the amplitude of individual EPSCs (■, stimulation at intervals of 4 s) and moving average (line) of 20 consecutive points. Moxonidine (100 nM to 10 μM) applied cumulatively at intervals of 5 min (as indicated by the vertical lines) decreased the amplitude of the evoked EPSCs in a concentration-dependent manner. *B*: percentage inhibition of the amplitude of evoked EPSCs by moxonidine in 4 cells (bars indicate means ± SE).

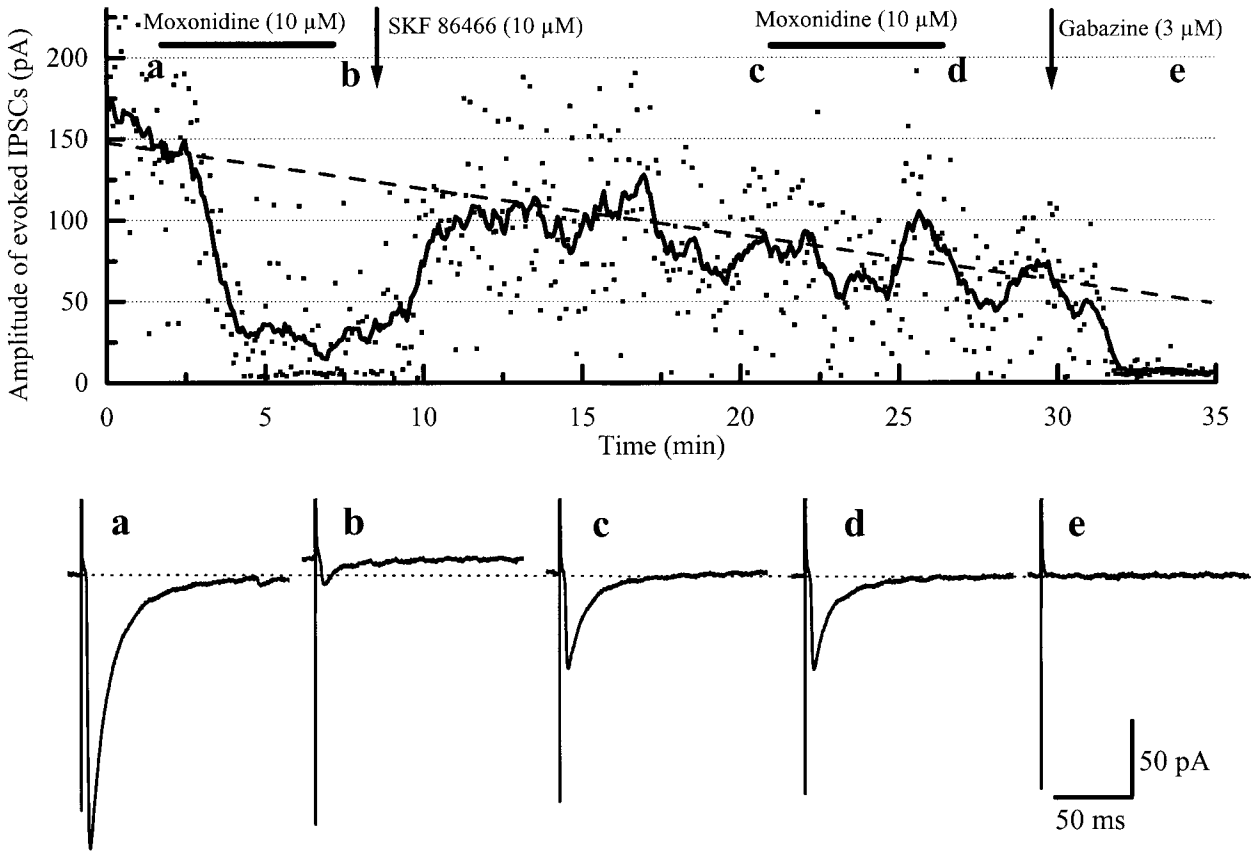

FIG. 7. Inhibition of the evoked inhibitory postsynaptic current (IPSC) in a bulbospinal RVL neuron by moxonidine: antagonism by SKF 86466. *Top*: amplitude of individual IPSCs (■, stimulation at intervals of 5 s) and moving average (line, 15 consecutive points). Moxonidine considerably decreased the amplitude of the evoked IPSCs. Application of the α2-AR antagonist SKF 86466 accelerated the recovery of the response on wash and attenuated the effect of a 2nd application of moxonidine. The $GABA_A$ receptor antagonist gabazine eliminated the evoked IPSCs. *Bottom traces* are averages of 12 consecutive evoked IPSCs with letters (*a*–*e*) referring to sampling periods indicated in the *top plot*. In this cell, moxonidine induced a large reduction in the amplitude of the evoked IPSCs, and produced a modest outward current (8 pA) indicated by the upward shift in the baseline of the evoked IPSCs (compare *b* and *a*). The outward current produced by moxonidine was also eliminated by SKF 86466 (compare *d* and *c*).

cases, the second exposure to moxonidine failed to produce a detectable inhibition of the IPSC. These data suggest a predominant contribution of α2-ARs to the inhibitory effect of moxonidine on the GABAergic inputs examined. The evoked IPSCs were blocked by additional application of gabazine (3 μM) in all three cells tested, confirming that these IPSCs were mediated by $GABA_A$ receptors (Hayar and Guyenet 1998; 1999).

DISCUSSION

The present study is the first to examine the effects of the prototypical I1-imidazoline ligand moxonidine in the RVL at the cellular level. The results suggest that moxonidine may contribute to sympathoinhibition and blood pressure reduction by inhibiting the activity of a group of RVL bulbospinal neurons that subserve a major sympathoexcitatory function in vivo. The evidence also suggests that the inhibitory effect of moxonidine on these bulbospinal neurons derives from a combination of postsynaptic hyperpolarization and presynaptic inhibition of glutamate release. Finally, the data indicate that moxonidine mimics all the effects of NE that are attributed to the activation of α2-ARs in the RVL (Hayar and Guyenet 1999). Because the effects of moxonidine were virtually eliminated by a selective antagonist of α2-ARs we conclude that the central sympatholytic action of moxonidine is likely to be due to its α2-AR agonist property.

Technical considerations and experimental limitations

Most of the experiments were carried out in rats younger than 8 days because in older animals the dense myelination of the RVL makes recording from visualized bulbospinal neurons extremely difficult. This difficulty is compounded by the fact that the total number of RVL bulbospinal neurons is ~200 per side, and only a fraction of these cells can be retrogradely labeled (Ruggiero et al. 1994). The practical limitation imposed by the small number of cells that can be recorded per rat, even in the neonate, compelled us to limit the number of experimental protocols to the strict minimum and, in particular, prevented us from testing the effects of multiple antagonists. Also we acknowledge the possibility that hypothetical effects of moxonidine mediated by I1 receptors could have been missed because of the predominant use of neonatal tissue.

Second, we cannot assert that all the RVL neurons selected for recording were presympathetic, although there are compel-

ling reasons to believe that this is true of most RVL bulbospinal neurons (for discussion see Li et al. 1995). In brief, the main evidence is that RVL neurons that are retrogradely labeled from the thoracic spinal cord are known to innervate selectively the intermediolateral cell column (Ross et al. 1984; Ruggiero et al. 1994). In addition, the majority of recorded neurons are catecholaminergic C1 cells which project monosynaptically to sympathetic preganglionic neurons (for review, see Guyenet et al. 1996). In any case, the uncertainty regarding the function of each individual bulbospinal neuron recorded is a minor concern in the present study because all neurons responded in a qualitatively similar manner.

Third, it is likely that we have underestimated the proportion of C1 cells recorded because prolonged recording in the whole cell mode attenuates the intensity of the TH immunoreactivity.

Finally, moxonidine may have some weak α1-AR agonist properties (Armah et al. 1988; Schlicker et al. 1990). In the present study, the effects of moxonidine were tested in the presence of the α1-AR antagonist prazosin. This was done to be able to compare the effects of moxonidine with those of NE previously examined in the same system, which in the presence of prazosin are entirely due to the activation of α2-ARs (Hayar and Guyenet 1999). However, because some glycinergic interneurons of the ventrolateral medulla are excited by α1-AR agonists (Hayar et al. 1997), we may have overlooked additional inhibitory mechanisms that may contribute to the central sympathoinhibitory action of moxonidine.

Moxonidine in the presence of prazosin mimics all the α2-AR–mediated actions of NE in RVL

With minor quantitative differences, moxonidine produced the same effects as NE (Hayar and Guyenet 1999 and present study). Both substances produced a small and variable barium-sensitive outward current that, in the case of NE, has been identified as an inwardly rectifying potassium current (Hayar and Guyenet 1999; Li et al. 1995). The average current produced by moxonidine (6.8 pA, this study) was slightly smaller than that produced by NE (11 pA) (Hayar and Guyenet 1999) or by the relatively selective α2-AR agonist α-methyl-norepinephrine at 30 μM (Li et al. 1995). The small amount of outward current produced by NE or moxonidine is in agreement with the finding that even saturating concentrations of these drugs fail to fully silence the majority of RVL bulbospinal neurons recorded extracellularly. This congruence indicated that the small magnitude of the outward current produced by either moxonidine or NE in whole cell recordings was not due to intracellular dialysis with the pipette solution. The magnitude of the outward current produced by NE and moxonidine is variable from cell to cell even within the narrow group of bulbospinal neurons identified as C1 cells (Hayar and Guyenet 1999). The cell-to-cell variability of the response to α2-AR agonists could be due to a heterogeneous level of expression of the α2-ARs or of their effectors (G proteins, potassium channels). This variability could also be due to a predominantly dendritic location of the α2-ARs because RVL bulbospinal neurons have long dendrites that are variably amputated by the slicing procedure. In any event, the small magnitude of the postsynaptic response to α2-AR agonists in vitro is unlikely to be a peculiarity of the neonate because the iontophoretic application of NE on RVL presympathetic neurons in vivo also produced an inhibition of firing that was modest and quite variable from one cell to another (Allen and Guyenet 1993). Collectively, these data suggest that the postsynaptic receptors may make a relatively small contribution to the inhibition of RVL presympathetic neurons by systemically administered hypotensive agents with α2-AR agonist properties.

In the presence of prazosin, moxonidine also mimicked the effect of NE on EPSCs evoked by focal stimulation in RVL bulbospinal neurons (moxonidine: 57 ± 4.6, $n = 15$; NE: $49 \pm 3\%$, $n = 22$) (Hayar and Guyenet 1999). Like NE (Hayar and Guyenet 1999), moxonidine reduced the amplitude of the evoked EPSCs without altering their decay time constant, suggesting that the properties of postsynaptic glutamate receptors were not affected. Moreover, in both cases the reduction in synaptic transmission by α2-ARs was not due to activation of barium-sensitive inwardly rectifying potassium channels because a similar degree of inhibition of glutamate release was obtained in the absence and presence of barium. Finally, both NE and moxonidine reduced the evoked IPSCs when this component was present (moxonidine: $60 \pm 16\%$, $n = 3$; NE: $59 \pm 19\%$, $n = 6$; Hayar and Guyenet 1999).

In brief, moxonidine reproduced all the effects of NE when applied in the presence of prazosin. Because the effects of NE in the presence of prazosin were totally inhibited by the highly potent and selective α2-AR antagonist methoxyidazoxan, they are clearly mediated by activation of α2-ARs (Hayar and Guyenet 1999). Thus, we conclude that in RVL, moxonidine produces qualitatively the same pre- and postsynaptic effects as those caused by other α2-AR agonists. We only found two quantitative differences. First, moxonidine produced somewhat less outward current and a somewhat smaller inhibition of the discharges of RVL neurons. Given that we used saturating doses of each agonist, the data suggest that moxonidine may be a partial agonist of α2-ARs in this system. Evidence that moxonidine may be a partial agonist has been obtained in other systems (Zhu et al. 1999). Second, moxonidine produced its effects in the slice significantly more slowly than NE. A possible explanation is that the access of moxonidine to α2-ARs may be delayed because of its hydrophobicity.

Are the actions of moxonidine in RVL fully accounted by its agonist activity at α2-ARs?

The fact that moxonidine is an α2-AR agonist with preferential affinity for the A subtype (α2D in rat) is well established (e.g., Gaiser et al. 1999; Szabo et al. 1996; Zhu et al. 1999). However, whether the α2-AR agonist activity of this drug accounts for its central sympatholytic action or whether this action is due to I1-imidazoline receptors is controversial (Ernsberger and Haxhiu 1997; Ernsberger et al. 1990). The present data are generally consistent with the first hypothesis, namely that the actions of moxonidine in RVL are mostly, if not exclusively, accounted for by its agonist activity at α2-ARs. In addition to the evidence discussed in the previous section, this interpretation is consistent with the ability of SKF 86466 to nearly or completely abolish the effects of moxonidine. SKF 86466 is a highly selective competitive α2-AR antagonist (Hieble et al. 1986) and is currently considered to have the least interaction with I1-imidazoline receptors among available α2-AR blockers (Ernsberger et al. 1990).

Both the inhibition of firing (extracellular recordings, Fig. 2) and the small outward current (whole cell recordings, Fig. 3) produced by moxonidine in RVL neurons were blocked by SKF 86466. These results are consistent with the study of Szabo et al. (1996) performed in the locus coeruleus of the adult rat in vitro. In this study, moxonidine inhibited neuronal discharges and the concentration-response relationship was shifted to the right by 10 μM SKF 86466 in a manner suggesting simple competitive antagonism. Finally, SKF 86466 attenuated the inhibitory effect of moxonidine on both evoked EPSCs and evoked IPSCs by 80–85%. The residual inhibition caused by 10 μM moxonidine in the presence of 10 μM SKF 86466 is consistent with the competitive nature of the antagonism and the fact that SKF 86466, although very selective, is not a very potent antagonist (Hieble et al. 1986; Szabo et al. 1996). However, we cannot exclude that this residual inhibition might be caused by an effect of moxonidine on other receptors including imidazoline receptors which may be present presynaptically in sympathetic ganglionic and other neurons (for a critical discussion of the evidence see Gaiser et al. 1999).

Another explanation of the present data is that the activation of imidazoline receptors by moxonidine causes the release of NE and a secondary activation of α2-ARs. Although we have no evidence to counter this argument, the possibility that moxonidine might release a catecholamine is contrary to existing data that unanimously demonstrated that moxonidine inhibits the release of catecholamines (e.g., Gaiser et al. 1999; Szabo and Urban 1995). Based on whole animals studies, Head and his collaborators (1997, 1998) have also suggested that the primary action of moxonidine could be to activate I1-imidazoline receptors but that simultaneous activation of α2-ARs (by moxonidine or by endogenous catecholamines) is necessary to turn this primary event into an effect on neuronal activity and blood pressure. This theory was designed to reconcile the high affinity of moxonidine for I1 binding sites and the fact that its effects are attenuated by various α2-AR antagonists in vivo (Chan and Head 1996; Head 1995). This theory could account for the inability of the I1 ligand AGN 192403 to change blood pressure (Munk et al. 1996) and its inactivity in vitro (present data).

Pharmacological implications: role of RVL in the sympatholytic effect of α2-AR agonists

The results suggest that the presence of an α2-AR agonist in the RVL contributes to sympathoinhibition and blood pressure reduction by inhibiting the activity of a group of RVL bulbospinal neurons that subserve a major sympathoexcitatory function in the whole animal (Guyenet et al. 1996). The evidence also suggests that the inhibitory effect of α2-AR agonists on these bulbospinal neurons is derived from the combined effects of a small and variable postsynaptic hyperpolarization and the presynaptic inhibition of glutamate release. Both mechanisms are consistent with the observed anatomic location of α2A-ARs in RVL (Guyenet et al. 1994; Milner et al. 1999). These anatomic data are especially relevant to the action of moxonidine which exhibits preferential affinity for α2A-ARs (Munk et al. 1996; Zhu et al. 1999). Furthermore, presynaptic inhibition of glutamate release by α2-AR agonists has been found in other brain stem neurons (Bertolino et al. 1997; Travagli and Williams 1996).

In agreement with our previous data (Hayar and Guyenet 1999), the present study also suggests that α2-AR agonists can reduce inhibitory synaptic transmission in RVL at the level of some of the GABAergic terminals that contact the bulbospinal neurons. Therefore even within RVL, the overall effect of α2-AR agonists represents a balance between presynaptic inhibition and presynaptic disinhibition. This notion can probably be extended to the effect of α2-AR agonists in many other brain stem structures that contribute to sympathetic regulation and have a high density of GABAergic receptors (nucleus of the solitary tract, dorsolateral pons, autonomic areas of the thoracic spinal cord). Accordingly, the α2-AR agonist clonidine can increase blood pressure, for example, when injected slightly caudal to the RVL in urethan-anesthetized rats (Sesoko et al. 1998) or intracerebroventricular in conscious rats. In each case, pharmacological evidence suggests that the pressor effects were caused by α2-AR stimulation. The degree of penetration of α2-AR agonists and antagonists to these various structures is certain to vary according to the lipid solubility of the individual agent and the route or volume of administration. Such differences, rather than an interaction with imidazoline receptors, may account for the slight differences that have been observed in whole animal preparations after treatment with individual representatives of this drug class (e.g., Chan and Head 1996).

In conclusion, the present data suggest that moxonidine exerts its effect within the RVL by virtue of its α2-AR agonist property. Because moxonidine is a preferential agonist at the A subtype of receptor and this receptor is abundantly represented in the cells we have investigated (Guyenet et al. 1994; Milner et al. 1999), we conclude that α2A-ARs probably make the main contribution to the sympatholytic effect of moxonidine in RVL. This conclusion is consistent with recent data on the D79N α2-AR mutant mouse, a strain that expresses only a low level of dysfunctional α2A-ARs (Link et al. 1996; MacMillan et al. 1996; Zhu et al. 1999). This mutation abolishes the hypotensive and bradycardic effect of imidazoline-related drugs (clonidine, moxonidine, rilmenidine).

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