Am J Physiol Cell Physiol 300: C58–C64, 2011.
First published October 27, 2010; doi:10.1152/ajpcell.00369.2010.

Angiotensin-(1–7) increases neuronal potassium current via a nitric oxide-dependent mechanism

Rui-Fang Yang,[1] Jing-Xiang Yin,[1] Yu-Long Li,[1,2] Matthew C. Zimmerman,[1,3]* and Harold D. Schultz[1,3]*

[1]*Cellular and Integrative Physiology,* [2]*Emergency Medicine, University of Nebraska Medical Center, Omaha;* [3]*Redox Biology Center, University of Nebraska-Lincoln, Lincoln, Nebraska*

Submitted 8 September 2010; accepted in final form 25 October 2010

Yang R, Yin J, Li Y, Zimmerman MC, Schultz HD. Angiotensin-(1–7) increases neuronal potassium current via a nitric oxide-dependent mechanism. *Am J Physiol Cell Physiol* 300: C58–C64, 2011. First published October 27, 2010; doi:10.1152/ajpcell.00369.2010.—Actions of angiotensin-(1–7) [Ang-(1–7)], a heptapeptide of the renin-angiotensin system, in the periphery are mediated, at least in part, by activation of nitric oxide (NO) synthase (NOS) and generation $NO^{\cdot}$. Studies of the central nervous system have shown that $NO^{\cdot}$ acts as a sympathoinhibitory molecule and thus may play a protective role in neurocardiovascular diseases associated with sympathoexcitation, such as hypertension and heart failure. However, the contribution of NO in the intraneuronal signaling pathway of Ang-(1–7) and the subsequent modulation of neuronal activity remains unclear. Here, we tested the hypothesis that neuronal NOS (nNOS)-derived $NO^{\cdot}$ mediates changes in neuronal activity following Ang-(1–7) stimulation. For these studies, we used differentiated catecholaminergic (CATH.a) neurons, which we show express the Ang-(1–7) receptor (Mas R) and nNOS. Stimulation of CATH.a neurons with Ang-(1–7) (100 nM) increased intracellular NO levels, as measured by 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) fluorescence and confocal microscopy. This response was significantly attenuated in neurons pretreated with the Mas R antagonist (A-779), a nonspecific NOS inhibitor (nitro-L-arginine methyl ester), or an nNOS inhibitor (*S*-methyl-L-thiocitrulline, SMTC), but not by endothelial NOS (eNOS) or inhibitory NOS (iNOS) inhibition {L-*N*-5-(1-iminoethyl)ornithine (L-NIO) and 1400W, respectively}. To examine the effect of Ang-(1–7)-$NO^{\cdot}$ signaling on neuronal activity, we recorded voltage-gated outward K^+ current (I_{Kv}) in CATH.a neurons using the whole cell configuration of the patch-clamp technique. Ang-(1–7) significantly increased I_{Kv}, and this response was inhibited by A-779 or *S*-methyl-L-thiocitrulline, but not L-NIO or 1400W. These findings indicate that Ang-(1–7) is capable of increasing nNOS-derived $NO^{\cdot}$ levels, which in turn, activates hyperpolarizing I_{Kv} in catecholaminergic neurons.

neuronal nitric oxide synthase; neuronal nitric oxide synthase; Mas receptor; differentiated catecholamine neurons; autonomic nervous system

NEURAL ABNORMALITY and heightened activity of the sympathetic nervous system are implicated in the pathogenesis of many cardiovascular diseases, including hypertension and heart failure. Possible mechanisms include repressed arterial baroreflex buffering of sympathetic nerve traffic induced by neurohumoral factors such as angiotensin II (ANG II) in autonomic control regions of the brain, including the subfornical organ (SFO), paraventricular nucleus (PVN), and rostral ventrolateral medulla (RVLM) (12, 39, 45).

The heptapeptide Ang-(1–7) is recognized as an important biologically active component of the renin-angiotensin system (RAS) in different tissues. In contrast to ANG II-mediated peripheral actions, including vasoconstriction and cell proliferation, Ang-(1–7) induces vasodilation and inhibition of cell growth mediated by nitric oxide ($NO^{\cdot}$) and prostaglandin release (30). In the brain, Ang-(1–7) can be produced and evoke functional effects in several areas of the forebrain and brain stem, including the paraventricular nucleus of the hypothalamus (PVN), nucleus tractus solitarii (NTS), and rostral ventrolateral medulla (RVLM), which underscore its role as a neuromodulator of cardiovascular function (29). Exogenous administration of Ang-(1–7) into the NTS or brain ventricular system facilitates arterial baroreflex bradycardia (4). In the caudal ventrolateral medulla, Ang-(1–7) lowered arterial pressure by inhibiting RVLM-mediated pressor responses (33). In spontaneously hypertensive rats, the pressor response induced by intrahypothalamic administration of ANG II is attenuated when Ang-(1–7) and ANG II are administered together (18). These studies suggest that Ang-(1–7) counteracts the hypertensive actions of ANG II in central nervous system (CNS).

The G protein-coupled receptor Mas (Mas R) has been identified as a receptor for Ang-(1–7) (32). In mammals, *Mas* gene is abundantly expressed in the testis and brain and less strongly but at detectable levels in the kidney, heart, and vascular endothelium (1, 2, 8). Recent studies suggest that Ang-(1–7), binding to Mas R, increases $NO^{\cdot}$ levels, which induces vasodilation and opposes ANG II-induced vasoconstriction and cardiomyocyte hypertrophy (14, 31). Moreover, *Mas*-deficient mice exhibit elevated blood pressure, decreased $NO^{\cdot}$ bioavailability, and increased levels of reactive oxygen species (ROS) in the vasculature (40). In the brain, Mas R is found in areas of the forebrain and medulla related to modulation of cardiovascular functions and is mainly located in neuronal cells (2).

The presence of Ang-(1–7) and Mas R in close proximity in the central areas that modulate cardiovascular function suggests a role for the Ang-(1–7)-Mas R axis in the regulation of sympathetic nerve activity. Indeed, a selective Mas antagonist, (D-Ala^7)-Ang-(1–7), given intracerebroventricularly or into the NTS, inhibits the improvements in baroreflex activity induced by Ang-(1–7) (24, 27). Gironacci and colleagues (13) reported that Ang-(1–7) attenuates the K^+-evoked neuronal norepinephrine (NE) release from rat hypothalamus through a $NO^{\cdot}$-dependent mechanism, partially mediated by Mas R signaling (13). Recently, Feng et al.(9) found that overexpression of angiotensin-converting enzyme type 2 (ACE2), which converts ANG II to Ang-(1–7), in the brain increases nitric oxide synthase (NOS) and $NO^{\cdot}$. Considering that Ang-(1–7)-Mas R axis plays an important role in regulation of baroreflex sensi-

* M. C. Zimmerman and H. D. Schultz contributed equally in this study.
Address for reprint requests and other correspondence: H. D. Schultz, Dept. of Cellular and Integrative Physiology, Univ. of Nebraska Medical Center, 985850 Nebraska Medical Center, Omaha, NE 68198-5850 (e-mail: hschultz@unmc.edu).

0363-6143/11 Copyright © 2011 the American Physiological Society

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 28, 2017

tivity and pressor responses in brain, we hypothesized that Ang-(1–7)-Mas- $NO^{\cdot}$ signaling regulates neuronal voltage-gated outward K^+ current (I_{Kv}) in catecholaminergic neurons. In the present study, we evaluated the Ang-(1–7)-Mas-$NO^{\cdot}$ signaling effects on I_{Kv} in CATH.a neurons, a catecholaminergic neuronal cell line.

MATERIALS AND METHODS

CATH.a neuronal cell culture. Catecholaminergic CATH.a neuronal cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured according to manufacturer's instructions. In brief, CATH.a neurons were grown in RPMI 1640 media supplemented with 8% normal horse serum, 4% fetal bovine serum, and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Before use in experiments, CATH.a neurons were differentiated for 6–8 days by adding N^6,2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (dbcAMP, 1 mM) to the culture media, as previously described (42).

Reverse-transcription PCR. Total RNA was isolated from differentiated CATH.a neurons with (TriReagent, MRC, Cincinnati, OH) according to the manufacturer's instructions. First-strand cDNA was reverse transcribed from 1 μg of total RNA using Reverse Transcription Reagents (TaqMan, Branchburg, NJ). Gene-specific primers for RT-PCR were as follows: neuronal NOS (nNOS): (s) AGATTGGCGTTCGTGATT and (as) GTGGAAGACAGGGGTGAT (sze 302 bp, Gene Bank no. NM_008712); endothelial NOS (eNOS): (s) CTGGCAAGACAGACTACACG and (as) GACATCGCCGCAGACAAA (size 280 bp, Gene Bank no. NM_008713); inducible NOS (iNOS): (s) CCCTAAGAGTCACCAAAATG and (as) TGATGGACCCCAAGCAAG (size 212 bp, Gene Bank no. NM_010927); Mas R: (s) GGGAGGAGGGAGCGAAAC and (as) GGAAGCAGAGGAACCAGAGGAG (size 365 bp, Gene Bank no. NM_008552). The same amount of cDNA was loaded for the PCR amplification of each gene and standard. PCR reactions were performed. Cycling conditions were 94°C for 10 min, and 35 cycles of 94°C for 30 s and 55°C for 1 min. PCR products were analyzed by gel electrophoresis on a 1.5% agarose gel stained with ethidium bromide. Band intensities were quantified using NIH Image J analysis software.

Western blot analysis. Protein expression for nNOS, eNOS, iNOS, Mas R, and actin in differentiated CATH.a neurons were determined using standard Western blot analysis. Cells were harvested in lysis buffer containing protease inhibitors (Roche, Indianapolis, IN). Cell lysates (100 μg) were separated by SDS-PAGE and transferred to nitrocellulose membrane. Membranes were incubated with appropriate primary antibodies for nNOS (Millipore, Billerica, MA), eNOS (Abcam, Cambridge, MA), iNOS (Cell signaling, Danvers, MA), Mas R (Alomone, Jerusalem, Israel), and actin (Sigma, St. Louis, MO). After incubation with appropriate horseradish peroxidase-conjugated secondary antibodies, immunoreactivity was detected by chemiluminescence using the Pierce ECL detection system (Thermo Scientific, Rockford, IL). Band intensities were analyzed using NIH Image J analysis software. The values were normalized to actin to correct for any differences in protein loading.

Measurement of $NO^{\cdot}$ levels. $NO^{\cdot}$ levels in differentiated CATH.a neurons were determined with the fluorescent dye 4-amino-5-methylamino-2′,7′-difluorofluorecein diacetate (DAF-FM; 5 μg/ml; Invitrogen) in buffer containing (in mM) 120 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 11 glucose, 15.9 NaH_2PO_4, and 1 L-arginine (pH 7.2) for 30 min at 37°C. Excess dye was removed, and cells were incubated with fresh buffer for an additional 20 min at 37°C to allow complete deesterification of the intracellular diacetates. Images were collected before and after adding Ang-(1–7) using a fluorescence confocal microscopy (Zeiss 510 Meta, Göttingen, Germany) with 488 nm wavelength excitation. In some experiments, CATH.a neurons were pretreated with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM, Sigma), *S*-methyl-L-thiocitrulline (SMTC, 1 μM, Cayman Chemical, Ann Arbor, MI), L-NIO (1 μM, Calbiochem, Gibbstown, NJ), 1400W (1 μM, Calbiochem) or A-779 (1 μM, Bachem, Torrance, CA) 20 min before Ang-(1–7) treatment. DAF-FM fluorescent intensity per cell was analyzed using the Zeiss LSM 510 analysis software. DAF-FM has a detection limit of 3 nM and does not react with other stable oxidized forms of $NO^{\cdot}$, such as NO_2, or ROS (20).

Electrophysiological recordings. Potassium channel currents were recorded by an Axopatch 200B amplifier (Axon Instruments) in the standard whole cell configuration of the patch-clamp technique. Patch pipette resistance ranged from 3 to 5 MΩ when filled with (in mM) 130 KCl, 2 $MgCl_2$, 0.25 $CaCl_2$, 5 EGTA, 1 Mg-ATP, 0.1 Tris-GTP, 10 HEPES, and 8 glucose, pH 7.2. The extracellular bath solution included (in mM) 137 NaCl, 5.4 KCl, 1.35 $CaCl_2$, 2 $MgCl_2$, 0.3 NaH_2PO_4, 10 HEPES, and 10 sucrose, pH 7.4. Na^+ and Ca^{2+} channels were blocked by TTX (1.5 μM) and $CdCl_2$ (0.3 mM), respectively. Current traces were sampled at 10 kHz and filtered at 5 kHz. Holding potential was −80 mV. Current-voltage (*I-V*) relations were elicited by test potential over the range of −80 to +80 mV with 200-ms duration in 20-mV increments. The pipette and series resistance and capacitance were compensated by more than 90% and checked during experiments for stability. Resulting data were acquired and analyzed with Clampfit 9.2 software (Axon Instruments). Recordings were performed at 22–24°C. In Ang-(1–7) experiments, 100 μM L-arginine was added to the bath solution. The effect of Ang-(1–7) on current density was tested by superfusing neurons with the peptide for 5 min and repeating the voltage-pulse regimen. To investigate the role of $NO^{\cdot}$ or Mas R activation, recordings of I_{Kv} were made under the following sequential treatments: *1*) superfusion of vehicle; *2*) superfusion of Ang-(1–7) (100 nM); Ang-(1–7) plus SMTC (1 μM), L-NIO (1 μM), 1400W (1 μM) or A-779 (1 μM); *3*) superfusion of $NO^{\cdot}$ donor *S*-nitroso-*N*-acetyl-*dl*-penicillamine (SNAP, 100 μM, Sigma); and *4*) washout of SNAP.

Statistical analysis. The data were analyzed in Prism software (GraphPad Software) and expressed as means ± SE. Analysis was performed with Student's *t*-test when comparing only two groups or by analysis of variance (ANOVA) followed by Newman-Keuls correction for multiple group comparisons. Significance is expressed by $P < 0.05$.

RESULTS

CATH.a neurons express NOS and Mas R. First, we assessed whether CATH.a neurons express mRNA and protein for Mas R and NOS isoforms. RT-PCR revealed relatively high levels of Mas R and nNOS mRNA in differentiated CATH.a neurons when compared with the modest levels of iNOS and eNOS mRNA (Fig. 1*A*). Protein expression of Mas R and nNOS in differentiated CATH.a neurons was confirmed by Western blot analysis (Fig. 1*B*). These results clearly show that nNOS and Mas R are expressed in differentiated CATH.a neurons.

Ang-(1–7) increases $NO^{\cdot}$ levels by activating Mas R-nNOS pathway in CATH.a neurons. To investigate whether Ang-(1–7) influences $NO^{\cdot}$ production in CATH.a neurons, intracellular $NO^{\cdot}$ levels were measured by DAF-FM fluorescence and confocal microscopy. As shown in representative confocal microscopy images and summary data, Ang-(1–7) (100 nM) significantly enhanced DAF-FM fluorescence intensity (Fig. 2; 134 ± 3% of baseline, $n = 66$ neurons on six coverslips, $P < 0.05$) compared with the vehicle-exposed control (104 ± 0.9% of baseline), suggesting that Ang-(1–7) stimulates $NO^{\cdot}$ production in CATH.a neurons. To study the role of Mas R and isoforms of NOS in Ang-(1–7)-induced $NO^{\cdot}$ generation, CATH.a neurons were pretreated with Mas R antagonist or NOS inhibitors before Ang-(1–7) stimulation. As illustrated in

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 28, 2017

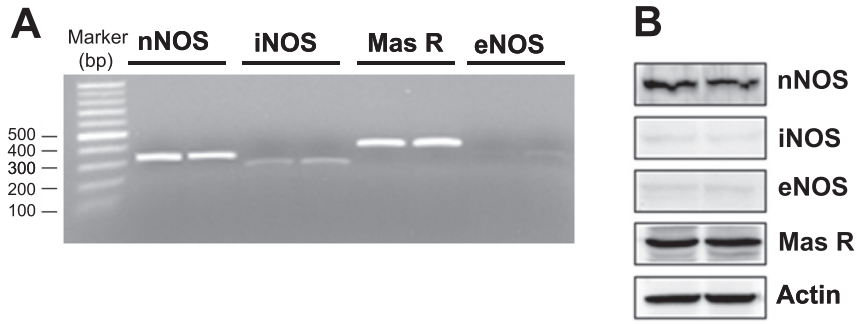

Fig. 1. Differentiated differentiated catecholaminergic (CATH.a) neurons express nitric oxide synthase (NOS) and Mas R. *A*: representative RT-PCR image from four separate experiments showing mRNA expression of neuronal (nNOS), inducible (iNOS), endothelial (eNOS) NOS, and Mas R in differentiated CATH.a neurons. The same amount of cDNA that was reverse transcribed from 1 μg of total RNA was loaded in PCR amplification for each gene. *B*: representative Western blot analysis images showing protein expression of nNOS, iNOS, eNOS, and Mas R in differentiated CATH.a neurons.

Fig. 2, the effect of Ang-(1–7) on $NO^{\cdot}$ production was blocked by nonspecific NOS inhibitor L-NAME (102 ± 6% of baseline, $n = 23$ neurons on 3 coverslips) or nNOS inhibitor SMTC (104 ± 3% of baseline, $n = 30$ neurons on 3 coverslips) and Mas R antagonist A-779 (109 ± 1% of baseline, $n = 28$ neurons on 3 coverslips). In contrast, the increased $NO^{\cdot}$ levels induced by Ang-(1–7) remained almost unchanged with application of iNOS inhibitor 1400W (140 ± 10% of baseline, $n = 44$ neurons on 4 coverslips) or eNOS inhibitor L-NIO (140 ± 4% of baseline, $n = 46$ neurons on 4 coverslips), suggesting that Mas R-nNOS pathway is responsible for Ang-(1–7)-induced $NO^{\cdot}$ generation in CATH.a neurons.

Ang-(1–7) activates K^+ current in CATH.a neurons. Voltage-gated K^+ channels play an important role in maintaining membrane potential and neuronal excitability. Figure 3*A* shows that Ang-(1–7) (100 nM) superfusion increased I_{Kv} in CATH.a neurons compared with vehicle. Ang-(1–7) increased steady-state K^+ current (I_{ss}) by 52 ± 9% (Fig. 3*B*; $P < 0.05$ vs. vehicle) and peak current (I_{peak}) by 49 ± 7% (Fig. 3*C*; $P < 0.05$ vs. vehicle, $n = 10$ neurons), indicating that acute Ang-(1–7) increases K^+ channel activity by enhancement of outward K^+ current density in neurons.

Ang-(1–7) increases K^+ current in CATH.a neurons via Mas R activation. To determine the role of Mas R in mediating the Ang-(1–7)-induced increase of I_{Kv}, a series of experiments were initiated by first recording increased I_{Kv} in response to Ang-(1–7), and then the same neurons were superfused with Ang-(1–7) in the presence of A-779. The increased I_{Kv} induced by Ang-(1–7) was reversed by A-779, as shown in I_{Kv} representative traces (Fig. 4*A*) and the summary data demonstrating the current density-voltage relationship for I_{ss} (Fig. 4, *B–C*, $n = 7$ neurons). To clarify the contribution of $NO^{\cdot}$ in modulating K^+ current and whether the neurons are responsive to $NO^{\cdot}$ stimulation after Ang-(1–7) plus A-779 treatment, the same neurons were then superfused with SNAP ($NO^{\cdot}$ donor). I_{Kv} was increased in the presence of SNAP (Fig. 4), which was restored after washout of SNAP (data not shown). Together, these data suggest that Mas R signaling pathway contributes to Ang-(1–7)-induced activation of I_{Kv}.

Ang-(1–7) increases K^+ current in CATH.a neurons via nNOS-$NO^{\cdot}$-dependent mechanism. $NO^{\cdot}$ has been implicated in reducing neuronal excitability by activating K^+ currents (15, 41). To elucidate whether $NO^{\cdot}$ is involved in the effect of Ang-(1–7) on I_{Kv} in CATH.a neurons, we tested increased I_{Kv}

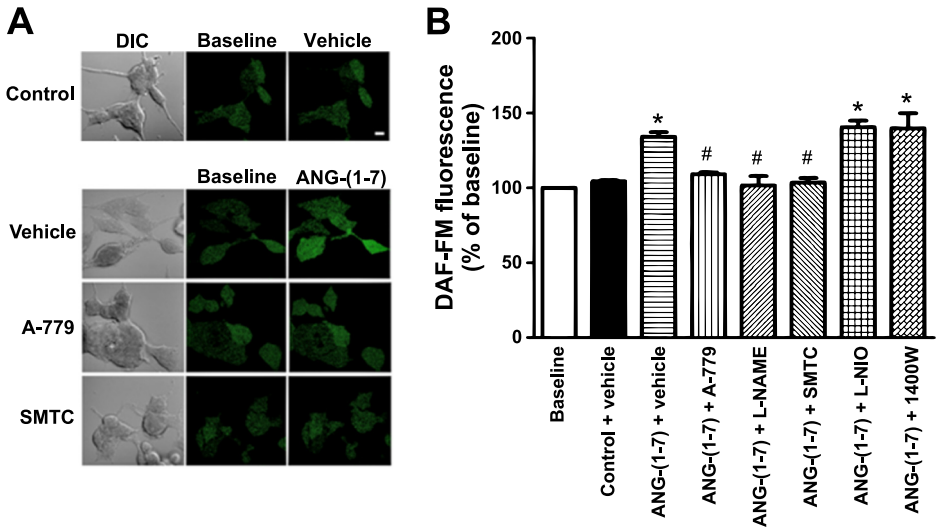

Fig. 2. Ang-(1–7) increases nitric oxide ($NO^{\cdot}$) levels in CATH.a neurons mediated by Mas R-nNOS signaling pathway. *A*: representative confocal microscopy images showing fluorescence of DAF-FM in CATH.a neurons pretreated with vehicle, Mas R antagonist A-779 (1 μM), or nNOS inhibitor *S*-methyl-L-thiocitrulline (SMTC, 1 μM) for 20 min before (baseline) and after vehicle or Ang-(1–7) stimulation (100 nM, 20 min). Scale bar = 10 μm. *B*: summary data of DAF-FM fluorescence intensity in CATH.a neurons with pretreatment of vehicle, A-779 ($n = 28$ neurons), nonspecific NOS inhibitor nitro-L-arginine methyl ester (L-NAME, 100 μM; $n = 23$ neurons), SMTC ($n = 30$ neurons), eNOS inhibitor L-*N*-5-(1-iminoethyl)ornithine (L-NIO, 1 μM; $n = 46$ neurons), or iNOS inhibitor 1400W (1 μM; $n = 44$ neurons), followed by vehicle ($n = 33$ neurons) or Ang-(1–7) stimulation ($n = 66$ neurons). DAF-FM fluorescence intensity was quantified using Zeiss LSM 510 software and expressed as percent increase from baseline [prevehicle or Ang-(1–7) stimulation]. *$P < 0.05$ vs. baseline and control + vehicle. #$P < 0.05$ vs. Ang-(1–7) + vehicle.

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 28, 2017

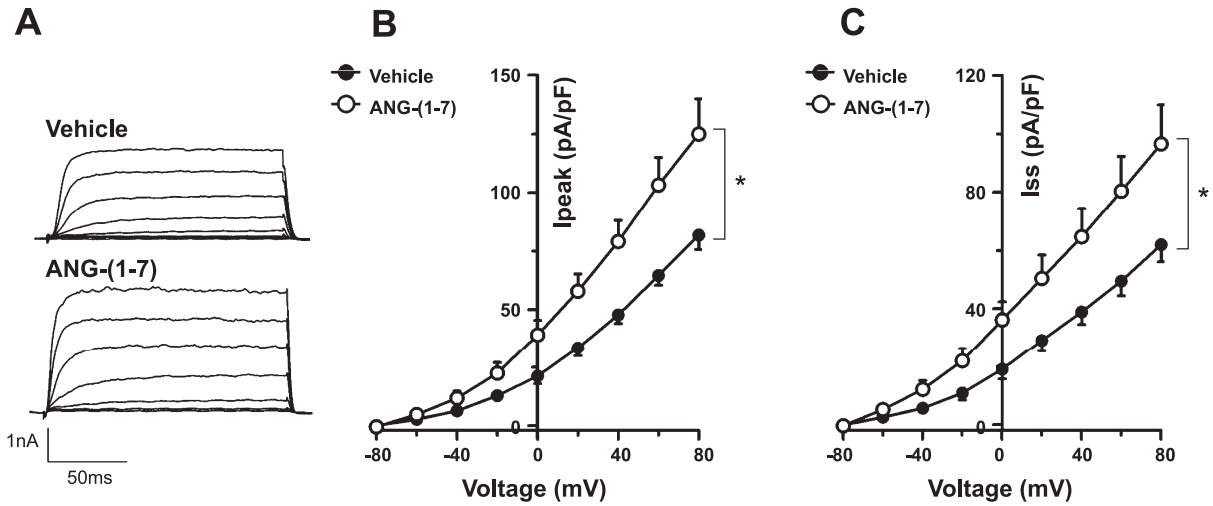

Fig. 3. Ang-(1–7) activates K^+ current in CATH.a neurons. *A*: representative K^+ current traces from CATH.a neurons superfused with vehicle or Ang-(1–7) (100 nM). Voltage-gated K^+ current (I_{Kv}) was recorded from a holding potential −80 to +80 mV during 200 ms. *B* and *C*: summary data of peak (I_{peak}) and steady-state (I_{ss}) current density-voltage relationships in CATH.a neurons ($n = 10$) superfused with vehicle or Ang-(1–7). *$P < 0.05$, vehicle vs. Ang-(1–7).

induced by Ang-(1–7) stimulation in the presence of SMTC. Ang-(1–7) superfusion led to a significant increase in I_{Kv}, and SMTC blocked this Ang-(1–7) response (Fig. 5). It should be noted that K^+ currents remained sensitive to NO· activation in SMTC-treated neurons as SNAP significantly increased I_{Kv} (Fig. 5, $n = 7$ neurons). To further verify the source of NO· in mediating the actions of Ang-(1–7), L-NIO and 1400W were used to inhibit eNOS and iNOS, respectively. The representative tracings show that enhanced I_{Kv} induced by Ang-(1–7) were unchanged by application of L-NIO (Fig. 6*A*) or 1400W (Fig. 6*B*). Taken together, these data show that the Ang-(1–7)-induced increase in I_{Kv} is mediated by activation of Mas R-nNOS-NO· pathway in CATH.a neurons.

DISCUSSION

Increasing evidence indicates that Ang-(1–7) plays an important role in modulating nerve activity. In this study, we observed that Ang-(1–7) increases I_{Kv} in differentiated CATH.a neurons. Furthermore, this response was blocked by Mas R antagonist A-779 and nNOS inhibitor SMTC, but not by eNOS inhibitor L-NIO or iNOS inhibitor 1400W, suggesting that Ang-(1–7) plays a role in inhibiting neuronal excitation by activating outward K^+ current through Mas R-nNOS-NO· signaling pathway.

Mas R has been identified as an Ang-(1–7) receptor and observed abundantly in central cardiovascular-related areas, such as the NTS, PVN, and RVLM, which are important sites that regulate sympathetic nerve outflow and baroreflex sensitivity. Furthermore, Mas R contributes to blood pressure regulation by Ang-(1–7) in the CNS (10, 32). Growing evidence indicates that some Ang-(1–7) actions are associated with the release of NO·. In vascular endothelium, Ang-(1–7)/Mas axis activates eNOS and increases NO· generation leading to vasodilation. Central administration of Ang-(1–7) reduces focal cerebral ischemia-reperfusion damage through stimulation of

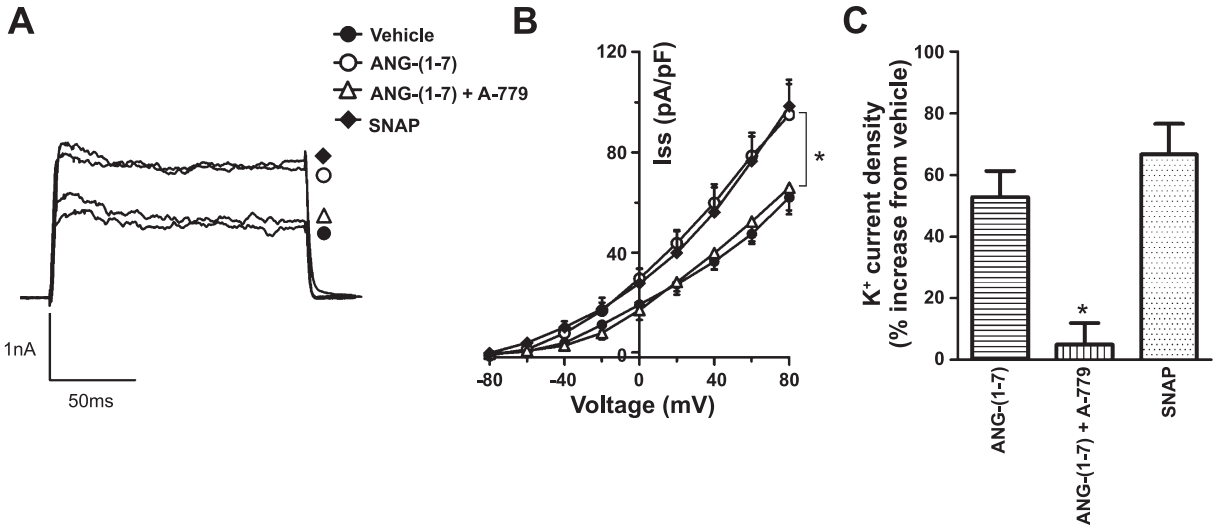

Fig. 4. Mas R mediates Ang-(1–7)-induced activation of K^+ current. *A*: representative K^+ current traces in the same CATH.a neuron induced by 1 episodic voltage step from −80 to +80 mV superfused with vehicle, Ang-(1–7) (100 nM), Ang-(1–7) plus A-779 (1 μM), and NO· donor SNAP (100 μM). *B* and *C*: summary data ($n = 7$ neurons) showing I_{ss} density-voltage relationships (*B*) and percent change of I_{Kv} current densities from baseline (*C*). *$P < 0.05$ vs. Ang-(1–7) alone.

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 28, 2017

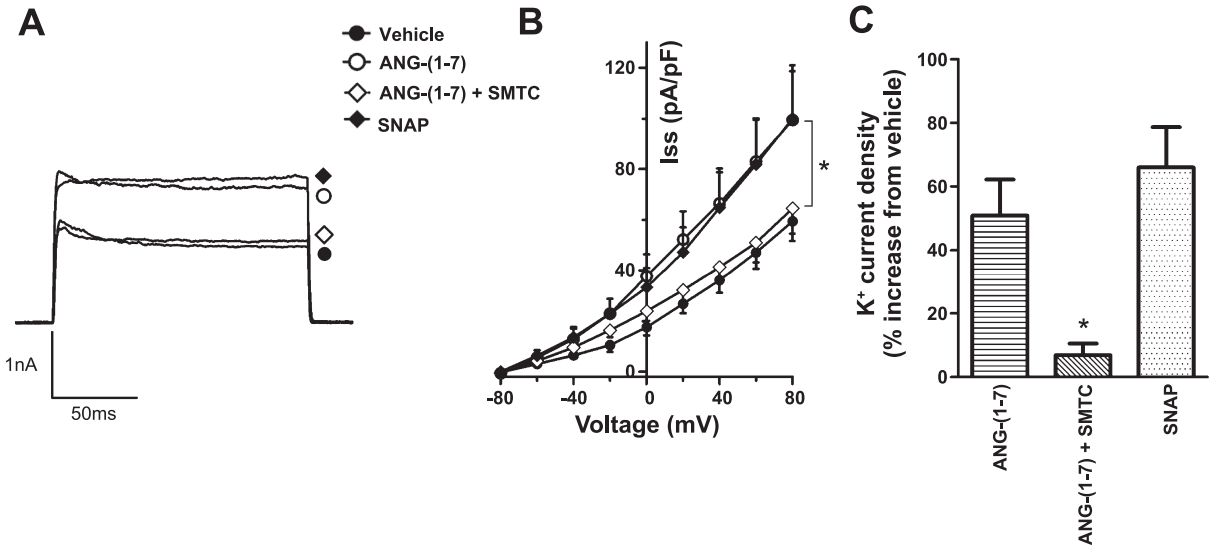

Fig. 5. nNOS-derived NO· mediates Ang-(1–7)-induced activation of K^+ current. *A*: representative K^+ current traces in the same CATH.a neuron induced by 1 episodic voltage step from −80 to +80 mV during superfusion of vehicle, Ang-(1–7) (100 nM), Ang-(1–7) plus SMTC (1 μM), and SNAP (100 μM). *B* and *C*: summary data ($n = 7$ neurons) showing I_{ss} density-voltage relationships (*B*) and percent change of I_{Kv} current densities from baseline (*C*). *$P < 0.05$ vs. Ang-(1–7) alone.

NO· release (44). Moreover, Ang-(1–7), partially mediated by Mas R, inhibits ANG II-mediated release of NE in hypothalamic nuclei (13). Taken together, these studies indicate that Mas R-NO· signaling pathway is involved in many of the beneficial effects of Ang-(1–7) in central neurons as well as the peripheral vasculature.

To clarify the functional role of Mas-NO· signaling in mediating the effects of Ang-(1–7) on neuronal activity, outward K^+ current was recorded from differentiated catecholaminergic CATH.a neurons. First, we assessed mRNA and protein levels of Mas R and three isoforms of NOS in differentiated CATH.a neurons. Our results show that Mas R mRNA and protein are expressed in differentiated CATH.a neurons under basal conditions. We also found nNOS to be highly expressed in CATH.a neurons in relation to eNOS and iNOS. Consistent with this observation, in the brain stem nNOS is more abundant than iNOS in NOS-positive autonomic neurons, whereas eNOS is mainly associated with vascular endothelial cells (11). Furthermore, our data indicated that nNOS is the primary isoform responsible for the increased NO· levels observed in CATH.a neurons following Ang-(1–7) stimulation. In addition, the Ang-(1–7)-induced increase in NO· is mediated by Mas R activation. Interestingly, nNOS but not eNOS, expression was reduced in ventricular myocytes from *Mas*$^{-/-}$ mice, suggesting that nNOS signaling is also associated with Ang-(1–7)/Mas R action in other cell types (8, 14).

In the present studies, we have not attempted to determine the intermediate pathways of Mas R-dependent NO· production induced by Ang-(1–7) nor the specific types of K^+ channels that are affected. However, a phosphatidylinositol 3′-kinase-protein kinase B (Akt) pathway is involved in Ang-(1–7) Mas R axis-dependent eNOS activation in endothelial cells (28). Whether a similar pathway and/or a Ca^{2+}-related mechanism is involved in Ang-(1–7)-induced increase in NO· levels in CATH.a neurons remain to be elucidated.

Neuronal firing rate depends on the activity of membrane ion channels. Using whole cell patch clamp recordings, Sumners and colleagues and our recent data showed that ANG II inhibits I_{Kv} in CATH.a neurons via superoxide ($O_2^{\cdot -}$)-dependent signaling (34, 42). In contrast to ANG II, in the present study, Ang-(1–7) increases I_{Kv} via activation of NOS and generation of NO·. Potassium channel modulation by NO· can be induced either indirectly through the classical pathway of sGC-cGMP-PKG activation (6), or direct effects, such as *S*-nitrosylation of target proteins (19). It has been shown that NO· can activate various types of K^+ channels. For example, NO· activates

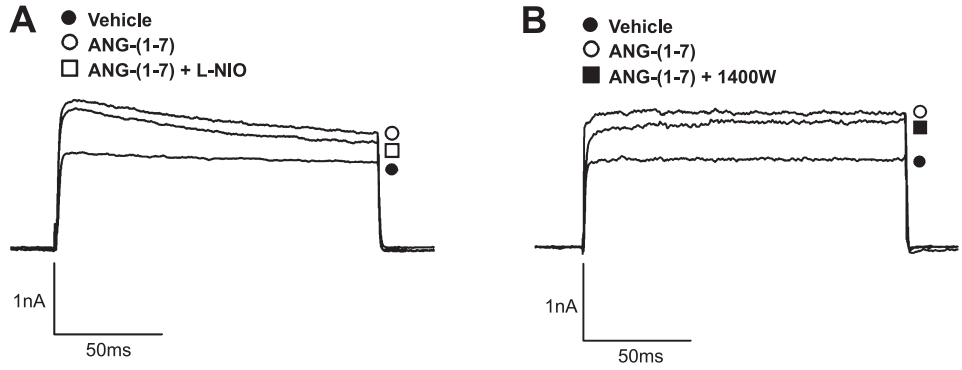

Fig. 6. eNOS and iNOS have no effect on Ang-(1–7)-induced activation of K^+ current. Representative K^+ current traces in CATH.a neuron induced by 1 episodic voltage step from −80 to +80 mV during superfusion of vehicle, Ang-(1–7) (100 nM), Ang-(1–7) plus L-NIO (1 μM, *A*), or 1400W (1 μM, *B*).

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 28, 2017

voltage-gated K^+ channels in PVN neurons and crustacean skeletal muscle via cGMP transduction pathway (15, 41). In a number of publications it was shown that that $NO^\cdot$ can activate calcium-activated K^+ channels in hippocampal CA1 neurons (37), as well as vascular and nonvascular smooth muscle cells (3, 36). $NO^\cdot$ also enhanced ATP-sensitive K^+ channels in cardiomyocytes (23) and sensory neurons by direct *S*-nitrosylation (19). The specific type(s) of voltage-sensitive K^+ channels and their mechanism of activation by $NO^\cdot$ in CATH.a neurons cannot be deduced from the present study and will require further investigation.

Chappell et al. first reported that endogenous Ang-(1–7) is present at concentrations approximately equivalent to ANG II and ANG I in the hypothalamus and medulla oblongata (6). Ang-(1–7) has been identified as a neuromodulator in the CNS, especially in those areas related to cardiovascular regulation. Microinjection of Ang-(1–7) into the NTS of normotensive rats not only decreased blood pressure but also produced facilitation of baroreceptor control of heart rate (7). Intrahypothalamic injection of Ang-(1–7) reduced blood pressure in sinoaortic denervated rats but not in sham animals (17). Together, these studies suggest that neuronal activity in central cardiovascular-control regions could be the potential target for Ang-(1–7) in regulation of blood pressure and baroreceptor sensitivity.

Central NOS inhibition increases blood pressure and central sympathetic outflow (38, 43), suggesting $NO^\cdot$ modulates autonomic neuronal activity associated with tonic restraint of sympathetic outflow from the CNS areas, including the PVN, NTS, and RVLM. The cellular sources contributing to $NO^\cdot$ production and release within these areas are not well understood. However, using cultured RVLM-projecting PVN neurons in vitro, Li et al. (22) found nNOS to be the major source of $NO^\cdot$ involved in inhibiting neuronal firing. Furthermore, the prevalence of nNOS over iNOS activity in the RVLM suggests that nNOS is a primary source of $NO^\cdot$ production in these neurons (5). Expression of nNOS in the NTS and RVLM is reduced in rats with heart failure and contributes to the enhanced sympathetic drive in this disease (16, 25), suggesting that nNOS-derived $NO^\cdot$ is associated with maintenance of sympathetic outflow. Our data demonstrate that nNOS-specific inhibitor SMTC prevents Ang-(1–7)-induced K^+ current activation; whereas eNOS- and iNOS-specific inhibitors do not alter Ang-(1–7)-induced increase in K^+ current in CATH.a neurons. These observations underscore the important role of nNOS in regulation of neuronal activity.

Data presented herein were obtained from catecholaminergic CATH.a neurons, a CNS neuronal cell line isolated from the brain stem, and this may be considered a limitation of this study. However, numerous characteristics of the CATH.a neurons make them an appropriate cell model to study Ang-(1–7) intraneuronal signaling. For example, CATH.a neurons express neuronal-specific markers, such as neurofilament, synaptophysin, and voltage-gated Na^+, K^+, and Ca^{2+} channels (21, 35). Morphologically, differentiated CATH.a neurons exhibit mature neuronal properties including neurites and synaptic vesicles (26). In addition, previous studies clearly identify CATH.a neurons as an appropriate cell model to study the intraneuronal signaling pathways of angiotensins. For example, many of the well-known ANG II-dependent intraneuronal signaling intermediates, including superoxide, PKC, and CaMKII, are similarly activated in both CATH.a neurons and primary neurons isolated from the hypothalamus or brain stem (34, 42). Together with our observation that CATH.a neurons express Mas R and nNOS, these studies support utilizing CATH.a neurons to investigate Ang-(1–7) intraneuronal signaling. Nevertheless, as with all cell culture-based studies, more studies are needed to confirm the role of nNOS-derived $NO^\cdot$ in mediating Ang-(1–7) action in primary neurons isolated from cardiovascular control brain regions, such as SFO, PVN, and/or RVLM, and in vivo experiments.

In summary, our findings demonstrate that nNOS and Mas R are highly expressed in relation to eNOS and iNOS in differentiated CATH.a neurons. We further identified nNOS as the primary source of elevated $NO^\cdot$ levels following Ang-(1–7) stimulation of neurons and that this response is mediated by Mas R activation. Finally, the Ang-(1–7)-induced $NO^\cdot$ production activates increased outward I_{Kv} current in CATH.a neurons, which may provide a cellular basis for the ability of Ang-(1–7) to restrain central sympathetic nerve activity.

ACKNOWLEDGMENTS

We acknowledge Janice A. Taylor and James R. Talaska of the Confocal Laser Scanning Microscope Core Facility at the University of Nebraska Medical Center for providing assistance with confocal microscopy and the Nebraska Research Initiative and the Eppley Cancer Center for their support of the Core Facility.

GRANTS

The present study was supported by National Institutes of Health Grants P01HL-062222 (to H. D. Schultz) and P20RR-017675 (to M. C. Zimmerman) and by a Scientist Development Grant (0930204N) from the American Heart Association (to M. C. Zimmerman).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

1. **Alenina N, Bader M, Walther T.** Imprinting of the murine MAS protooncogene is restricted to its antisense RNA. *Biochem Biophys Res Commun* 290: 1072–1078, 2002.
2. **Becker LK, Etelvino GM, Walther T, Santos RA, Campagnole-Santos MJ.** Immunofluorescence localization of the receptor Mas in cardiovascular-related areas of the rat brain. *Am J Physiol Heart Circ Physiol* 293: H1416–H1424, 2007.
3. **Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, Cohen RA.** Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* 368: 850–853, 1994.
4. **Campagnole-Santos MJ, Diz DI, Santos RA, Khosla MC, Brosnihan KB, Ferrario CM.** Cardiovascular effects of angiotensin-(1–7) injected into the dorsal medulla of rats. *Am J Physiol Heart Circ Physiol* 257: H324–H329, 1989.
5. **Chang AY, Chan JY, Chan SH.** Differential distribution of nitric oxide synthase isoforms in the rostral ventrolateral medulla of the rat. *J Biomed Sci* 10: 285–291, 2003.
6. **Chappell MC, Brosnihan KB, Diz DI, Ferrario CM.** Identification of angiotensin-(1–7) in rat brain. Evidence for differential processing of angiotensin peptides. *J Biol Chem* 264: 16518–16523, 1989.
7. **Chaves GZ, Caligiorne SM, Santos RA, Khosla MC, Campagnole-Santos MJ.** Modulation of the baroreflex control of heart rate by angiotensin-(1–7) at the nucleus tractus solitarii of normotensive and spontaneously hypertensive rats. *J Hypertens* 18: 1841–1848, 2000.
8. **da Costa Goncalves AC, Leite R, Fraga-Silva RA, Pinheiro SV, Reis AB, Reis FM, Touyz RM, Webb RC, Alenina N, Bader M, Santos RA.** Evidence that the vasodilator angiotensin-(1–7)-Mas axis plays an important role in erectile function. *Am J Physiol Heart Circ Physiol* 293: H2588–H2596, 2007.
9. **Feng Y, Xia H, Cai Y, Halabi CM, Becker LK, Santos RA, Speth RC, Sigmund CD, Lazartigues E.** Brain-selective overexpression of human

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 28, 2017

Angiotensin-converting enzyme type 2 attenuates neurogenic hypertension5. *Circ Res* 106: 373–382, 2010.

10. **Fontes MA, Silva LC, Campagnole-Santos MJ, Khosla MC, Guertzenstein PG, Santos RA.** Evidence that angiotensin-(1–7) plays a role in the central control of blood pressure at the ventro-lateral medulla acting through specific receptors. *Brain Res* 665: 175–180, 1994.
11. **Forstermann U, Kleinert H, Gath I, Schwarz P, Closs EI, Dun NJ.** Expression and expressional control of nitric oxide synthases in various cell types. *Adv Pharmacol* 34: 171–186, 1995.
12. **Gao L, Wang W, Li YL, Schultz HD, Liu D, Cornish KG, Zucker IH.** Sympathoexcitation by central ANG II: roles for AT1 receptor upregulation and NAD(P)H oxidase in RVLM. *Am J Physiol Heart Circ Physiol* 288: H2271–H2279, 2005.
13. **Gironacci MM, Vatta M, Rodriguez-Fermepin M, Fernandez BE, Pena C.** Angiotensin-(1–7) reduces norepinephrine release through a nitric oxide mechanism in rat hypothalamus. *Hypertension* 35: 1248–1252, 2000.
14. **Gomes ER, Lara AA, Almeida PW, Guimaraes D, Resende RR, Campagnole-Santos MJ, Bader M, Santos RA, Guatimosim S.** Angiotensin-(1–7) prevents cardiomyocyte pathological remodeling through a nitric oxide/guanosine 3′,5′-cyclic monophosphate-dependent pathway. *Hypertension* 55: 153–160, 2010.
15. **Hermann A, Erxleben C.** Nitric oxide activates voltage-dependent potassium currents of crustacean skeletal muscle. *Nitric Oxide* 5: 361–369, 2001.
16. **Hirooka Y, Shigematsu H, Kishi T, Kimura Y, Ueta Y, Takeshita A.** Reduced nitric oxide synthase in the brainstem contributes to enhanced sympathetic drive in rats with heart failure. *J Cardiovasc Pharmacol* 42, *Suppl* 1: S111–S115, 2003.
17. **Hocht C, Gironacci MM, Mayer MA, Schuman M, Bertera FM, Taira CA.** Involvement of angiotensin-(1–7) in the hypothalamic hypotensive effect of captopril in sinoaortic denervated rats. *Regul Pept* 146: 58–66, 2008.
18. **Hocht C, Opezzo JA, Gironacci MM, Pena C, Taira CA.** Hypothalamic cardiovascular effects of angiotensin-(1–7) in spontaneously hypertensive rats. *Regul Pept* 135: 39–44, 2006.
19. **Kawano T, Zoga V, Kimura M, Liang MY, Wu HE, Gemes G, McCallum JB, Kwok WM, Hogan QH, Sarantopoulos CD.** Nitric oxide activates ATP-sensitive potassium channels in mammalian sensory neurons: action by direct S-nitrosylation. *Mol Pain* 5: 12, 2009.
20. **Kojima H, Nakatsubo N, Kikuchi K, Kawahara S, Kirino Y, Nagoshi H, Hirata Y, Nagano T.** Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal Chem* 70: 2446–2453, 1998.
21. **Lazaroff M, Dunlap K, Chikaraishi DM.** A CNS catecholaminergic cell line expresses voltage-gated currents. *J Membr Biol* 151: 279–291, 1996.
22. **Li Y, Zhang W, Stern JE.** Nitric oxide inhibits the firing activity of hypothalamic paraventricular neurons that innervate the medulla oblongata: role of GABA. *Neuroscience* 118: 585–601, 2003.
23. **Ljubkovic M, Shi Y, Cheng Q, Bosnjak Z, Jiang MT.** Cardiac mitochondrial ATP-sensitive potassium channel is activated by nitric oxide in vitro. *FEBS Lett* 581: 4255–4259, 2007.
24. **Oliveira DR, Santos RA, Santos GF, Khosla M, Campagnole-Santos MJ.** Changes in the baroreflex control of heart rate produced by central infusion of selective angiotensin antagonists in hypertensive rats. *Hypertension* 27: 1284–1290, 1996.
25. **Patel KP, Zhang K, Zucker IH, Krukoff TL.** Decreased gene expression of neuronal nitric oxide synthase in hypothalamus and brainstem of rats in heart failure. *Brain Res* 734: 109–115, 1996.
26. **Qi Y, Wang JK, McMillian M, Chikaraishi DM.** Characterization of a CNS cell line, CAD, in which morphological differentiation is initiated by serum deprivation. *J Neurosci* 17: 1217–1225, 1997.
27. **Sakima A, Averill DB, Gallagher PE, Kasper SO, Tommasi EN, Ferrario CM, Diz DI.** Impaired heart rate baroreflex in older rats: role of endogenous angiotensin-(1–7) at the nucleus tractus solitarii. *Hypertension* 46: 333–340, 2005.
28. **Sampaio WO, Souza dos Santos RA, Faria-Silva R, da Mata Machado LT, Schiffrin EL, Touyz RM.** Angiotensin-(1–7) through receptor Mas mediates endothelial nitric oxide synthase activation via Akt-dependent pathways. *Hypertension* 49: 185–192, 2007.
29. **Santos RA, Campagnole-Santos MJ, Andrade SP.** Angiotensin-(1–7): an update. *Regul Pept* 91: 45–62, 2000.
30. **Santos RA, Ferreira AJ, Pinheiro SV, Sampaio WO, Touyz R, Campagnole-Santos MJ.** Angiotensin-(1–7) and its receptor as a potential targets for new cardiovascular drugs. *Expert Opin Investig Drugs* 14: 1019–1031, 2005.
31. **Santos RA, Frezard F, Ferreira AJ.** Angiotensin-(1–7): blood, heart, and blood vessels. *Curr Med Chem Cardiovasc Hematol Agents* 3: 383–391, 2005.
32. **Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de B, I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T.** Angiotensin-(1–7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci USA* 100: 8258–8263, 2003.
33. **Silva LC, Fontes MA, Campagnole-Santos MJ, Khosla MC, Campos RR, Jr, Guertzenstein PG, Santos RA.** Cardiovascular effects produced by micro-injection of angiotensin-(1–7) on vasopressor and vasodepressor sites of the ventrolateral medulla. *Brain Res* 613: 321–325, 1993.
34. **Sun C, Du J, Raizada MK, Sumners C.** Modulation of delayed rectifier potassium current by angiotensin II in CATH.*a* cells. *Biochem Biophys Res Commun* 310: 710–714, 2003.
35. **Suri C, Fung BP, Tischler AS, Chikaraishi DM.** Catecholaminergic cell lines from the brain and adrenal glands of tyrosine hydroxylase-SV40 T antigen transgenic mice. *J Neurosci* 13: 1280–1291, 1993.
36. **Thornbury KD, Ward SM, Dalziel HH, Carl A, Westfall DP, Sanders KM.** Nitric oxide and nitrosocysteine mimic nonadrenergic, noncholinergic hyperpolarization in canine proximal colon. *Am J Physiol Gastrointest Liver Physiol* 261: G553–G557, 1991.
37. **Tjong YW, Li M, Hung MW, Wang K, Fung ML.** Nitric oxide deficit in chronic intermittent hypoxia impairs large conductance calcium-activated potassium channel activity in rat hippocampal neurons. *Free Radic Biol Med* 44: 547–557, 2008.
38. **Tseng CJ, Liu HY, Lin HC, Ger LP, Tung CS, Yen MH.** Cardiovascular effects of nitric oxide in the brain stem nuclei of rats. *Hypertension* 27: 36–42, 1996.
39. **Veerasingham SJ, Raizada MK.** Brain renin-angiotensin system dysfunction in hypertension: recent advances and perspectives. *Br J Pharmacol* 139: 191–202, 2003.
40. **Xu P, Costa-Goncalves AC, Todiras M, Rabelo LA, Sampaio WO, Moura MM, Santos SS, Luft FC, Bader M, Gross V, Alenina N, Santos RA.** Endothelial dysfunction and elevated blood pressure in MAS gene-deleted mice. *Hypertension* 51: 574–580, 2008.
41. **Yang Q, Chen SR, Li DP, Pan HL.** Kv1.1/1.2.channels are downstream effectors of nitric oxide on synaptic GABA release to preautonomic neurons in the paraventricular nucleus. *Neuroscience* 149: 315–327, 2007.
42. **Yin JX, Yang RF, Li S, Renshaw AO, Li YL, Schultz HD, Zimmerman MC.** Mitochondria-produced superoxide mediates angiotensin II-induced inhibition of neuronal potassium current. *Am J Physiol Cell Physiol* 298: C857–C865, 2010.
43. **Young CN, Fisher JP, Gallagher KM, Whaley-Connell A, Chaudhary K, Victor RG, Thomas GD, Fadel PJ.** Inhibition of nitric oxide synthase evokes central sympatho-excitation in healthy humans. *J Physiol* 587: 4977–4986, 2009.
44. **Zhang Y, Lu J, Shi J, Lin X, Dong J, Zhang S, Liu Y, Tong Q.** Central administration of angiotensin-(1–7) stimulates nitric oxide release and upregulates the endothelial nitric oxide synthase expression following focal cerebral ischemia/reperfusion in rats. *Neuropeptides* 42: 593–600, 2008.
45. **Zucker IH.** Novel mechanisms of sympathetic regulation in chronic heart failure. *Hypertension* 48: 1005–1011, 2006.

Downloaded from http://ajpcell.physiology.org/ by 10.220.33.6 on May 28, 2017