Angiotensin AT1-Receptors Depolarize Neonatal Spinal Motoneurons and Other Ventral Horn Neurons Via Two Different Conductances

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Oz, Murat, and Leo P. Renaud. Angiotensin AT1-receptors depolarize neonatal spinal motoneurons and other ventral horn neurons via two different conductances. J Neurophysiol 88: 2857–2863, 2002; 10.1152/jn.00978.2001. Angiotensin receptors are highly expressed in neonatal spinal cord. To identify their influence on neuronal excitability, we used patch-clamp recordings in spinal cord slices to assess responses of neonatal rat (5–12 days) ventral horn neurons to bath-applied angiotensin II (ANG II; 1 μM). In 14/34 identified motoneurons tested under current clamp, ANG II induced a slowly rising and prolonged membrane depolarization, blockable with Losartan (n = 5) and (Sar1, Val5, Ala8)-ANG II (Saralasin, n = 4) but not PD123319 (1 μM each; n = 4). Under voltage clamp (VH = −65 mV), 7/22 motoneurons displayed an ANG-II-induced tetrodotoxin-resistant inward current (−128 ± 31 pA) with a similar time course, an associated reduction in membrane conductance and net current reversal at −98.8 ± 3.9 mV. Losartan-sensitive ANG II responses were also evoked in 27/78 tested ventral horn “interneurons.” By contrast with motoneurons, their ANG-II-induced inward current was smaller (−39.9 ± 5.2 pA) and reversed at −37.9 ± 3.6 mV. In 12 cells, I-V lines remained parallel with no reversal within the current range tested. Intracellular dialysis with GTP-γ-S significantly prolonged the ANG II effect in seven responsive interneurons and GDP-B-S significantly reduced the ANG II response in four other cells. Peak inward currents were significantly reduced in all 13 responding neurons recorded in slices incubated in pertussis toxin (5 μg/ml) for 12–18 h or in 12 neurons perfused with N-ethylmaleimide. Of 29 interneurons sensitive to pertussis toxin or N-ethylmaleimide treatment, 9 cells displayed a decrease in membrane conductance that reversed at −101.3 ± 3.8 mV. In eight cells, membrane conductance increased and reversed at −38.7 ± 3.4 mV. In 12 cells, the I-V lines remained parallel with no reversal within the current range tested, suggesting that both conductances are modulated by pertussis toxin-sensitive G proteins. These observations reveal a direct, G-protein-mediated depolarizing action of ANG II on neonatal rat ventral horn neurons. They also imply involvement of two distinct conductances that are differentially distributed among different cell types.

I N T R O D U C T I O N

In the peripheral circulation, angiotensin (ANG II) is not only a powerful modulator of vasomotor activity but can act through neurons in the subfornical organ, a circumventricular organ, to initiate a behavioral response, i.e., drinking, a response dependent on a central renin-angiotensin system whose roles also include modulation of cardiovascular reflexes (Phillips 1987; Summers et al. 1994). In CNS, angiotensin-like immunoreactive neurons and fibers as well as ANG II receptors, mainly of the AT1 type, have a wide but differential distribution (see Gehlert et al. 1986; Lind et al. 1984; Mendelsohn et al. 1984). That the latter participate in regulating neuronal excitability is inferred from electrophysiological observations of neuronal responsiveness to exogenous ANG II (e.g., Bai and Renaud 1998; Ferguson and Washburn 1998; Li and Guyenet 1996; Ono et al. 2001; Yang et al. 1992). These features indicate considerable regional and cell-specific expression of ANG II receptors.

Binding studies and receptor-expression analyses also indicate plasticity within the central renin-angiotensin system. A high level of expression during the first and second postnatal week and change during ontogeny (Tsutsumi and Saavedra 1991) suggest a physiologically important role in CNS development. One of these sites may be in spinal cord, where ANG II-immunoreactive fibers and receptors have been observed (Gehlert et al. 1986; Lind et al. 1984; White et al. 1988) and where ANG II has a depolarizing action on motoneurons (Suzue et al. 1981) and lateral horn neurons (Lewis and Coote 1993). There are few details on mechanisms of ANG II action on spinal neurons.

During patch-clamp recordings to evaluate functional evidence for peptide receptors on thoracolumbar neurons in neonatal spinal cord, we observed that a subpopulation of motoneurons and unidentified ventral horn neurons were responsive to exogenous ANG II. We now report that these neurons display ANG-II-induced membrane depolarization and inward currents that engage two separate conductances: a potassium conductance in motoneurons and/or a presumed nonselective cationic conductance in interneurons.

M E T H O D S

The spinal cords of methoxyflurane-anesthetized Sprague-Dawley rats of either sex (5–12 days old) were removed after a dorsal thoracolumbar laminectomy and placed in ice-cold (4°C) artificial cerebrospinal fluid (ACSF). ACSF was composed of (in mM) 127 NaCl, 26 NaHCO3, 3.1 KCl, 1.2 MgCl2, 2.4 CaCl2, and 10 D-glucose (pH 7.35; osmolarity 290–305 mosmol) and was gassed with 95% O2–5% CO2. Transverse 350–450 μm sections from the Th3 to L5
segments were cut on a vibratome, equilibrated in ACSF at room temperature, and continuously superfused at 4–6 ml/min in a recording chamber.

Using the blind whole cell patch-clamp technique, neurons were recorded using Axopatch 1A or an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). Micropipettes were filled with (in mM) 130 K-Gluconate, 10 KCl, 10 NaCl, 1 MgCl₂, 10 HEPES, 1 EGTA, 1 GTP, and 2 Mg-ATP, adjusted to a pH of 7.3 with Tris buffer. Lucifer yellow (1 mg/ml) was included for later visualization and morphological identification using methods described earlier (Kolaj and Renaud 1998). Corrections of the liquid-junction potentials (approximately −10 mV) were performed off-line. Data was filtered on-line at 2 kHz. Digidata 1200 interface and version 7 of pCLAMP software were used on-line to generate clamp commands. Motoneurons were identified by their all-or-none antidiromic responses to ventral stimulation applied with a concentric bipolar electrode (1–10 V, duration 0.02 s) and/or by their morphology and evidence of an axon projecting toward the ventral root. In this study, we applied the term interneuron to any other neuron.

ANG II, a peptide receptor antagonist (Sar¹, Val⁵, Ala⁸)-ANG II (Saralasin), a nonpeptide AT₂ receptor antagonist PD123319, GTP, GTP-γ-S, GDP-β-S, pertussis toxin, and tetrodotoxin were from Sigma-RBI (St. Louis, MO). Agents were dissolved in ACSF at their final concentrations, and delivered by bath application at a perfusion rate of 4 ml/min. Losartan, a nonpeptide AT₁ receptor antagonist, was obtained from Merck (Rathway, NJ). For statistical evaluation, we used paired or unpaired Student’s t-test as indicated in the text. Results are presented as means ± SE.

RESULTS

Motoneurons

Data obtained from 56 motoneurons located in (within Rexed laminae VIII and IX) displayed a mean resting membrane potential of −71.4 ± 1.9 mV and input resistance of 60.9 ± 4.3 MΩ.

ANG-II-INDUCED POSTSYNAPTIC MEMBRANE DEPOLARIZATION AND INWARD CURRENT. In 14/34 neurons tested while recording in current-clamp mode, bath application of ANG II (1 µM; 30 s) initiated a slowly rising (60–90 s) to peak) membrane depolarization that reached a plateau of 11.6 ± 3.2 mV and was sufficient to trigger a burst of action potentials in five cells (Fig. 1A). Some desensitization seems likely since washout intervals of 30–45 min were required to regain full recovery. Application of an AT₁ receptor antagonist Losartan, (1 µM; n = 5 cells) was without effect on resting membrane properties but completely blocked the ANG-II-induced membrane depolarizations. Similar results were achieved with Saralasin (1 µM; n = 4 cells). However, an AT₂ receptor antagonist PD123319 (1 µM; n = 5, and specific AT-receptor antagonist (Sar¹, Val⁵, Ala⁸)-angiotensin II (1 µM; n = 4) on ANG-II-induced membrane depolarizations (n = total of 14 cells). Asterisk: P < 0.05, compared with control values obtained without pretreatments (paired Student’s t-test for each group control values).

Under voltage clamp (V_{m} −65 mV) and in the presence of 1 µM TTX, 1 µM ANG II applications to 22 motoneurons yielded seven cells that responded with a slowly developing postsynaptic response characterized by inward current (peak of −128 ± 31 pA) that slowly recovered over 8–10 min (Fig. 2A). Comparison of instantaneous I-V plots before and at the peak of the ANG II responses revealed net ANG II currents (difference between control and peak ANG II effect) whose slope indicated a 19.1% reduction in membrane conductance (from 11.3 ± 1.8 to 8.9 ± 1.3 nS). The mean net ANG II current reversed at −98.8 ± 3.9 mV, approximating the estimated equilibrium potential for potassium ions under these conditions (Fig. 2B). Increasing extracellular concentration of K⁺ to 10 mM shifted the reversal potential to −68 ± 2.3 mV (Fig. 2C; n = 3 cells) implying mediation via potassium channels.

Interneurons

A population of 78 cells labeled as interneurons based on failure of antidiromic activation displayed a significantly less negative resting membrane potential (−59.8 ± 1.4 mV; P < 0.05, Student’s t-test) and higher input resistance (179.8 ± 14.3 MΩ; P < 0.05, Student’s unpaired t-test) than motoneurons.

ANG-II-INDUCED POSTSYNAPTIC RESPONSES. Recordings from 78 neurons in voltage-clamp mode in the presence of TTX yielded 27 neurons that responded to ANG II (1 µM) with a mean inward current of −37.9 ± 5.2 pA (Fig. 3A). Although
different in magnitude from the response in motoneurons, the
time course of these ANG-II-induced current was virtually
identical (compare Figs. 2A and 3A). Similarly, responses to
ANG II were blocked by prior application of Saralasin (1 μM;
3/3 cells) and Losartan (1 μM; 4/4 cells), but not by PD123319
(1 μM, 4/4), all without effect on resting membrane properties
(Fig. 3A).

Analyses of voltage-current relationships obtained at the
peak of the responses for the population of cells indicated a small
decrease in membrane conductance (from a control value of
4.9 ± 0.8 to 4.6 ± 1.2 nS; P > 0.05, paired t-test). However,
a comparison of the net ANG-II-induced currents for individ-
ual cells revealed three significantly differing patterns (Fig.
3B). One group of eight neurons demonstrated a net ANG-II-
induced conductance that decreased from a control of 4.5 ±
0.7 to 3.8 ± 0.8 nS (15.6% decrease) and reversed close to
−100 mV (−102.6 ± 4.3 mV). Thus the data from this group
resembled that from the motoneuron population, suggesting
mediated of the ANG II response via reduction in conductance
for potassium ions. By contrast, in another seven cells, the
slope of the net ANG II current reflected an increase
in conductance (from 4.8 ± 0.6 to 5.3 ± 1.2 nS, 11.3% increase)
with current reversal close to −40 mV (−38.4 ± 4.1 mV).
These values could reflect mediation of the ANG II response
via increase in a nonselective cationic conductance. In the
remaining 12 cells, the mean net ANG-II-induced current
displayed a slight reduction (only 3.6% decrease) in membrane
conductance (from 5.1 ± 1.2 to 4.9 ± 1.1 nS), with no reversal
within the voltage range tested.

Because ANG II receptors belong to family of G-protein-
coupled receptors, we first compared the control data (contain-
ing 1 mM GTP) with data obtained 5 min after establishment
of seals using pipettes that contained GTP-γ-S (0.4 mM), a
nonhydrolyzable derivative of GTP that activates G protein in
an irreversible manner (Gilman 1987). Of 18 cells recorded
and tested with ANG II, 7 cells responded. The maximum
amplitudes of their response (−38.7 ± 6.1 pA) were not
significantly different from control, but the recovery phase was
greatly prolonged by the presence of GTP-γ-S in the pipette
(Fig. 4A). Of 7 cells treated with GTP-γ-S, membrane conduc-
tance increased in two cells (reversed close to E_K), decreased
in two cells (reversed close to −40 mV), and was unchanged
in the remaining three cells (with no reversal potential detected
in the range of −10 to −120 mV). We also diazylized four cells
with GDP-β-S (1 mM for 10 min), a stable analogue of GDP
that competitively inhibits G protein binding by GTP (Gilman
1987). The data from four cells indicated that mean ANG-II-
induced inward current was significantly reduced (−17.4 ± 3.7
vs. −37.9 ± 5.2 pA in 27 control cells; P < 0.05, Student’s
unpaired t-test, Fig. 4B).
containing PTX, and the magnitudes maximal ANG-II-induced currents were compared. In the control cells, we also tested bath-applied NEM (50 μM), a sulphydryl-alkylating agent shown to block G-protein-effector interactions by alkylating the α-subunits of PTX-sensitive GTP-binding protein (Shapiro et al. 1994; Viana and Hille 1996). The advantage of using NEM was that it allowed us to examine ANG-II-induced currents before and after inhibition of PTX-sensitive G proteins within the same cell.

In control slices, mean ANG-II-induced inward currents in 13 of 29 cells tested were 38.4 ± 4.1 pA. The mean ANG-II-induced inward current was significantly reduced to 16.8 ± 2.1 in PTX treated neurons (Fig. 4C, P < 0.05, Student’s unpaired t-test). Further analysis of the net ANG-II-induced currents for individual cells revealed three differing patterns (Fig. 5). One group of four neurons demonstrated a net ANG-II-induced conductance that decreased from a control of 4.4 ± 0.5 to 3.7 ± 0.6 nS and reversed close to −100 mV (−101.2 ± 4.1 mV). In another four cells, the slope of the net ANG-II-induced current reflected an increase in conductance (from 4.6 ± 0.6 to 5.1 ± 1.1 nS) with current reversal close to −40 mV (−39.4 ± 3.7 mV). In the remaining five cells, the mean net ANG-II-induced current displayed a slight reduction in membrane conductance (from 4.9 ± 1.2 to 4.7 ± 0.9 nS), with no reversal within the voltage range tested.

In PTX-incubated slices, 16 of 34 neurons responded to ANG II. Among these cells, one group of five neurons demonstrated a net ANG-II-induced conductance that decreased from a control of 2.8 ± 0.6 to 2.1 ± 0.5 nS and reversed close to −100 mV (−102.9 ± 3.4 mV). In another four cells, the slope of the net ANG-II current showed an increase in conductance (from 2.7 ± 0.5 to 3.1 ± 0.8 nS) with current reversal close to −40 mV (−37.7 ± 2.8 mV). In the remaining seven cells, the mean net ANG-II-induced current displayed a slight reduction in membrane conductance (from 2.9 ± 0.7 to 2.6 ± 0.8 nS), with no reversal within the voltage range tested.

In 12 of 13 cells in the control group described earlier, a 5-min exposure treatment with 50 μM NEM was then observed. The ANG-II-induced inward current was then converted to 31.4% of control values (12.2 ± 3.6 vs. 38.1 ± 4 pA in controls, P < 0.05, Student’s paired t-test, Fig. 4C). Further analysis revealed that all patterns of conductance were sensitive to NEM pretreatment (Fig. 5C). In four cells with current reversal close to −100 mV, ANG-II-induced inward currents were significantly reduced from 39.2 ± 4.3 (controls) to 14.6 ± 3.5 pA (P < 0.05, Student’s paired t-test). In another four cells with current reversal close to −40 mV, ANG-II-induced inward currents were significantly reduced from 36.9 ± 4.1 (controls) to 11.6 ± 3.2 pA (P < 0.05, Student’s paired t-test). In the remaining four cells with no current reversal within the voltage range tested, ANG-II-induced inward currents were significantly reduced from 40.2 ± 4.3 (controls) to 12.9 ± 3.4 pA (P < 0.05, Student’s paired t-test).

**DISCUSSION**

The observation that exogenous ANG II induces membrane depolarization and inward currents in a subpopulation of neonatal spinal ventral horn neurons, including identified motoneurons, implies the presence of functional angiotensin receptors. These receptors are of the AT1 subtype because responses were sensitive to pretreatment with Losartan, a selective AT1 receptor antagonist, but not with PD123319, which selectively blocks angiotensin AT2 type receptors.

Our analyses of I-V relationships suggest that more than one membrane conductance underlies the depolarizing action of ANG II. In both motoneurons and a subpopulation of unidentified neurons, the ANG-II-induced inward currents were con-
sistently associated with reduction in a membrane conductance that reversed close to the potassium equilibrium potential and shifted appropriately with the transmembrane potassium gradient. In these neurons, the linearity and reversal potential for the net ANG-II-induced currents indicate involvement of a voltage-independent potassium conductance that contributes to resting membrane potential, often referred to as a leak conductance. Similar potassium-mediated responses to ANG II can be seen in rat bulbospinal (Li and Guyenet 1996) and median preoptic neurons (Bai and Renaud 1998) and hamster submandibular ganglion neurons (Ikegami et al. 2000). In rat adrenal glomerulosa cells, ANG II suppresses a potassium conductance

FIG. 5. ANG-II-regulated conductances are sensitive to PTX and N-ethylmaleimide. In 13 control neurons, after overnight incubation in ACSF, net ANG-II-induced inward current (● in A–C) analyses indicated 3 patterns in I-V relationships: current that reversed at −101.2 ± 4.1 mV (A; n = 4 cells); current that reversed at −39.4 ± 5.7 mV (B; n = 4 cells); and current that demonstrated no obvious reversal potential (C; n = 5 cells). In the same control cells, tested again after 30–45 min, incubation with 50 μM N-ethylmaleimide (n = 12) for 5 min significantly reduced the current sizes in all 3 types of ANG II responses (● in A–C). In another 16 cells, recorded after overnight incubation in ACSF containing PTX (5 μg/ml), current sizes were significantly reduced compared with control neurons (● in A–C; n = 13). In 5 of 16 cells, currents were reversed at −102.9 ± 3.4 mV (A); in 4 cells, currents were reversed at −37.7 ± 2.8 mV (B); in 7 cells, currents did not show an apparent reversal potential (C).

FIG. 4. ANG-II-induced inward currents are mediated through pertussis toxin-sensitive G proteins in interneurons. A: in voltage-clamp mode (Vh = −65 mV) and in presence of tetrodotoxin, the top trace displays a control ANG-II-induced current that recovers after several minutes. The bottom trace from another cell dialyzed with GTP-γ-S (0.4 mM, for 10 min) illustrates inward current that fails to recover. The plot below the current traces illustrates the averaged data for the time course of the normalized inward current induced by 1 μM ANG II. The maximal currents were defined as the difference between the resting currents and maximal amount of ANG II induced inward current. Currents from the cells recorded with GTP are depicted as closed symbols (control, n = 12), those recorded with GTP-γ-S as ○ (n = 5). B: ANG-II-induced currents are mediated by pertussis toxin (PTX)-sensitive G proteins. Histograms (left to right): normalized control ANG-II-induced current recorded after overnight incubation in ACSF (control, n = 13), in ACSF containing PTX (5 μg/ml, n = 16), after 5 min incubation with 50 μM N-ethylmaleimide (n = 12), and in cells recorded with electrodes containing GDP-β-S (1 mM for 10 min, n = 4). *, P < 0.05, compared with values obtained without pretreatment (paired or unpaired Student’s t-test compared with control values of each group).
that in brain stem motoneurons is attributed to a family of two-pore domain pH-sensitive channels, named TASK-1 (Czirjak and Enyedi 2002; Czirjak et al. 2000; Talley and Bayliss 2002; Talley et al. 2000, 2002). Whether similar channels are responsible for ANG-II-induced inward currents in neonatal spinal motoneurons remains to be established.

By contrast, the ANG-II-induced inward currents in a population of interneurons were associated with an increase in membrane conductance that reversed around $-40\,\text{mV}$, suggestive of a nonselective cationic conductance. This is similar to the ANG-II-induced responses in rat supraoptic neurons where net currents also reverse close to $-40\,\text{mV}$ (Yang et al. 1992). In a subpopulation of rat adrenal glomerulosa cells, net ANG II currents reverse around $-10\,\text{mV}$ (Lotshaw and Li 1996), and in ANG-II-responsive subfornical organ neurons, a net current that reverses closer to $-30\,\text{mV}$ has been reported (Ono et al. 2001). Whether these differences reflect variations in underlying cationic conductances remains to be clarified, but they do suggest cell specificity in coupling of ANG II receptors to ion channels. Moreover, we speculate that in some neurons, ANG II receptors can couple to both potassium and cationic conductances, in which situation the net ANG II-induced conductance could be reflected in a nearly parallel shift, as observed here in a subpopulation of ventral horn interneurons.

The binding of angiotensin to AT$_1$ receptors can activate a number of intracellular signaling pathways, mediated through heterotrimeric G proteins (see Sumners et al. 1994 for review). These may include G$_{q/11}$ to stimulate phosphoinositide hydrolysis, subsequent activation of protein kinase C and a rise in intracellular calcium, G$_i$ to inhibit adenylate cyclase, and a G$_{q/11}$/Ras/Raf-1 pathway that increases mitogen-activated protein (MAP) kinase activities. While it remains undefined as to the identity of G proteins linking AT$_1$ receptors to the conductances described above, the linkage must involve PTX-sensitive G proteins given the marked reduction in both types of conductances after treatment with PTX or NEM (Fig. 5).

The present observations bear a striking resemblance to the response of neonatal spinal neurons to other peptides including thyrotropin-releasing hormone (Kolaj et al. 1997), vasopressin (Kolaj and Renaud 1998; Oz et al., 2001), and substance P (Yasuda et al., 2001). Two issues that derive from these observations include the sources for ligands for these receptors, and the physiological roles that peptide receptors may have in neuronal function in neonatal spinal cord. One source may derive from the cerebrospinal fluid, which is known to contain a variety of neuropeptides, including some that display a circadian rhythmicity in their concentrations (Reppert et al. 1987). Neuronal pathways may provide a source since angiotensin-like immunoreactivity has been observed in adult spinal cord (Lind et al. 1984); however, information is lacking for neonatal tissue. Whatever the origin, angiotensin receptors may have specific functional links to neuronal development and survival. This is suggested by their ability to undergo developmental changes (Milas et al. 1991), to participate in recovery following axotomy (Palkovits 1995), and to modulate neurite outgrowth from cultured embryonic neurons (Iwasaki et al. 1991; Sood et al. 1990) and by their neurotrophic action in cultured explants of spinal cord (Ikeda et al. 1989; Yang et al. 2001). It is possible that their influence on excitability of neonatal spinal cord neurons is important for their subsequent development.
ANG II RECEPTORS IN NEONATAL VENTRAL SPINAL CORD


