Adrenomedullin influences magnocellular and parvocellular neurons of paraventricular nucleus via separate mechanisms

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Follwell, Matthew J., and Alastair V. Ferguson. Adrenomedullin influences magnocellular and parvocellular neurons of paraventricular nucleus via separate mechanisms. Am J Physiol Regul Integr Comp Physiol 283: R1293–R1302, 2002.—We previously reported that adrenomedullin (AM) decreases blood pressure following microinjection into the paraventricular nucleus of the hypothalamus (PVN) of the rat. With the use of whole cell recordings in rat hypothalamic slice preparations, we characterized the effects of AM on electrophysiologically identified PVN neurons and described the membrane events underlying such actions. AM hyperpolarized magnocellular (type I) neurons in a dose-dependent manner, a response associated with an increase in the frequency and amplitude of inhibitory postsynaptic potentials. Blockade of action potentials with tetrodotoxin (TTX) abolished AM effects on membrane potential and synaptic activity in magnocellular neurons, suggesting direct actions on inhibitory interneurons. Furthermore, blockade of inhibitory synaptic transmission with the GABAA receptor antagonist bicuculline methiodide also abolished AM effects on membrane potential in magnocellular neurons. In contrast, parvocellular (type II) neurons depolarized following AM receptor activation. AM effects on parvocellular neurons were dose dependent and were maintained in the presence of TTX, indicating direct effects on this population of neurons. Voltage-clamp recordings from parvocellular neurons showed AM enhances a nonselective cationic conductance, suggesting a potential mechanism through which AM influences membrane potential. These observations show clear population-specific actions of AM on separate identified groups of PVN neurons. Such effects on magnocellular neurons likely contribute to the hypertensive actions of this peptide in PVN. Although the effects on parvocellular neurons may also contribute to such cardiovascular effects of AM, it is more likely that actions on this population of PVN neurons underlie the previously demonstrated activation effects of AM on the hypothalamic-pituitary-adrenal axis.

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(15, 29). These observations led us to undertake studies in which AM was microinjected directly into the PVN to assess actions of this peptide on cardiovascular regulation. Contrary to our original hypothesis, we observed that such microinjections resulted in a decrease, rather than the predicted increase in BP (27). These observations did, however, clearly establish that AM acts in the PVN to control autonomic output, although they did not, at this stage, address the cellular mechanisms underlying such actions.

The PVN is a bilateral, periventricular structure situated in the hypothalamus that contains separate subpopulations of neurons that regulate both pituitary hormone secretion and autonomic output. Although neurosecretory corticotropin-releasing hormone (CRH)-, AVP-, and oxytocin (OXT)-synthesizing neurons play critical obligatory roles in controlling pituitary hormone secretion (11), PVN neurons that send caudal projections to medullary and spinal autonomic centers play equally important roles in controlling autonomic output. Recent patch-clamp studies in hypothalamic slices have described electrophysiological properties of magnocellular PVN neurons (AVP or OXT immunopositive) that allow specific classification as a distinct subpopulation different from the parvocellular population (CRH-synthesizing and caudally projecting neurons) (2, 32). This study used patch-clamp recording techniques in an in vitro slice preparation in an effort to identify the cellular mechanisms through which AM causes changes in BP as a result of specific actions on separate subpopulations of PVN neurons.

METHODS

**Slice preparation.** Male Sprague-Dawley rats (150–250 g, Charles River, P. Q., Canada) were decapitated, and the brain was quickly removed from the skull and immersed in cold (1–4°C) artificial cerebrospinal fluid (aCSF). The hypothalamus was blocked and 400-μm slices including the PVN were prepared as described previously (18). Slices were incubated in oxygenated aCSF (95% O2-5% CO2) for at least 90 min at room temperature. Thirty minutes before recording, the slice was transferred into an interface-type recording chamber and continuously perfused with oxygenated aCSF at a rate of 1 ml/min.

**Electrophysiological techniques.** Electrophysiological experiments were performed using whole cell configuration of the “blind” patch-clamp technique to record from the PVN neurons. Patch pipettes were pulled to a resistance of 5–8 MΩ filled with a solution containing (in mM): 140 K-gluconate, 0.1 CaCl2, 2 MgCl2, 1.1 EGTA, 10 HEPES, 2 Na2ATP, and adjusted to pH 7.25 with KOH. An Ag-AgCl electrode connected to the bath solution via a KCl-agar bridge served as the reference. The aCSF composition was (in mM): 124 NaCl, 2 KCl, 1.25 KPO4, 2.0 CaCl2, 1.3 MgSO4, 20 NaHCO3, and 10 glucose. An 8- to 12-mV junction potential correction has been applied to the data presented.

**Bath application of AM.** Male Sprague-Dawley rats (150–250 g, Charles River, P. Q., Canada) were decapitated, and the brain was quickly removed from the skull and immersed in cold (1–4°C) artificial cerebrospinal fluid (aCSF). The hypothalamus was blocked and 400-μm slices including the PVN were prepared as described previously (18). Slices were incubated in oxygenated aCSF (95% O2-5% CO2) for at least 90 min at room temperature. Thirty minutes before recording, the slice was transferred into an interface-type recording chamber and continuously perfused with oxygenated aCSF at a rate of 1 ml/min.

Patch clamp recordings were processed using an Axoclamp-2A amplifier, filtered at 3 kHz, digitized using the CED 1401 Plus interface at 5 kHz, and stored on computer for off-line analysis. Data were collected using the Signal (episode-based capture) or Spike2 (continuous recording) packages (CED, Cambridge, UK).

**Analysis.** All cells included in our analysis had a stable resting membrane potential more negative than -40 mV and action potential amplitude of greater than 50 mV. A series of hyperpolarizing current pulses was then applied to determine the identity of the neuron based on its electrophysiological fingerprint (32). Neurons were required to maintain a stable resting membrane potential before application of the agents (minimum 5 mV). After peptide application, responses were assigned to one of three groups: 1) depolarization, characterized as an increase in membrane potential of at least 5 mV followed by a return to baseline; 2) hyperpolarization, characterized as a decrease of at least 5 mV followed by a return to baseline; and 3) no response, characterized as the failure to generate a change in membrane potential of more than 5 mV.

We also assessed changes in input resistance (IR) in response to AM application. I-V curves were plotted from data obtained following a series of current pulses (I-V protocol). I-V protocols were run before AM application, following AM application, and following the return to baseline. If a response was observed, current was applied to return the resting membrane potential to the original baseline before collection of I-V data.

Inhibitory postsynaptic potentials (IPSPs) and miniature IPSPs (mIPSPs) were analyzed using Mini Analysis Program 4.1.1 (Synaptosoft) and quantified based on amplitude (minimum 2 mV) and shape (fast falling phase and slow decay). Each detected event was inspected visually to exclude obvious false IPSPs. The data generated from these analyses were used to graph frequency plots. Changes in IPSP frequency in response to different conditions were compared using the paired Student’s t-test, with P < 0.05 being set as the level for significance. All values are plotted as means ± SE.

**Statistics.** In current-clamp recordings, a change in membrane potential of greater than 3 mV from a baseline, established during the 30 s before peptide application, was arbitrarily determined as indicative of an effect on the recorded cells. For statistical analysis of effects of AM on PVN neuronal properties, means were calculated from cells that were determined to have been affected using these criteria. Changes in IR and peak and steady-state conductance in response to AM were compared using the Student’s t-test. All values are plotted as means ± SE. Linear regression was used to analyze I-V relationships, and the dose-response curve was constructed from sigmoidal function of nonlinear regression.

**RESULTS**

Whole cell patch-clamp recordings were obtained from a total of 99 PVN neurons. These neurons had a mean resting membrane potential of -53.3 ± 2.7 mV, displayed action potentials with a mean amplitude of 78.6 ± 3.4 mV, and had a mean IR of 926 ± 57 MΩ. Cells that possessed a linear I-V relationship and a prominent transient outward rectification were classified as magnocellular (n = 30), whereas those exhibiting an inward rectification at hyperpolarized potentials and a low threshold rebound action potential were classified as parvocellular (n = 46) (32). The remaining
23 neurons could not be accurately classified as either magnocellular or parvocellular and served as a separate control population.

**AM hyperpolarizes magnocellular neurons.** Thirty magnocellular neurons were tested for the effects of bath application of AM in aCSF under current-clamp configuration. The majority of these cells (n = 21) hyperpolarized in response to 50–500 nM AM application (−5.6 ± 0.4 mV, P < 0.01; Fig. 1), while five cells showed small depolarizations (4.2 ± 2.7 mV, P < 0.05), and the remaining cells showed no response. These hyperpolarizations usually occurred within 30 s of AM reaching the slice, and these responses lasted for 5–15 min before a return to baseline membrane potential as shown in Fig. 1A. Cells were tested with doses of AM ranging from 1 to 500 nM, and effects on membrane potential were found to be dose dependent with an EC_{50} of 47 nM as shown in the dose-response relationship presented in Fig. 1B. Two cells tested with 1 nM AM did not show any change in membrane potential. Although not systematically analyzed (recordings were not usually maintained for over 1 h), cells did not appear to show desensitization to AM as single cells were found to respond to more than a single application of AM.

To establish whether the observed actions of AM were due to direct actions of the peptide on the recorded cell or were the result of modified synaptic input from local interneurons, we recorded from 5 AM-responsive magnocellular neurons in the presence of TTX (5.0 μM). Although all cells responded to AM applied in normal aCSF, bath administration of AM during TTX application failed to elicit a response in four of five of the cells tested (Fig. 1A), while the remaining cell showed a small depolarization (3.2 mV).

![Fig. 1](http://ajpregu.physiology.org/)

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**Fig. 1.** Adrenomedullin (AM) hyperpolarizes magnocellular neurons. **A:** bath application of 100 nM AM (represented by horizontal bar above traces) hyperpolarized this cell (top trace). This response was abolished in the presence of 5.0 mM tetrodotoxin (TTX) (bottom trace). The downward deflections in the bottom trace represent voltage responses to 20-pA hyperpolarizing pulses. **B:** change in membrane potential measured during responses to 1.0 (n = 2), 10 (n = 2), 50 (n = 2), 100 (n = 9), and 500 (n = 4) nM AM. Data change presented as means ± SE. Data were applied to a sigmoid dose-response function and the corresponding curve was overlaid. **C:** AM did not have a significant effect on passive membrane properties, as no significant change in input resistance was observed (P > 0.5, paired t-test, n = 8).
The hyperpolarization of magnocellular neurons in response to AM was not a function of changes in passive membrane properties, as there was not a significant change in IR during AM application (1,011.2 ± 70.4 vs. 953.7 ± 92.7 MΩ, control vs. AM, respectively; paired Student’s t-test, n = 8, P > 0.5; Fig. 1C), as indicated by measuring membrane potential changes in response to hyperpolarizing current injection.

**AM increases IPSP frequency in magnocellular neurons.** The observation that TTX abolished effects of AM on magnocellular neurons suggested that these effects were the result of modified input from other neurons in our hypothalamic slice. We therefore examined the effects of AM administration on postsynaptic potentials in four magnocellular neurons. In all cases, the AM-induced hyperpolarization was accompanied by an increase in the frequency of IPSPs (0.68 ± 0.12 vs. 2.11 ± 0.17 Hz, control vs. AM, respectively; paired Student’s t-test, n = 4, P < 0.01; Fig. 2C). This increase was no longer observed following bath application of 5.0 mM TTX. In addition, there was no change in mIPSP frequency during bath application of 5.0 mM TTX and 100 nM AM in the presence of 5.0 mM TTX (0.43 ± 0.07 vs. 0.39 ± 0.09, TTX vs. TTX and AM, respectively; paired Student’s t-test, n = 4, P > 0.5; Fig. 2C), suggesting that the effects of AM on magnocellular neurons are due to activity-dependent changes in cells providing inhibitory input to the nucleus.

To investigate the possibility that enhanced inhibitory synaptic input underlies the observed AM effects on magnocellular neurons, we recorded from four (AM responsive) magnocellular neurons in the presence of BMI (10 μM). Although all cells responded to AM applied in normal aCSF, bath administration of AM during BMI application failed to elicit a response in any of the cells tested (AM, −5.3 ± 0.4 mV vs. AM + BMI, −0.6 ± 0.3 mV, P < 0.001, n = 4, paired t-test; Fig. 3).

**AM depolarizes parvocellular neurons.** Parvocellular neurons of the PVN have prominent roles in autonomic control and represent an additional subpopulation within the nucleus that can be identified electrophysiologically. We obtained recordings from 46 parvocellular neurons that were tested for the effects of bath application of AM. A total of 22 cells showed rapid reversible depolarizations in response to such peptide application as illustrated in Fig. 4A (top trace). Cells did not appear to show desensitization to AM, as multiple applications of AM to the same cell elicited similar responses. AM-mediated depolarizations of parvocellular neurons were also found to be dose dependent as illustrated in Fig. 5 in
doses ranging from 1.0 to 500 nM, with an EC_{50} of 75 nM. To establish whether observed AM actions were direct effects on these neurons, four neurons that responded to 0.1 μM AM were tested with AM during the blockade of action potentials by bath administration of TTX. AM elicited TTX-resistant depolarizations in all four neurons tested as shown in Fig. 4A (bottom trace).

To rule out the possibility that the effects observed were the result of AM binding at a CGRP family receptor, neurons that had previously depolarized following AM administration were bathed with the CGRP receptor antagonist CGRP_{8–37} (1.0 μM) for 10 min before and during subsequent AM application. After CGRP_{8–37} exposure, AM application resulted in a depolarization in three of four cells tested (Fig. 4B), suggesting this response was not mediated by activation of the CGRP receptor.

The depolarizations were also accompanied by a small yet statistically significant decrease in IR as measured by the maximum voltage response to a hyperpolarizing current pulse (1,128.2 ± 76.3 vs. 1,034.2 ± 65.3 MΩ, control vs. AM, respectively; paired Student’s t-test, P < 0.01, n = 10; Fig. 6A). The IR of two cells was unaffected despite the fact that they depolarized (6.4 ± 2.1 mV). In some cells, complete I-V

Fig. 3. Bicuculline methiodide (BMI) blocks membrane potential effects in magnocellular neurons. A: application of 100 nM AM hyperpolarized this magnocellular neuron (top trace; a), an effect that was not observed following pretreatment with 10 μM BMI (bottom trace; b). B: IPSPs are no longer observed following pretreatment with BMI. C: summary bar graph illustrating that the membrane potential response is abolished by BMI (AM, −5.3 ± 0.4 mV vs. AM + BMI, −0.6 ± 0.3 mV, **P < 0.001, n = 4, paired t-test).
curves were constructed during AM application, and extrapolation of a linear fit to these relationships showed a reversal potential near $-36.5$ mV ($n = 8$; Fig. 6B), suggesting that the conductances responsible for the AM effect reverse around this potential and the potential involvement of a nonselective cationic conductance (NSCC).

**AM enhances NSCC.** To test this possibility, eight parvocellular neurons were tested for the effects of bath application of AM under voltage-clamp configuration using slow voltage ramps (10 mV/s, −80 to 20 mV), which primarily activate only steady-state conductances including the NSCC (4). Bath application of AM caused an increase in conductance over the voltage range tested ($n = 5$), as shown in Fig. 7A. The proportion of the current sensitive to AM was obtained by subtracting the current response during control and 100 nM AM (Fig. 7B). The difference current ($I_{AM}$) is linear throughout the voltage range tested ($r^2 = 0.99$), and the mean reversal potential ($E_{AM}$) was found to be $-37.6 \pm 2.1$ mV ($n = 5$), obtained from a dose of 100 nM AM (Fig. 7B).

**AM does not alter unclassified PVN neurons.** Twenty-three PVN neurons that could not be electrophysiologically classified as magnocellular or parvocellular did not exhibit any change in membrane potential following bath application of 100 nM AM. Furthermore, four of these unclassified neurons were tested in voltage clamp configuration, and no change in the peak or steady-state conductances was observed following 100 nM AM application.
DISCUSSION

The present study was undertaken to examine the cellular mechanisms through which AM acts in the PVN to impact autonomic outputs controlling BP. Although our observations demonstrate AM effects on the PVN neuronal subgroups that may explain effects of this peptide on the cardiovascular system, they have also identified effects of AM that may explain effects of this peptide on the hypothalamic-pituitary-adrenal (HPA) axis.

Our experiments examined the effects of AM on type I (magnocellular) neurons in the PVN and demonstrated repeatable, dose-dependent membrane hyperpolarizations in the majority of magnocellular neurons tested. The fact that this hyperpolarization was blocked when magnocellular neurons were placed in synaptic isolation (TTX) suggested that these increases were not the result of direct effects of the peptide on these neurons. In accordance with such a hypothesis, we were also able to demonstrate an increase in IPSP frequency in magnocellular neurons of the PVN in response to AM, effects that were blocked in the presence of TTX. In addition, the effects of AM on membrane potential in magnocellular neurons were no longer observed following pretreatment with the GABA$_A$ antagonist BMI, confirming the role of inhibitory input in mediating AM effects on this population of neurons. Together, these observations support the conclusion that AM hyperpolarizes PVN magnocellular neurons as a secondary consequence of excitatory effects on inhibitory interneurons in the region of the PVN. Whether these effects are the result of presynaptic...
The stimulation of GABAergic nerve terminals or AM actions on GABA cell bodies will require additional voltage-clamp analysis of mini postsynaptic currents. Finally, intracerebroventricular injection of AM has been shown to increase NO production within the PVN (25), suggesting the potential involvement of the NO-driven GABAergic previously reported in the PVN (2). The potential importance of such inhibitory circuits in regulating magnocellular neuron excitability is highlighted by anatomic data demonstrating that approximately 50% of all synaptic connections made in the PVN are GABAergic in nature (9). The stimulation of GABAergic neurons, likely in the perinuclear region, in response to AM appears to enhance inhibitory input to magnocellular neurons, an effect that will result in a decrease in AVP and OXT release from the posterior pituitary. These observations are in accordance with evidence demonstrating that intracerebroventricular injections of AM inhibit AVP release in conscious rats (36). However, despite the fact that AVP is a potent vasoconstrictor and a mediator of fluid and electrolyte homeostasis, it is unlikely that modulation of AVP levels would translate to the rapid decrease in BP we have recently reported in response to AM microinjection into the PVN (27). A second possibility, which cannot be ruled out, is that a proportion of these type I neurons may project caudally to sympathetic preganglionic neurons in the intermediolateral column (IML) of the spinal cord. Inhibition of these cells would result in rapid decreases in BP. The fact that type I cells have been defined according to immunocytochemical properties (OXY or AVP) rather than anatomic projections (13) at least forces consideration of this possibility as PVN neurons projecting to the spinal cord have previously been demonstrated to be either OXY or AVP immunoreactive (28). Recent work recording from PVN neurons identified as projecting to IML is, however, suggestive of the conclusion that these cells do not show type I characteristics (3). We also examined the effects of AM on other subpopulations of PVN neurons that may represent the primary target for AM actions. Parvocellular (type II) neurons comprise a large proportion of the nucleus and include neurosecretory cells projecting to the median eminence (16), preautonomic neurons projecting to medullary and spinal autonomic centers (30), as well as GABA and glutamate interneurons (8, 19). Bath application of AM elicited dose-dependent depolarizations in the majority of parvocellular neurons tested, effects that were maintained in the presence of TTX, suggesting the activation of postsynaptic receptors. The observation that these effects were also maintained in the presence of the CGRP receptor antagonist led us to conclude that these effects are mediated by AM rather than CGRP receptors (31).

Our current-clamp recordings demonstrated an AM-induced decrease in IR, with a reversal potential of approximately −36.5 mV, which is consistent with the activation of an NSCC (4). We next used voltage-clamp techniques to specifically test the hypothesis that effects of AM application resulted from the activation of such an NSCC. Specifically, we were able to demonstrate, using slow voltage ramps (10 mV/s), that the AM-sensitive current recorded from parvocellular neurons presented displayed characteristics similar to the NSCC described by Washburn et al. (35) in subfornical organ neurons. It has been shown that the gating of the NSCC is voltage independent and that the modulation this conductance produces is a linear difference current, similar to \( I_{AM} \) in parvocellular neurons. Enhancement of these conductances has been shown to mediate neuronal depolarization and repetitive action potential.

**Fig. 7.** Ramp currents activated by AM in parvocellular neurons. **A:** instantaneous current-voltage relation elicited by slow voltage ramps from −80 to +20 mV during bath application of control (a), 100 nM AM (b), and wash (c). AM increased conductance throughout the voltage range \( n = 5 \). **Inset:** current activated by AM \( (I_{AM}) \) is obtained by subtracting the currents \( (b - a) \) shown in A. The current was voltage independent, as indicated by the linearity \( r^2 = 0.99 \). The reversal potential for this example current was −38.7 mV (arrow), suggesting a nonselective cation conductance. **B:** summary of \( I_{AM} \) over the entire voltage range tested, reversal potential = 37.6 ± 2.1 mV; data presented as means ± SE, \( n = 5 \).
firing of neurons at rest or near threshold for action potential generation (5, 35).

Our observations of direct effects of AM on parvocellular (type II) neurons in the PVN clearly focus attention on a physiological correlate. Parvocellular neurons represent a multifunctional heterogenous population of neurons that contribute to endocrine and autonomic output as well as intranuclear regulation (34). The largest population of parvocellular PVN neurons has been characterized as CRH-synthesizing neurons projecting to the median eminence. Their ultimate role is the maintenance of ACTH secretion and thus maintenance of the HPA axis (11). Although an increase in these hormones would not be expected to mediate the observed decrease in BP in response to AM (27), this observation may provide an electrophysiological correlate to AM modulation of the output function of CRH neurons. Our in vitro results mirror previous studies demonstrating a three- to fivefold increase in CRH and ACTH levels following intracerebroventricular administration of AM in vivo (6).

In addition to playing a neuroendocrine function, PVN parvocellular neurons have been implicated in modulating sympathetic outflow to the spinal cord and medulla (7, 10). However, previous studies have shown that activation of PVN neurons projecting to the IML results in increases in BP (14), whereas our electrophysiological data show activation of parvocellular neurons in response to AM, which would thus be predicted to increase BP. Therefore, it is unlikely that the activation of parvocellular type II neurons observed here is responsible for the decreases in BP observed in response to AM microinjection (27).

The present study demonstrates that AM receptor activation results in population-specific effects on the PVN neurons. AM hyperpolarized magnocellular (type I) neurons in a dose-dependent manner, a response associated with an increase in the frequency and amplitude of IPSPs. Blockade of action potentials with TTX and GABAergic receptors with BMI abolished AM amplitude of IPSPs. Blockade of action potentials with BMI associated with an increase in the frequency and amplitude of IPSPs, associated with an increase in the frequency and amplitude of IPSPs. Blockade of action potentials with BMI associated with an increase in the frequency and amplitude of IPSPs. Blockade of action potentials with BMI associated with an increase in the frequency and amplitude of IPSPs. Blockade of action potentials with BMI associated with an increase in the frequency and amplitude of IPSPs. Blockade of action potentials with BMI associated with an increase in the frequency and amplitude of IPSPs. Blockade of action potentials with BMI associated with an increase in the frequency and amplitude of IPSPs. Blockade of action potentials with BMI associated with an increase in the frequency and amplitude of IPSPs. Blockade of action potentials with BMI associated with an increase in the frequency and amplitude of IPSPs. Blockade of action potentials with BMI associated with an increase in the frequency and amplitude of IPSPs. Blockade of action potentials with BMI associated with an increase in the frequency and amplitude of IPSPs.


