α2-Adrenergic autoreceptors in A5 and A6 neurons of neonate rats

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Huangfu, Donghai, and Patrice G. Guyenet. α2-Adrenergic autoreceptors in A5 and A6 neurons of neonate rats. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H2290–H2295, 1997.—A5 noradrenergic neurons control sympathetic outflow, nociception, and respiration. The presence of α2-adrenergic receptors (α2-ARs) in A5 cells has been suggested by immunohistochemistry. In the present experiments, we analyze the response of spinal projecting A5 cells to α2-AR agonists, and we compare it with that of locus ceruleus (A6) neurons. Whole cell recordings were obtained from 52 spinal projecting neurons in the ventrolateral pons of neonate rats. Immunohistochemistry showed that 60% of the recorded cells were A5 cells. In A5 cells clamped at −55 mV, norepinephrine (NE) in the presence of the α2-AR antagonist prazosin produced a Ba2+ sensitive outward current (20.4 ± 2.6 pA, n = 28). The α2-AR-induced current reversed at the K+ equilibrium potential (E_K) at three different extracellular K+ concentrations. Replacement of 82% of the extracellular Na+ concentration with N-methyl-D-glucamine did not change the reversal potential. The 19 presumably noncatecholaminergic neurons responded weakly or not at all to NE (2.5 ± 0.6 pA outward current). Pontospinal A6 neurons (n = 11) were also recorded. Six A6 cells displayed large tetrodotoxin (TTX)-resistant membrane oscillations. In these cells, the current induced by α2-AR stimulation did not reverse over the voltages tested (−50 to −130 mV) or reversed at potentials more negative than E_K (less than −114 mV). In A6 neurons that did not display large oscillations (n = 5), the α2-AR-induced current reversed at or close to the E_K (−90 ± 1.6 mV). In conclusion, A5 cells, like locus ceruleus neurons, have α2-ARs that may function as autoreceptors. In both cases, α2-AR activation increases an inward rectifying K+ conductance. In A5 cells, we found no evidence that α2-AR activation decreases a resting Na+ conductance. The inhibition of A5 cells by clonidine and other agonists with α2-AR agonist activity is likely to contribute to the ability of these drugs to decrease sympathetic tone and arterial pressure.

A5 noradrenergic cells; locus ceruleus; α2-adrenergic receptors; autonomic regulations; sympathetic tone

THE RETICULAR FORMATION of the caudal ventrolateral pons receives most of its input from brain stem areas that are involved in autonomic regulation (8). Stimulation of this region by microinjection of excitatory amino acids produces large cardiovascular changes in anesthetized rats (12, 22, 28) and changes in nociception (6). Some of these effects are attributed to activation of the large group of noradrenergic neurons known as the A5 cells. A5 cells project predominantly to brain stem autonomic centers (8). They are among a very small number of brain stem neurons that establish monosynaptic connections with sympathetic preganglionic neurons (15, 16, 29). The discharges of the bulbospinal neurons of the A5 region are influenced by afferent inputs from the cardiopulmonary region, including arterial baroreceptors and peripheral chemoreceptors (9, 13). Many of these bulbospinal cells are very powerfully inhibited by systemic administration of the α2-adrenergic-receptor (α2-AR) agonist clonidine or by iontophoretic application of catecholamines (5, 13). A prior immunohistochemical study from this laboratory suggested that A5 neurons, like the C1 adrenergic cells of the rostral ventrolateral medulla, have postsynaptic α2A-ARs (27), a subtype of adrenergic receptor (10), the integrity of which is required for the efficacy of clonidine-like hypotensive agents (21). The objectives of the present study were threefold. The first was to determine whether A5 cells have α2-ARs. The second was to determine whether the presence of these receptors is of diagnostic value to distinguish between A5 cells and the noncatecholaminergic component of the spinal projection of the ventrolateral pons. The last objective was to analyze whether α2-adrenergic-receptor stimulation produces the same or different effects in A5 cells and in the reference noradrenergic cell group, the locus ceruleus.

METHODS

Whole cell recordings were obtained at room temperature in thin coronal brain slices (120 μm) from neonate rats (age 5–10 days). The technique was identical to that previously used to record from C1 adrenergic neurons (19, 20). Briefly, ventrolateral pontine neurons were retrogradely labeled with fluorescent microbeads injected on postnatal day 3 into the thoracic spinal area. Individual retrogradely labeled neurons were visualized with a water-immersion ×40 objective via epifluorescence and Hoffman modulation optics. All pertinent technical details regarding the preparation of the slices can be found in the companion paper (11a). For recording, the slice was continuously superfused at the rate of 2–3 ml/min with a medium of the following composition (in mM): 124 NaCl, 26 NaHCO3, 5 KCl, 1 NaH2PO4, 2 MgSO4, 2 CaCl2, and 10 glucose. This medium was equilibrated with 95% O2–5% CO2 (pH 7.4). In some experiments, NaCl (124 mM) was replaced by an equimolar concentration of N-methyl-D-glucamine (NMDG) titrated with HCl. Drugs and solutions of different ionic content were applied to the slice by switching the perfusion solution with a series of electronic valves. Time to onset of drug action was −30 s. Patch pipettes were filled with a solution of the following composition (in mM): 114 K gluconate, 17.5 KCl, 4 NaCl, 4 MgCl2, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 0.2 ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid, 3 MgATP, and 0.3 Na2GTP and 0.02% lucifer yellow (Molecular Probes). Osmolality was adjusted to 270 mosmol, and pH was adjusted to 7.3. Electrode resistance was 5–7 MΩ. Whole cell current- and voltage-clamp recordings were made with an Axodamp-2A amplifier. The liquid junction potential was measured (8–12 mV), and all reported voltage measurements have been corrected for this potential. Series resistance
compensation was not employed because the recorded currents were small enough (<100 pA) that voltage errors due to series resistance should have been negligible. Current and voltage data were collected through a DigiData-1200 interface with pCLAMP software version 6.0 (Axon Instruments) and were stored on videotape for off-line analysis. In some experiments, the SD of the membrane potential was also used to quantify the variability of the resting membrane potential. In this case, a 100-s segment was used (sampling every 10 ms), and the signal was filtered from 0.1 to 50 Hz because power density spectral analysis revealed that most of the power was distributed within this range.

After a recording was made, every slice was fixed in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). Immunostaining for tyrosine hydroxylase (TH) was done with a previously described technique (19, 20). The procedure is an avidin-biotin-based reaction (mouse anti-TH monoclonal antisera from Chemicon, dilution 1:750; biotinylated goat anti-mouse antisera from Vector, 1:150 dilution; and avidin-conjugated Texas red from Molecular Probes, 1:200 dilution). Recorded cells were identified by the presence of lucifer yellow and bulbospinal cells by the presence of microbeads. TH-immunoreactive (TH-ir) neurons were considered to be A5 neurons.

Drugs and chemicals. Tetrodotoxin (TTX), norepinephrine bitartrate (NE), clonidine HCl, 2-methoxyidazoxan (MOI), and NMDG were obtained from Sigma Chemical (St. Louis, MO).

Statistics. Results are expressed as means ± SE. Data were analyzed by either paired t-tests or analysis of variance. Significance was set at P < 0.05.

RESULTS

Except where indicated, the use of the term A5 neuron is restricted to a spinally projecting neuron of the ventrolateral pons shown by post hoc immunohistochemistry to contain TH.

Effects of α2-AR activation on spinally projecting neurons of the A5 region. Postsynaptic α2-ARs were selectively stimulated by exposing the cells to NE (30 µM, 30 s) in the continuous presence of prazosin (1 µM) to block α2-adrenergic receptors and TTX (1 µM) to reduce synaptic activity. The experiment was carried out in 52 spinally projecting cells that were voltage clamped at their resting membrane potential (holding potential −50 to −60 mV) unless otherwise indicated. After histology, 28 of 47 cells were found to be TH-ir (A5) and 19 of 47 were not TH-ir. The 19 presumably noncatecholaminergic neurons (without detectable TH-ir) responded weakly or not at all to NE (2.5 ± 0.6 pA outward current, range 0–8 pA; P < 0.01 compared with the response of A5 cells to be described below). In contrast, in all A5 cells (n = 28), NE produced a relatively large outward current (20.4 ± 2.6 pA, range 6–60 pA) that peaked within 1 min of switching perfusion media and lasted 5–7 min (Fig. 1A). In A5 cells, donidine (1 µM) also elicited an outward current (13.3 ± 3.6 pA; n = 3) that lasted for up to 15 min (not illustrated). The effect of NE was repeatable without significant attenuation (first vs. second application, 23.0 ± 2.6 vs. 21.5 ± 2.5 pA; P = 0.45; n = 7 neurons; Fig. 1A). In seven neurons, NE was applied once, and after full recovery the catecholamine was reapplied in the presence of the selective α2-AR antagonist MOI (10 µM). The outward current induced by NE was blocked by the antagonist (22.8 ± 4.8 pA before and −0.8 ± 0.8 pA in presence of MOI; P < 0.01; n = 7 neurons; Fig. 1, B and C). Responsiveness to NE recovered partially after a 0.5- to 1-h washout (38.6 ± 4.0% of control value; n = 5 neurons). MOI alone did not change the holding current. The outward current induced by 30 µM NE at a holding potential of −50 to −60 mV was greatly attenuated by the addition of 1 mM Ba2+ to the perfusion solution (26.2 ± 6.5 pA before and 3.0 ± 2.0 pA in presence of Ba2+; P < 0.01; n = 6 neurons; an example of 1 cell is shown in Fig. 2A), suggesting that this current was due mostly to the activation of K+ channels. To examine this point further, the voltage dependence of the NE-induced current was examined with a slow ramp paradigm (−80-mV linear ramp from resting potential in 1 s). The current induced by NE was determined by subtracting the control current-voltage (I-V) curve from that determined in the presence of the catecholamine. Examples of I-V curves generated in the presence and absence of NE are illustrated in Fig. 2B. The NE-induced current was inwardly rectifying, and its polarity reversed at −83 mV. The slope conductance measured in the linear part of the I-V curve (from −130 to −110 mV) was significantly increased by NE (from 4.2 ± 0.3 to 6.4 ± 0.4 nS; P < 0.01; n = 22 cells). The relationship between the reversal potential (Erev) and the K+ equilibrium potential (EK) was examined by applying NE in the presence of up to three different K+ concentrations per cell as illustrated in Fig. 2C. As indicated in Fig. 2D, Erev was linearly related to the logarithm of the external K+ concentration (2.5 mM K+: −98.8 ± 1.3 mV, n = 8 cells; 5 mM K+: 84.5 ± 0.6 mV, n = 22 cells; 10 mM K+: 64.6 ± 1.6 mV, n = 8 cells). These Erev values were very close to the calculated EK values (−102, −84, and −66 mV for 2.5, 5, and 10 mM external K+, respectively).

In the noradrenergic neurons of the locus ceruleus, α2-AR stimulation may also reduce a Na+ conductance.
(3). This effect, along with the increase in K⁺ conductance, may contribute to the outward current observed around the resting membrane potential. We searched for the presence of a similar mechanism in spinally projecting A5 neurons by determining the effect of lowering extracellular Na⁺ concentration (82% substitution with NMDG) on the NE-induced response in five cells. No histology was done in these experiments. The five cells were presumed to be A5 neurons because they were spinally projecting and located in the A5 area and all displayed a large outward current in response to NE, which we considered to have diagnostic value at this stage of the experiments. As illustrated in Fig. 3A, at a holding potential close to the resting level (−55 mV), Na⁺ substitution produced a small positive shift in the holding current and reduced the outward current induced by NE by −50%. The effect of NMDG on the NE-induced current was examined in more detail with the slow ramp paradigm illustrated in Fig. 2, B and C. The NE-induced current was determined by subtracting the I-V relationships obtained before and during NE application. The NE-induced current was measured while the slice was perfused in control medium and was remeasured 10 min after the start of perfusion with low-Na⁺ medium (Fig. 3B). The two difference curves (NE-induced current) were very similar except for a reduction in the outward component of the NE-induced current. This experiment was replicated four times with a similar outcome. On average, NMDG did not change the E<sub>rev</sub> (−86.1 ± 1.2 mV in control solution; 83.4 ± 1.6 mV in NMDG) nor the magnitude of the inward current (−72.1 ± 8.3 vs. −69.3 ± 6.9 pA measured at −125 mV). However, NMDG decreased the outward portion of the current (from 20.3 ± 3.1 to 11.2 ± 2.5 pA measured at −55 mV; P < 0.01 by paired t-test; n = 5 cells).

Effect of α₂-AR stimulation on A6 cells in the neonate. We found two types of spinally projecting noradrenergic neurons in the locus ceruleus. Five cells had low-amplitude, irregular membrane oscillations similar to those present in A5 neurons (SD of membrane current of five cells clamped at −55 mV, 3.1 ± 0.4 pA). The remainder (n = 6 cells) exhibited large, voltage-independent membrane oscillations at rest (SD of membrane current at −55 mV, 5.3 ± 0.5 pA). The large-amplitude regular oscillations of locus ceruleus neurons were suppressed reversibly by application of 30 µM NE (30 s; experiments performed in the continuous presence of 1 µM of prazosin; Fig. 4A). To examine whether application of NE changed the oscillations of the membrane current recorded in voltage-clamp mode in A5 cells, we measured the SD of the current 2 min before applying 30 µM NE and during the 2 min corresponding to the peak of the response to the catecholamine. The SD of the membrane current (holding potential 56.7 ± 1.9 mV; band pass 0.1–50 Hz; 1 µM TTX present) was unchanged by the presence of NE (2.9 ± 0.2 vs. 3.0 ± 0.3 pA; P > 0.05; n = 17 cells).

The voltage dependence of the current induced by 30 µM NE (determined in the presence of 1 µM each prazosin and TTX) was examined in each of the 11 locus ceruleus cells with the slow voltage-ramp paradigm (−55 to −135 mV in 1 s). In the six cells that exhibited large-amplitude oscillations, the NE-induced current either did not reverse within the range of voltages examined (n = 3; Fig. 4A) or reversed at potentials
more negative than the predicted $E_K$ ($E_{rev}$ less than $-114$ mV). In the remaining five cells that did not exhibit the large regular oscillations, the NE-induced current reversed close to the predicted $E_K$ ($E_{rev} \approx -90 \pm 1.6$ mV; Fig. 4B). The mean slope conductance (measured between $-110$ and $-130$ mV) of the cells exhibiting the large oscillations ($7.0 \pm 1.7$ nS; $n = 6$) tended to be larger than in the other five neurons ($3.7 \pm 0.6$ nS), but the difference did not reach statistical significance ($P = 0.127$).

**DISCUSSION**

The study provides electrophysiological confirmation that A5 noradrenergic neurons have $\alpha_2$-ARs. It is also the first comparative study of A5 and A6 (locus ceruleus) cells at the same stage of development. Finally, the study indicates that A5 neurons can be distinguished from surrounding non-A5 spinally projecting neurons based on the magnitude of their response to $\alpha_2$-AR stimulation.

A5 cells can be identified by the presence of a larger outward current in response to $\alpha_2$-receptor stimulation. As indicated in the companion paper (11a), the intrinsic properties of A5 cells are not noticeably different from those of the surrounding nonaminergic bulbospinal cells, at least under the present experimental conditions. However, the presence of a substantial outward current in response to $30 \mu$M NE ($>15$ pA in cells held at $-55$ mV in presence of prazosin) had a diagnostic value to identify A5 neurons within the limited area of the pons that was explored.

$\alpha_2$-ARs activate a $K^+$ conductance in A5 cells. Under voltage clamp at or close to the resting membrane potential, application of NE in the presence of the selective $\alpha_2$-AR antagonist prazosin (32, 33) produced an outward current in all A5 neurons. Subsequent tests were consistent with the interpretation that the effect of NE was due to the activation of postsynaptic $\alpha_2$-ARs.
and that the NE-induced current resulted predominately, if not exclusively, from the activation of an inwardly rectifying K\(^+\) conductance.

The outward current elicited by NE was abolished by the selective \(\alpha_2\)-AR antagonist MOI (24). The NE-induced current reversed polarity in every cell within 2 mV of the calculated value of the \(E_K\). Also, the \(E_{rev}\) changed according to the Nernst equation when the concentration of \(K^+\) in the bath was changed from 2.5 to 10 mM. Finally the NE-induced current was attenuated by \(>90\%\) by Ba\(^{2+}\), a blocker of inwardly rectifying K\(^+\) channels. Ba\(^{2+}\) also blocks some Ca\(^{2+}\)-activated K\(^+\) currents, but this type of current is unlikely to be involved in the response to \(\alpha_2\)-AR stimulation because, in neurons, Ca\(^{2+}\) currents (specifically high voltage-activated currents) are typically inhibited by \(\alpha_2\)-AR stimulation, and \(\alpha_2\)-ARs are not known to influence phosphatidylinositol hydrolysis (7).

A hyperpolarization-activated current (\(I_h\)) (25) is present in A5 cells [see companion paper (11a)]. At potentials more negative than \(-80\,\text{mV}\), this current is recruited during the slow ramp voltage-clamp paradigm used to measure the NE-induced current. However, it is improbable that a significant portion of the inward current produced by NE at potentials more negative than \(-100\,\text{mV}\) could be due to activation of \(I_h\). Indeed, the expected effect of \(\alpha_2\)-AR stimulation in the brain is a reduction in the level of adenosine 3',5'-cyclic monophosphate, and \(I_h\) is thought to be upregulated by this cyclic nucleotide (25). In addition, Na\(^+\) substitution with NMDG did not significantly change the inward current produced by NE at hyperpolarized potentials (Fig. 3B). Finally, in dopaminergic neurons, another group of autoactive catecholaminergic cells, autoreceptor stimulation via D\(_2\) receptors inhibits \(I_h\), consistent with the inhibitory effect of autoreceptors (17).

In short, the current produced by \(\alpha_2\)-AR stimulation in A5 neurons under our experimental conditions is most likely due to the activation of an inwardly rectifying K\(^+\) conductance.

Differences between A5 and A6 cells. The NE-induced current always reversed close to \(E_N\) in A5 cells, whereas in \(-50\%\) of A6 neurons the NE-induced current reversed at potentials significantly more negative than \(E_K\) or did not reverse at all. Similar left shifts in the apparent \(E_{rev}\) of the current induced by opiate agonists have been observed in A6 cells. Two interpretations of this phenomenon have been proposed, namely, a space-clamp problem (14, 30) or the additional contribution of an Na\(^+\) conductance to the opiate-induced response (1, 3, 31). According to the latter theory, A6 cells have a resting Na conductance that is activated by adenosine 3',5'-cyclic monophosphate (2, 4). This conductance would therefore be reduced by receptors coupled to G\(_{\alpha}\) such as \(\alpha_2\)- or \(\mu\)-opiate receptors, and this effect would cause the \(E_{rev}\) of the agonist-induced current to be more hyperpolarized than the \(E_K\) (3). In our hands, the NE-induced current reversed at an abnormally negative potential only in A6 cells that exhibited large-amplitude oscillations. Because these oscillations may be due to dendritic coupling (14), our results tend to support the notion that space-clamp problems or active dendritic conductances could account for the unusually negative \(E_{rev}\) of the NE-induced current. In any event, we found no trace of a similar anomaly in A5 cells. In these cells, the NE-induced current reversed close to the calculated \(E_K\), and an 82% reduction in the extracellular Na\(^+\) did not significantly change the \(E_{rev}\) (Fig. 3B). Na\(^+\) substitution with NMDG did not significantly reduce the inward component of the NE-induced current in A5 cells, but the outward component was attenuated (Fig. 3). This may be due to an increased rectification of the K\(^+\) current activated by \(\alpha_2\)-ARs. Lowering extracellular Na\(^+\) causes intracellular acidification and a rise in intracellular Ca\(^{2+}\) (18, 26). Intracellular acidification can change the rectification of some inwardly rectifying K\(^+\) currents either directly or via changes in the ionization of polyamines (e.g., Ref. 26; for a review, see Ref. 23).

In summary, A5 and A6 cells display an equally marked sensitivity to \(\alpha_2\)-AR stimulation, which is consistent with prior immunohistochemical evidence of \(\alpha_2\)-ARs in both cell types (e.g., Ref. 27). The response of A5 cells is consistent with the activation of an inwardly rectifying K\(^+\) current, and it resembles qualitatively and quantitatively that found in about one-half of the locus ceruleus cells. The response of the other half of the locus ceruleus neurons is more complex, and the main difference appears related to the presence or absence of large membrane oscillations that were not observed in A5 cells. We cannot exclude the possibility that a different slice configuration or thickness could reveal the presence of similar oscillations and anomalies of the NE-induced response in A5 cells because both phenomena may reside in distal dendrites (30).

Finally, it should also be kept in mind that the present results were obtained in a thin slice of neonate brain and that whole cell recording, as opposed to a perforated-patch technique, was used. It is unlikely that the major mechanism for A5 cell hyperpolarization by \(\alpha_2\)-AR agonists (activation of an inwardly rectifying K\(^+\) conductance) would disappear in the adult because it remains in A6 cells. On the other hand, additional membrane effects of \(\alpha_2\)-AR stimulation could have been overlooked by the use of the whole cell recording.

Functional significance. The presence of inhibitory \(\alpha_2\)-ARs in A5 cells suggests that, like other groups of aminergic neurons, A5 neurons may autoregulate their activity or that they are subject to collateral inhibition by neighboring noradrenergic cells. An other possibility is that the \(\alpha_2\)-ARs of A5 cells mediate the effect of an extrinsic catecholaminergic innervation. Possible candidates include the C1 or C3 neurons of the medulla oblongata because terminals immunoreactive for phenylethanolamine N-methyltransferase are present in the immediate vicinity of A5 neurons (11).

The A5 noradrenergic cells are the second major group of bulbo spinal neurons with monosynaptic and presumably excitatory projections to sympathetic preganglionic neurons that have electrophysiologically and anatomically demonstrated inhibitory \(\alpha_2\)-ARs. The other cell group is the C1 adrenergic neurons (19). The
inhibition of both A5 and C1 cells by clonidine and other agents with \( \alpha_2 \)-AR agonist activity is likely to contribute to the ability of these drugs to decrease sympathetic tone and arterial pressure. Consistent with this view, both A5 and C1 neurons contain immunoreactivity for the \( \alpha_2A \)-subtype adrenergic receptor (10), the integrity of which is required for the efficacy of clonidine-like hypotensive agents (21).

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