Area-Specific Differences in Transmitter Release in Central Catecholaminergic Neurons of Spontaneously Hypertensive Rats

Anja G. Teschemacher, Sheng Wang, Mohan K. Raizada, Julian F.R. Paton, Sergey Kasparov

Abstract—The link among blood pressure, sympathetic output, and brain neurons producing catecholamines is well documented. Nevertheless, their intrinsic properties and any alterations in signaling characteristics between normotensive and hypertensive phenotypes remain unknown. Here, we directly compared neurophysiological properties of catecholamine release of C1 and A2 neurons of the spontaneously hypertensive rat and Wistar rat in organotypic slices. C1 and A2 areas were studied because both are widely implicated in the pathophysiology of hypertension. Catecholaminergic neurons were visualized using viral vectors to express green fluorescent protein. Microamperometry revealed that C1 axonal varicosities of spontaneously hypertensive but not normotensive Wistar rats release a transmitter predominantly (≈86%) in very large quanta, comparable in catecholamine load to adrenal chromaffin granules. Because quantal size affects the spread of transmitter in the extracellular space, this may enhance the impact of C1 varicosities on their downstream targets and increase sympathetic drive in the hypertensive rat. Electrophysiological properties and Ca\(^{2+}\) handling were studied using patch clamp and confocal imaging. Although overall electrophysiological characteristics of C1 and A2 neurons were comparable between strains, the characteristic angiotensin-II–induced Ca\(^{2+}\) mobilization was reduced in A2 neurons of the spontaneously hypertensive rat. Because A2 neurons are a part of a homeostatic antihypertensive circuit, this could reduce their restraining influence on blood pressure. Thus, we have revealed an increased quantal size in C1 varicosities and a reduced responsiveness of A2 neurons of the spontaneously hypertensive rat to angiotensin II. Both effects could contribute to elevated sympathetic activity and blood pressure in the spontaneously hypertensive rat. (Hypertension. 2008;52:351-358.)

Key Words: norepinephrine ■ epinephrine ■ medulla ■ hypertension ■ blood pressure ■ neurotransmitter release ■ patch clamp

Essential hypertension in both human patients and an animal model of this disease (the spontaneously hypertensive rat [SHR]) is accompanied characteristically by increased central sympathetic drive. The link among brain stem catecholaminergic (CAergic) activity, sympathetic output, and blood pressure is documented very extensively (reviewed in Reference 5) but as yet not well understood. Based on previous studies, 3 main differences between CAergic transmission in the normotensive rat and SHR may be hypothesized.

First, noradrenaline (NA) or adrenaline (ADR) in SHRs may be stored and released differently. This hypothesis is based on evidence of morphological changes in storage vesicles in SHRs and their increased ability to take up \(^{3}\)H-NA,\(^{6}\) higher expression of norepinephrine transporter in SHR brain stem cultures,\(^{7}\) and increased tyrosine hydroxylase expression in the SHR ventrolateral medulla.\(^{8}\) Structural differences in NA-storing vesicles and/or NA synthesis and transport could translate into enhanced packaging and release of catecholamine (CA) in SHRs. Thus, it could be that, in SHRs, CAergic volume transmission is amplified, increasing the effect of CA on various cellular targets in areas critical for control of sympathetic outflow.

Second, central CAergic neurons in the SHR could be electrically “hyperactive” and generate action potentials at higher rates than in normotensive animals. Higher discharge rates were detected in neurons (some of which could be CA containing) of the rostral ventrolateral medulla of neonatal SHRs.\(^{9}\) The third hypothetical difference is that the excitatory effect of angiotensin II (Ang II) on these neurons or a characteristic Ang II–induced Ca\(^{2+}\) mobilization\(^{10}\) may be enhanced in SHRs. Central Ang II has been implicated in pathogenesis of excessive sympathetic activity and hyperten-
sion by numerous studies11–13 (reviewed in Reference 14). In rat hypothalamus/brain stem primary cultures, Ang II excited a significant proportion of neurons,15 and Ang II effects were enhanced in primary cultures of neonatal SHRs.16,17 It is, therefore, conceivable that stronger activation of CAergic neurons by central Ang II could lead to a higher transmitter release in SHRs.

Finally, CAergic neurons are located within several discrete clusters within the brain stem (referred to as “A” groups, which release norepinephrine, and “C” groups, which have the biochemical machinery for synthesis of epinephrine), and it appears from the literature that the roles of distinct CAergic cell groups in control of blood pressure are different or even opposite. For example, C1 neurons are widely believed to play an important role in maintaining sympathetic outflow,18–22 whereas A2 neurons are likely to be a part of the central homeostatic “antihypertensive” mechanism.23–26 It follows that if the hypertensive phenotype is associated with altered signaling in central CAergic neurons, the changes must be cell group specific rather than universal.

Although the first clear links between hypertension and central CA transmission were made >30 years ago, to date it has not been possible to pinpoint any differences in the signaling in these neurons between hypertensive and normotensive phenotypes, because suitable methods were lacking. We have recently developed transgene-based approaches that allow us, for the first time, to study intracellular signaling and quantal characteristics of CA release from identified clusters of CA neurons in organotypic slices of rat brain stem.27,28 Using these strategies, we have been able to directly test the hypotheses listed above. The present study compared the quantal transmitter release characteristics, electrophysiological properties, and Ca2+ handling in C1 and A2 neurons in response to Ang II from SHRs and the normotensive Wistar rat (WR).

Methods

Methods Summary
For a detailed account of the methods please see the data supplement (available online at http://hyper.ahajournals.org). In brief, all of the experiments were performed in organotypic cultured slices of rat brain stem from P7–9 rat pups, where NA/ADR-producing neurons were induced to express enhanced green fluorescent protein (EGFP) by an adenoviral vector with a PRSx8 promoter.26–30 For each part
of the study, pups from ≥3 different litters have been used. It has been demonstrated by numerous earlier studies that changes in the activity and biochemistry of central neurons are detectable in tissues from postnatal SHRs in vitro and that there are alterations in sympathetic activity in juvenile SHRs before the development of hypertension, consistent with recent unpublished observations from this laboratory. We have focused on 3 main characteristics of either C1/A1 or A2 neurons. The transverse brain stem slices for C1 were taken >1 mm more rostrally than for A2, but it is acknowledged that the distributions of phenylethanolamine-N-methyltransferase–positive, ie, putative adrenergic, and of phenylethanolamine–N-methyltransferase–negative but dopamine-β-hydroxylase (DBH)–positive, ie, noradrenergic, neurons partially overlap in the ventrolateral medulla. Transmitter release and packaging characteristics were assessed using microamperometry (see Figure 1) as described previously. Membrane potential, ongoing firing activity, and electric and intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) responses to Ang II were assessed using patch clamp and confocal fluorescence imaging of Rhod-2, essentially as described previously (Figure 2).

**Drugs and Their Application**

In amperometric and electrophysiological/Ca\(^{2+}\) imaging studies, Ang II (Sigma) and other drugs were introduced into the perfusion media, as described previously. For the experiments presented here we used a 200 nmol/L concentration, which is similar to or lower than those used in many previous in vitro studies. In a few preliminary experiments we applied 50 and 100 nmol/L Ang II and observed qualitatively similar effects, albeit smaller (data not shown).

**Statistical Analysis**

All of the values in this text are expressed as means±SEMs unless indicated otherwise. Statistical evaluation was carried out using Microsoft Excel and GraphPad Prism 4.

**Results**

**Quantal Characteristics of Transmitter Release**

Microamperometric currents appeared as either separate single-peaked events with monophasic rising and falling phases (Figure 1B and 1C, top) or complex events (Figure 1B and 1C, bottom). Single events (Figure 1C, top) could be subdivided into relatively frequent small-to-medium-sized quanta (referred to as “small” events below; median quanta: \(≈0.02\) pC; main populations on Figure 3A and 3B; examples shown in Figure 1C) and more rare “large” events \(>0.5\) pC (median quanta: \(≈1\) pC; average: \(≈2.1\) pC; events within the right part of the distributions in Figure 3A and 3B; examples shown in Figure 1C). Event frequencies varied widely between individual axonal varicosities (0.01 to 2.50 Hz) and were not significantly different between CAergic areas or rat strains (n=6 varicosities for each area in each strain). Because the size of a release event determines the spread of the CA in the extracellular space, we analyzed the characteristics and relative contributions of various types of events to the overall amount of transmitter released (Table 1).

**C1 Varicosities**

Total CA release (in pC/min) from C1 SHR varicosities was larger than in WRs (\(+55\%)\). Median quantal content or \(I_{1/2}\) of single small events did not significantly differ between WRs and SHRs (Table 1). Although the kinetics of large events were similar between WRs and SHRs, their frequency increased \(≈1.5\) times in SHRs (Table 1 and Figure 3A). More importantly, the relative contribution of large and small
events to total CA release was different between SHRs and WRs ($\chi^2$ test values $<0.05$ for all fractions; Figure 3C). Although in WRs single and complex large events delivered $1.6 \times 10^6$ CA molecules per minute, this parameter more than doubled in SHRs ($3.7 \times 10^6$ molecules per minute). This was because of a combination of both higher incidence of large events in C1 varicosities of SHRs (Figure 3A) and a much higher transmitter load of such events ($\approx 270\%$; $P<0.01$; Table 2). Thus, whereas in WRs large events were only responsible for $57\%$ of the total release, in SHRs they delivered $86\%$ of the total CA because of a dramatic increase in both single and complex fractions. In contrast, release from small events in SHRs decreased from $1.2 \times 10^6$ to $5.9 \times 10^5$ molecules per minute, corresponding with a fall from $43\%$ to $14\%$ of the total release. As a result, the bulk of the transmitter released from C1 varicosities in SHRs arrives in large packages (Figure 1C and 3C) in excess of 0.5 pC, or $1.6 \times 10^6$ molecules.

**A2 Varicosities**

There were no significant differences in median quantal content between the populations of single small events at A2 release sites of WRs versus SHRs, but small events in SHR had a significantly longer $\tau_{1/2}$ ($P<0.05$; Table 1). Kinetics of large quanta were not different between WRs and SHRs. The total quantity of NA released from the pool of 6 A2 varicosities was $35\%$ greater in SHRs because of slightly higher frequencies of all of the fractions of events. However, the relative contribution of various types of quanta to the total transmitter output was similar between the strains (Figure 3C). Moreover, the transmitter load of the large release events (Figure 1C) in SHR A2 varicosities tended to be smaller than in WRs (Table 2).

**Electrophysiological Characteristics**

**C1 Area Neurons**

More C1 cells were active (ie, showed ongoing action potential firing) in WRs (47% of 43 neurons) than in SHRs (9% of 11 neurons; Figure 4A). “Quiescent” neurons in WRs and SHRs had identical resting membrane potential ($-54.5\pm0.9$ mV, $n=23$, versus $-54.6\pm2.0$ mV, $n=10$). Actively firing C1 WR neurons had a resting membrane potential of $-47.9\pm1$ mV ($P<0.01$ compared with silent C1 WRs). Because only 1 C1 SHR cell had ongoing discharge, such a comparison is not possible for that strain.

**A2 Neurons**

A2 cells (silent or quiet cells) of WRs and SHRs had similar resting membrane potential ($-56\pm2$ versus $-58\pm3$ mV, $n=8$ and 9, correspondingly; $P>0.05$). A total of $38\%$ of WR and $22\%$ of SHR A2 neurons were active (Figure 4A).

**Responses to Ang II**

**C1 Area Neurons**

Ang II (200 nmol/L) evoked a larger depolarization in the active rather than in silent C1 WR cells ($+4.8\pm0.6$ mV, $n=20$ and 2.8$\pm0.7$ mV, $n=23$, correspondingly; $P<0.05$; Figures 4B and 5). The average amplitude of depolarization in silent SHR C1 cells ($2.2\pm0.6$) was not different from the silent WR cells ($P>0.1$). Only silent WR neurons were used for comparison because we only found 1 active cell in slices from SHRs. Ang II evoked distinct [Ca$^{2+}$], increases in $61\%$ silent C1 neurons from WRs and $55\%$ silent cells from SHRs. There was a trend of a higher
Ang II depolarized the majority of WR and SHR A2 neurons (4.3 ± 0.5 mV), but there was no difference between rat strains (P > 0.05; Figure 4B). Ang II triggered a clear increase in [Ca\(^{2+}\)] in 75% of WR A2 neurons (average increase: +589 ± 151 mV in 6 responding neurons), but in SHR A2 cells we only found a response in 2 of 9 cells (ie, 22%). Thus, the average increase in [Ca\(^{2+}\)], in WRs was greater than in SHRs (129 ± 9% versus 106 ± 3%; P < 0.05; Figure 4C). All of the effects of Ang II were reversible and/or could be abolished or prevented by 1 μmol/L of losartan (Figures 5 and 6).

To assure that our conclusions were not dependent on the way the data have been analyzed, we also evaluated them by grouping cells in a variety of different ways, such as “Ang II” responsive and “nonresponsive” categories. However, this did not affect our conclusions (data not shown). In addition, we did not find significant differences in passive input resistance (measured by hyperpolarizing current pulses) of comparable populations of WR and SHR neurons (data not shown).

Discussion

This study is the first to directly compare various properties of identified CAergic neurons from C1 and A2 areas of SHRs with a normotensive WR strain. Although a number of differences have been documented, we believe that the most important finding of this study is the confirmation of the first hypothesis posed in the Introduction. It appears that C1 varicosities in SHRs release almost double the amount of CA via large quanta that have transmitter loads comparable to those found in peripheral chromaffin granules. We have also revealed that, whereas CAergic neurons of A2 and C1 cell groups in SHRs are not electrically “hyperactive,” they are differentially sensitive to Ang II. Unexpectedly, SHR A2 neurons were less sensitive to this critical neuromodulator, whereas an opposite trend was evident in C1 cells. Taken together, these observations confirm that the changes associated with the hypertensive phenotype are not universal across all of the central neurons equipped to synthesize CA. Instead, the differences revealed in C1 are in some respects the opposite of those that we found in the A2 area.

Looking for the differences in central CA neurons in a hypertensive and a normotensive rat strain has been made possible by the introduction of new approaches, such as virally mediated genetic targeting of these neurons, which allows their direct identification and microamperometric analysis of CA release from their fluorescently labeled varicosities in organotypic slices of rat brain stem.28,29,41 Consistent with our previous study,28 release of CA from axonal varicosities of A2 and C1 neurons occurred in 2 types of quanta. The most frequent release events have a median of 0.28 pC\(^{2+}\) (0.022 pC), corresponding with ∼70 000 CA molecules. This is compatible with vesicles with an average size of 75 nm, assuming a 0.5-mol/L intravesicular CA concentration.28,42 In addition, there are rare but very large quanta28 for which the neuroanatomical substrate in the brain has not yet been identified, but they may be related to vesicular organelles described in sympathetic neurons.43

Complex events, small and large, make a considerable contribution to total release. Overall in A2 neurons, large events composed a similar fraction of total CA release in WRs and SHRs, whereas release from C1 varicosities of SHRs was altered: the contribution of large quanta rose from 57% in WRs to 86% in SHRs, whereas the fraction of small events decreased. Higher output from large events was a combination of their much higher transmitter load (∼270%) and higher frequency relative to WRs. Increase in the size of these events may be because of a higher norepinephrine transporter activity in the SHR brain stem; an increased expression of tyrosine hydroxylase, the key enzyme of NA/ADR biosynthesis, in the SHR ventral medulla,44; and/or alterations in the

Table 1. Characteristics of Single Small and Large (<0.5 pC) Events in Areas C1 and A2 of WRs and SHRs

<table>
<thead>
<tr>
<th>Event Type</th>
<th>Median Quantal Content, pA</th>
<th>1/2, ms</th>
<th>Q, fC</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1</td>
<td>A2</td>
<td>C1</td>
<td>A2</td>
</tr>
<tr>
<td>WR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single small events</td>
<td>4.0 ± 0.3</td>
<td>5.2 ± 0.5</td>
<td>3.6 ± 0.3</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td>Single large events</td>
<td>109.1 ± 29.5</td>
<td>165.0 ± 98.6</td>
<td>4.5 ± 1.1</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>SHR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single small events</td>
<td>5.7 ± 0.8</td>
<td>4.9 ± 0.7</td>
<td>3.0 ± 0.2</td>
<td>3.9 ± 0.2</td>
</tr>
<tr>
<td>Single large events</td>
<td>186.6 ± 63.9</td>
<td>138.5 ± 39.8</td>
<td>5.2 ± 1.3</td>
<td>4.3 ± 0.5</td>
</tr>
</tbody>
</table>

Individual amperometric spikes were characterized by their medial quantal content, half-times and charge (Q in femtoCoulomb). Differences were not significant, apart from time courses (1/2) of small events: A2 of WR vs SHRs P < 0.01, WR C1 vs A2 P < 0.05, and SHR C1 vs A2 P < 0.01 (unpaired Student t test). Please note that kinetic parameters of the complex events such as shown in Figure 1C were not evaluated.

Table 2. Charges (in fC) of Large Events (single and complex) in C1 and A2 Varicosities of WR and SHR

<table>
<thead>
<tr>
<th>Rat Strain</th>
<th>C1</th>
<th>A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>WR</td>
<td>1460 ± 220</td>
<td>3810 ± 940</td>
</tr>
<tr>
<td>SHR</td>
<td>3970 ± 760</td>
<td>2860 ± 510</td>
</tr>
</tbody>
</table>

Note the highly significant increase in the transmitter load of large release events in the C1 area varicosities of SHRs (Student 2-tailed unpaired t test). Interestingly, the trend in the A2 area was the opposite. Q in charge in femtoCoulomb.
vesicular storage apparatus. These changes, when combined, possibly alter the way the vesicles are formed or filled. Interestingly this was area specific and not evident in A2 varicosities.

We believe that such reorganization of the CA vesicular pools may have a major impact on downstream signaling by C1 varicosities. Because large release events are very fast in the brain, they deliver copious quantities of CA into the extracellular space essentially instantaneously. Consistent with the “volume” mode of transmission, varicosities of C1 neurons in the spinal cord seldom form tight oppositions with preganglionic sympathetic neurons. Thus, CA released in the volume mode of transmission may spread further from the release site than when released in small quanta. When released within synaptic appositions, a high quantal load may result in synaptic overspill and add to volume transmission.

The RVLM provides tonic stimulatory input to many central nervous system sites of autonomic control, including the sympathetic preganglionic neurons. Hence, by releasing bulk loads of the transmitter, C1 varicosities could signal to a wider range of cellular targets in SHRs. In addition, by analogy with granules in chromaffin cell, large vesicles are likely to release copious amounts of cotransmitters (such as

Figure 4. Ongoing activity and responses to Ang II of C1 and A2 neurons. A, Distribution of silent vs active neurons in C1 and A2 areas. In both areas fewer cells in SHR exhibited ongoing action potential activity. B, Effect of Ang II (200 nmol/L) on membrane potential of C1 and A2 neurons. Because we have only encountered 1 C1 cell with ongoing activity in SHR slices, the effect of Ang II is not shown here. C, Effect of Ang II (200 nmol/L) on [Ca\(^{2+}\)] estimated using Rhod-2. In A2 cells of SHR, Ang II evoked much weaker Ca\(^{2+}\) response. Note that, in C1 cells, the trend was opposite.

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ATP, chromogranin, and neuropeptide Y), which could play an additional important signaling role.

The total CA release/time was higher in SHRs than WRs for both C1 and A2 varicosities. This observation is at odds with the lower levels of ongoing action potential activity in SHRs and may suggest a prevalence of action potential–independent release in SHRs. However, at present, we have insufficient evidence to support this hypothesis, because microamperometry does not take into account the balance between the numbers of release-active and “dormant” varicosities.

Regarding the second objective of this study, we found no evidence that either C1 or A2 neurons of SHRs are “hyperexcitable” or “hyperactive,” at least under our in vitro conditions. In fact, fewer C1 cells of SHRs had an ongoing discharge compared with WRs (10% versus 53%). The techniques used here required genetic labeling of the CA neurons with EGFp and their optical identification. Therefore, this study could only be conducted in vitro, and this limitation needs to be acknowledged. Obviously, within the context of the intact brain, CA neurons receive various synaptic and paracrine signals, which may well affect their firing frequencies. Therefore, differences in neuronal activity in slice cultures predominantly reflect limited synaptic inputs and the intrinsic features of these cells, which suggests that neither A2 nor C1 neurons are electrically hyperactive.

The third hypothesis tested was that the effects of Ang II on electric activity or Ca2+i of CA neurons mobilization are enhanced in SHRs. Here, we only analyzed the fast, non-genomic effects of Ang II, which occur within minutes and involve effects on ion channels, Ca2+i stores, and transmitter release, similar to those effects described previously in chromaffin cells. Consistent with the results obtained in peripheral models, Ang II depolarized many A2 and C1 neurons and triggered [Ca2+i] elevations. Based on previous evidence10,47 and the present experiments, such [Ca2+i] elevations are largely attributable to Ca2+i release from the intracellular stores. Interestingly, WR A2 neurons responded to Ang II more vigorously than SHRs, both in terms of depolarization and Ca2+i release. A2 neurons are most likely a part of an antihypertensive homeostatic mechanism, as several groups including us have shown,23,25,26 This may involve NA release from A2 neurons within the nucleus tractus solitarius and/or from A2 projections to the hypothalamus,48,49 as reviewed previously. Thus, reduced sensitivity of the A2 cells to Ang II might further compromise their homeostatic role in the SHR.

Perspectives

This study has uncovered previously unsuspected changes in central CA transmission in SHRs that are area specific. A2 cells of SHRs have a reduced sensitivity to Ang II, which could indicate their weaker restraining influence on blood pressure. C1 varicosities of the SHR, but not WR, preferentially release CA in large quanta. This is likely to translate into enhanced impact on the cellular recipients of C1 signals throughout the central nervous system. Both changes may potentially lead to elevated sympathetic activity and hypertension in SHRs. The molecular approaches of this study, in particular, the cell-specific viral vector–mediated gene expression, will be instrumental for future investigations into how differences at the single cell level in vitro translate into a genetic predisposition to hypertension in vivo.

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Disclosures

None.

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