Dysregulation of Neuronal Ca\textsuperscript{2+} Channel Linked to Heightened Sympathetic Phenotype in Prohypertensive States

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Hypertension is associated with impaired nitric oxide (NO)–cyclic nucleotide (CN)-coupled intracellular calcium (Ca\textsuperscript{2+}) homeostasis that enhances cardiac sympathetic neurotransmission. Because neuronal membrane Ca\textsuperscript{2+} currents are reduced by NO-activated S-nitrosylation, we tested whether CNs affect membrane channel conductance directly in neurons isolated from the stellate ganglia of spontaneously hypertensive rats (SHRs) and their normotensive controls. Using voltage-clamp and cAMP–protein kinase A (PKA) FRET sensors, we hypothesized that impaired CN regulation provides a direct link to abnormal signaling of neuronal calcium channels in the SHR and that targeting cGMP can restore the channel phenotype. We found significantly larger whole-cell Ca\textsuperscript{2+} currents from diseased neurons that were largely mediated by the N-type Ca\textsuperscript{2+} channel (Cav\textsubscript{2.2}). Elevating cGMP restored the SHR Ca\textsuperscript{2+} current to levels seen in normal neurons that were not affected by cGMP. cGMP also decreased cAMP levels and PKA activity in diseased neurons. In contrast, cAMP–PKA activity was increased in normal neurons, suggesting differential switching in phosphodiesterase (PDE) activity. PDE2A inhibition enhanced the Ca\textsuperscript{2+} current in normal neurons to a conductance similar to that seen in SHR neurons, whereas the inhibitor slightly decreased the current in diseased neurons. Pharmacological evidence supported a switching from cGMP acting via PDE3 in control neurons to PDE2A in SHR neurons in the modulation of the Ca\textsuperscript{2+} current. Our data suggest that a disturbance in the regulation of PDE-coupled CNs linked to N-type Ca\textsuperscript{2+} channels is an early hallmark of the prohypertensive phenotype associated with intracellular Ca\textsuperscript{2+} impairment underpinning sympathetic dysautonomia.

Key words: autonomic nervous system; cGMP; dysautonomia; hypertension; N-type Ca\textsuperscript{2+} channel; sympathetic

Significance Statement

Here, we identify dysregulation of cyclic-nucleotide (CN)-linked neuronal Ca\textsuperscript{2+} channel activity that could provide the trigger for the enhanced sympathetic neurotransmission observed in the prohypertensive state. Furthermore, we provide evidence that increasing cGMP rescues the channel phenotype and restores ion channel activity to levels seen in normal neurons. We also observed CN cross-talk in sympathetic neurons that may be related to a differential switching in phosphodiesterase activity. The presence of these early molecular changes in asymptomatic, prohypertensive animals could facilitate the identification of novel therapeutic targets with which to modulate intracellular Ca\textsuperscript{2+}. Turning down the gain of sympathetic hyperresponsiveness in cardiovascular disease associated with sympathetic dysautonomia would have significant therapeutic utility.

Introduction

Increased activity of the sympathetic nervous system is a well-established contributing factor to the pathophysiology of human hypertension (Esler, 2010; Grassi et al., 2015) and to that observed in the spontaneously hypertensive rat (SHR) (Heaton et al., 2006; Shank et al., 2013a; Mancia and Grassi, 2014). In par-
ticular, the renal nerves (Lundin et al., 1984) and the postganglionic nerves innervating the heart are sympathetic beds associated with heightened activity. Interestingly, this sympathetic impairment occurs in young, prohypertensive animals (Li et al., 2012; Shanks et al., 2013c), suggesting that it is an early hallmark of the disease itself, although the exact mechanisms that underpin the phenotype remain poorly understood. When postganglionic sympathetic stellate neurons (PGSNs) that predominantly innervate the heart from age-matched prohypertensive SHR s are compared with PGSNs from normotensive rats (WKY), the SHR neurons have increased intracellular calcium transients ($[^{2+}]Ca$) (Li et al., 2012) and enhanced neurotransmission (Zugck et al., 2003; Li et al., 2007; Shanks and Herrig, 2013; Shanks et al., 2013c). This translates into increased postsynaptic excitability of the sinoatrial node due to a greater conductance of $[^{2+}]Ca$ and an increase in heart rate (Heaton et al., 2006). The observed differences in $[^{2+}]Ca$ may be partially explained by altered mitochondrial Ca$^{2+}$ handling (Li et al., 2012), but it is unclear whether there are additional changes at the membrane level that contribute to the greater calcium transients.

Recently, Tu et al. (2014) demonstrated enhanced activity of the N-type voltage-gated Ca$^{2+}$ ($Cav_{2.2}$) channel in the PGSNs of a rat model of heart failure. Another study found that inhibition of this channel restores cardiac autonomic imbalance and prevents lethal arrhythmias in a mouse model of heart failure (Yamada et al., 2014). Similarly, we also observed increased whole-cell neuronal Ca$^{2+}$ currents in the prohypertensive SHR (Lu et al., 2015), suggesting that channel dysregulation is an early molecular event that may be highly conserved over several developmental stages from the predisposed phenotype to advanced heart failure (Li and Paterson, 2016). What links ion channel dysregulation to sympathetic hyperresponsiveness? One suggestion is that early metabolic-oxidative stress could be coupled to abnormal cyclic nucleotide (CN) regulation (Krukoff, 1998; Danson and Paterson, 2005; Danson et al., 2005). Voltage-gated Ca$^{2+}$ channels are strongly regulated by CN-coupled protein kinases (Catterall, 2000; Simms and Zamponi, 2014) that are dysfunctional in hypertension (Paterson, 2001; Heaton et al., 2007; Li et al., 2007; Wang et al., 2007; Li et al., 2013). Specifically, PGSNs have reduced neuronal nitric oxide synthase (nNOS) activity, low level expression of soluble guanylyl cyclase (sGC) (Li et al., 2013) and high phosphodiesterase 2A (PDE2A) activity (Li et al., 2015), leading to reduced levels of cGMP (Li et al., 2007, 2013). Together, this cascade of events could provide a link to ion channel dysregulation. However, emerging evidence suggests that the inhibitory action of NO donors on $Cav_{2.2}$ in HEK 293 cells is via cGMP-independent pathways coupled to S-nitrosylation of the N-type channel (Jin et al., 2011; Zhou et al., 2015).

Here, we tested the hypothesis that impaired CN regulation provides a direct link to abnormal signaling of neuronal calcium channels in prohypertensive rats and that targeting cGMP can restore the normal channel phenotype in the diseased PGSN.

Materials and Methods

**Animals.** Four-week-old male prohypertensive SHR s and normotensive WKY and Wistar (hereafter referred to as control) rats were obtained from Harlan Laboratories and Charles River Laboratories. The SHR s begin to show development of clinical symptoms of hypertension at 6 weeks of age (Dickhout and Lee, 1998). The rats were housed in standard plastic cages and artificial lighting was fixed to a natural 12 h light/dark cycle. Food and water were available ad libitum. All experiments were performed in accordance with the UK Home Office Animals Scientific Procedures Act of 1986 (PPL 30/2360 and 30/3131, David J. Paterson).

**Reagents.** All drugs and reagents were sourced from Sigma-Aldrich unless stated otherwise.

**Adenovirus generation.** The complete open reading frame of the cAMP-sensitive FRET-based sensor H187 was obtained from pcDNAs using double digestion with the restriction enzymes EcoRI and HindIII and introduced in the pDual vector. Generation and amplification of the viral particles were outsourced to Vector Biosials.

**Cell isolation.** The sympathetic stellate neurons that predominately innervate the heart were isolated as described previously (Shanks et al., 2013b). Briefly, the stellate neurons from 4-week-old prohypertensive SHR and normotensive control rats were dissected out and digested with collagenase (1 mg/ml) and trypsin (2 mg/ml) to create a single-cell suspension. The PGSNs were then grown on 6 mm poly-n-lysine/laminin-covered coverslips (37°C 5% CO$_2$) for 3 h before electrophysiological recordings were made. For measurements of intracellular CAMP and protein kinase A (PKA), PGSNs were transfected with relevant adenosinal particle-forming units (H187:1.4 × 10$^7$, AKAR4: 4 × 10$^7$) for 24 h. The cells were incubated for 24–48 h before FRET imaging.

**Immunofluorescence.** Cells were fixed with 4% formaldehyde for 10 min before blocking and permeabilization using a solution containing 10% goat serum, 0.3% bovine serum albumin, and 0.1% Triton X-100 in PBS for 1 h at room temperature. After washes with PBS, primary antibodies against tyrosine hydroxylase (TH, 1:250, T2199; Abcam) and Cav$_{2.2}$ (1: 250, ACC-002; Alomone Laboratories) were incubated overnight at 4°C. Appropriate secondary antibodies (Alexa Fluor 1:1000) were added for 2 h before the coverslips were mounted using mounting medium containing DAPI (Vectashield; Vector Laboratories). Cells were imaged on a confocal microscope (Live Cell; Olympus) within 1 week of mounting.

**Whole-cell voltage clamp of acutely isolated neurons.** Voltage-clamp experiments were conducted using the Axopatch 200B amplifier and Clamplex software. The neurons were held at −90 mV before a set of 50 ms voltage steps from −50 to +50 were applied with 10 mV, 5 s intervals. The bath was grounded using an Ag/AgCl silver pellet wire connected to the bath via a 3 m agar salt bridge. Cells were perfused with tyrode or Ca$^{2+}$-isolating solution using a custom-built perfusion system into a heated microscope stage chamber maintained at 37°C. Access to the cell was obtained in normal tyrode solution containing the following (in ms): 135 NaCl, 4.5 KCl, 11 glucose, 20 HEPES, 1 MgCl$_2$, 2 CaCl$_2$, pH 7.4.

Then, the solution was changed to the Ca$^{2+}$-isolating solution containing no Na$^+$ and K$^+$ channel blockers (TEA and Cs$^+$) with Ba$^{2+}$ as the charge carrier to isolate the Ca$^{2+}$ currents containing the following (in ms): 135 TEACl, 4.5 KCl, 1 MgCl$_2$, 4 glucose, 1 NaHCO$_3$, 2 Ba$^{2+}$, pH 7.40, either in the presence or absence of drugs (1 μM ω-conotoxin GVIA or 100 μM b-homouguanosine-cGMP, 8b-cGMP). AKAR4 (Lepery et al., 2011; Lethkinowitz et al., 2013) for 24 h. FRET inactivation (Imredy and Yue, 1994). The internal solution contained the following (in ms): 140 CsCl, 10 HEPES, 0.1 CaCl$_2$, 1 MgCl$_2$, 4 MgATP, 1 EGTA, pH 7.30. All solutions had osmolarities of 300 mOsm/L. A 75–90% series resistance compensation was applied to all cells. Peripheral neurons portray significant axonal branching, making an effective voltage clamp difficult to achieve (Bar-Yehuda and Korngreen, 2008). To minimize these space-clamp errors, the cells were held at −90 mV, low concentrations of the charge carrier was used, cells were patched acutely, and large bore, blunt electrodes (1.5–2 MΩ) were used in addition to strict exclusion criteria. Only cells that had a series resistance of <10 MΩ throughout the recording, a holding potential of >−300 pA, and cells in which no visible contusion of the currents were visible were included in this study. Due to significant run-down of the currents in both animal models, which was found to be neither time nor activity dependent, unpaired experiments were performed to unmask differences between cells.

**FRET measurements of cytosolic cAMP and PKA activity.** For intracellular measurements of cAMP and PKA activity, cells were infected 0–24 h after culture with the FRET-based cAMP sensors Epac-H30 (Ponsioen et al., 2004), Epac-H187 (Klarenbeek et al., 2015) or the PKA sensor (Lepery et al., 2011; Lethkinowitz et al., 2013) for 24 h. FRET sensor-expressing neurons were imaged 3–4 d after isolation on an inverted Nikon microscope. This was connected to an OptoLED fluores-
cence imaging system (Cairn Research) equipped with a 40× oil-immersion objective, a CoolSnap HQ2 digital CCD camera (Photometrics), and a beam-splitter (DV2; Photometrics), which included the emission filters for CFP and YFP acquisition (dichroic mirror 505DCXR). The cells were excited at 430 nm for 100 ms every 15 s. CFP and YFP emissions were measured and the ratio between 480/535 nm fluorescent emission intensities were automatically calculated using Optofluor software. Background fluorescence was subtracted from emission intensities and intensity ratios were plotted against time. Mean FRET responses were expressed as the percentage change from baseline (\(R/R_0\) where \(R_0 = R - R_0\). \(R_0\) is the ratio of intensity over a 30 s period in the absence of drugs and \(R\) is the mean ratio over 30 s in the presence of the respective drug treatment).

During FRET experiments, cells were perfused continuously with Tyrode’s solution and flow rate was controlled at 2–3 ml/min. Pharmacological agents were diluted in Tyrode’s solution and perfused at the following concentrations: forskolin, 0–25 \(\mu\)M; 3-isobutyl-1-methylxanthine (IBMX), 1–100 \(\mu\)M; the cGMP analog 8b-cGMP, 100 \(\mu\)M; the PDE3 inhibitors cilostamide, 10 \(\mu\)M, or milrinone, 10 \(\mu\)M; and the PDE2 inhibitor BAY-60-7550, 1 \(\mu\)M (Cayman Chemicals). For comparisons between cells, the average percentage FRET change over a 30 s period was calculated once equilibrium was reached. In all experiments, the maximal FRET change of each cell was recorded by exposing the cells to saturating concentrations of an adenylyl cyclase (AC) activator and a PDE inhibitor (25 \(\mu\)M forskolin and 100 \(\mu\)M IBMX, respectively) to ensure that the cells responded similarly to the sensor. The H30 cAMP sensor responded differently in the SHR and control cells, so these data were normalized to the IBMX/forskolin maximum FRET response to allow for comparisons between the control and SHR neurons.

**Figure 1.** The whole-cell \(Ca^{2+}\) current is larger in the prohypertensive SHR. Whole-cell voltage clamp was performed on the cardiac sympathetic stellate ganglion innervating the heart to investigate the whole-cell \(Ca^{2+}\) properties of 4-week-old prohypertensive SHR and normotensive control rats; 50 ms, 10 mV voltage steps from −50 to +50 were applied to the cell before the resulting current was measured. Immunofluorescence showed TH positivity, confirming sympathetic phenotype of the neurons (A). B, Representative voltage-clamp traces of control and SHR neurons showing significantly larger currents in the SHR neurons. C, Peak current (−10 mV) was −127.5 ± 5.94 pA/pF (n = 10) in the SHR and −108.0 ± 6.80 pA/pF (n = 10, \(p < 0.045\)) in the control. D, Current–voltage relationship showing significantly larger currents in the SHR compared with the control at multiple voltages. E, There were no changes in the voltage dependency of the channel, as shown by the activation curve, suggesting that the biophysical properties of the channels are unaffected. Data are represented as the mean ± SEM.
Protocols. Specifically, we looked at the cells’ ability to generate cAMP and resulting PKA activity by administering the AC activator forskolin. Further, we assessed the cells’ ability to hydrolyze cAMP by pharmacologically inhibiting the predominant PDE subtypes (PDEs 1–7, 10–11) with the nonspecific PDE inhibitor IBMX. To test the involvement of the CNs in the regulation of the N-type channel, 8b-cGMP was perfused before the voltage-clamp experiment was repeated. In addition, to investigate whether the cGMP dysfunction seen in the prohypertensive state interferes with the cAMP pathway (through cross-talk via PDEs), we administered 8b-cGMP to the control and SHR neurons and monitored the resulting cAMP levels and PKA activity. Finally, to test the relative involvement of PDE2A and PDE3 in the cells, the cells were incubated with selective PDE2 (160–7550) and PDE3 inhibitors (cilostamide or milrione) while the cAMP levels were monitored.

Statistical analysis. All data were analyzed using GraphPad Prism software. When the data passed normality tests, unpaired two-tailed t tests were used; when they did not, nonparametric tests were used with the specific test reported in the figure legend. All data are expressed as the mean ± SEM. Statistical significance was accepted at p < 0.05.

Results
Neuronal Ca²⁺ currents of the prohypertensive SHR are larger than that of the normotensive control
Immunofluorescence analysis of the cardiac stellate neurons confirmed their sympathetic phenotype by their TH positivity (Fig. 1A). The whole-cell voltage-gated Ca²⁺ currents of the prohypertensive SHR (−127.5 ± 5.94 pA/pF, n = 10) were significantly larger than that of the normotensive control animals (−108.0 ±
Increased whole-cell Ca\textsuperscript{2+} currents are attributed to changes in Cav\textsubscript{2.2}

Immunofluorescence analysis confirmed the expression of the N-type Ca\textsuperscript{2+} channels on the membrane of the TH-positive cells (Fig. 2A). We tested their functional relevance by perfusing the cells with 1 μM ω-conotoxin GVIA and observed a reduction in the whole-cell currents by 75% and 83%, respectively, down to similar levels in both normotensive and hypertensive phenotypes (control −26.88 ± 1.70, n = 6; SHR −22.04 ± 1.60 pA/pF, n = 5, p = 0.072), suggesting that Cav\textsubscript{2.2} is the Ca\textsuperscript{2+} channel predominantly carrying the Ca\textsuperscript{2+} current in PGSNs (Fig. 2B–E).

Increasing the intracellular cGMP concentrations significantly lowers Ca\textsuperscript{2+} currents and reverses the channel phenotype

To test the involvement of the CNs in the regulation of the N-type channel, the cell-permeant 8b-cGMP was perfused before the voltage-clamp experiment was repeated. Elevating intracellular cGMP did not alter the Ca\textsuperscript{2+} currents in the normotensive control animals, but reduced the SHR currents (−127.5 ± 5.94 pA/pF, n = 10 to −105.2 ± 7.79 pA/pF, n = 7, p = 0.035) down to control levels (−108.0 ± 6.80 pA/pF, n = 10, p = 0.79; Fig. 3A–E). No changes were observed in the activation curve (Fig. 3F).
To investigate whether the cGMP dysfunction seen in the prohypertensive state was interfering with the cAMP pathway (through cross-talk via PDEs), we administered 8b-cGMP to the neurons and monitored cAMP levels. We then established the functional relevance of this by assessing PKA activity. Application of 8b-cGMP led to a decrease in cAMP levels (4.82 ± 2.02%, n = 14), with no change in PKA activity (1.09 ± 0.57%, n = 8) in the SHR neurons (Fig. 4A,B). In contrast, when normotensive neurons were treated with 8b-cGMP, we observed a significant increase in both cAMP levels (11.68 ± 1.54%, n = 16) and PKA activity (19.15 ± 3.51%, n = 6) (Fig. 4C,D). This finding suggests a differential involvement of PDE isoforms in response to cGMP signaling in the normotensive versus prohypertensive PGSNs.

Differential response of the SHR and control neurons to PDE2A blockade

PDE2A has been shown recently to play a major role in the control of neurotransmission in sympathetic neurons. To assess whether PDE2A plays a role in regulating Ca²⁺ channels, we bath applied the specific PDE2A inhibitor BAY 60–7550 and repeated the voltage-clamp experiments. The application of 1 μM BAY 60–7550 significantly increased the channel currents (108.0 ± 6.803 pA/pF, n = 9 to 138.7 ± 9.610 pA/pF, n = 10, p = 0.0169) in the normotensive neurons. Interestingly, the SHR neurons responded to the same treatment with a slight, nonsignificant decrease of currents (127.5 ± 5.937 pA/pF, n = 10 to 118.0 ± 6.673 pA/pF, n = 9). After PDE2A inhibition, the control currents were trending toward being larger than the SHR, but this was not quite significant (138.7 ± 9.610 pA/pF to 118.0 ± 6.673 pA/pF, p = 0.052, Mann–Whitney test) (Fig. 5A-C).

Sympathetic stellate neurons of the SHR and control rats functionally express both PDE2A and PDE3

To test the relative involvement of PDE2A and PDE3 in the cells, the cells were incubated with selective PDE2A (1 μM BAY 60–7550) and PDE3 inhibitors (10 μM cilostamide or milrinone) while the cAMP levels were monitored. Pharmacological inhibition of PDE2A (Fig. 6A) led to significant increases in cAMP in both the control and SHR neurons that was not significantly different (19.69 ± 5.16%, n = 10 vs 36.55 ± 21.47%, n = 8, p = 0.94). The cAMP responses to two separate, clinically tested PDE3 inhibitors led to similar increases in cAMP generation (Fig. 6B) that was not significantly different between the SHR and control (milrinone: 22.98 ± 6.81%, n = 10 vs 31.14 ± 6.25%, n = 16, p = 0.41; cilostamide: 28.15 ± 15.45%, n = 5 vs 33.23 ± 8.18%, n = 8, p = 0.58, Mann–Whitney test).

Differential cGMP–cAMP cross-talk in the SHR

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Figure 5. PDE2A blockade suggests differential involvement of PDEs in the control and SHR neurons. Current–voltage relationships of the control (A) and SHR (B) neurons after the application of 1 μM BAY 60–7550, a PDE2A-selective inhibitor. BAY 60–7550 significantly increased the peak control channel currents (−108.0 ± 6.803 pA/pF to −138.7 ± 9.610 pA/pF, n = 9–10, p = 0.0169), but showed a slight, nonsignificant decrease on the SHR currents (−127.5 ± 5.937 pA/pF to −118.0 ± 6.673 pA/pF, p = 0.052 n = 10 and 9). After PDE2A inhibition, the control currents were trending toward being larger than the SHR, but this was not quite significant (138.7 ± 9.610 pA/pF to −118.0 ± 6.673 pA/pF, p = 0.052; C).

Figure 6. Evidence for the functional expression of PDE2 and PDE3 in the sympathetic neurons of the SHR and WKY. To assess the functional expression of PDE2 and PDE3, FRET imaging of cAMP levels (H187) in response to well characterized PDE-selective blockers was performed. Pharmacological inhibition of PDE2A (1 μM BAY 60–7550; A) and PDE3 (10 μM milrinone; B) increased the cAMP levels in both the control and SHR neurons. This was found to be nonsignificant between the two strains (n = 8–16; PDE2: 19.69 ± 5.16%, n = 10 vs 36.55 ± 21.47%, n = 8, p = 0.94, PDE3: milrinone; 22.98 ± 6.81%, n = 10 vs 31.14 ± 6.25% n = 16, p = 0.41, cilostamide; 28.15 ± 15.45%, n = 5 vs 33.23 ± 8.18% n = 8, p = 0.58, Mann–Whitney test). The presence of PDE2A in sympathetic stellate neurons is known, but this provides the first evidence for the functional expression of PDE3 in these cells.
cAMP–PKA signaling pathway remains unchanged in prohypertensive animals

To determine the cells’ ability to generate cAMP and activate PKA, we administered the AC activator forskolin and measured the FRET responses of the two sensors. FRET experiments in which the PGSNs were challenged with increasing doses of forskolin (0.1–25 mM) revealed no significant difference between the generation of cytosolic cAMP (n = 15–18/21–28), and PKA activity (n = 20 –23/16 –17) between 4-week normotensive control and pro-SHR, respectively (Fig. 7 A, B). This indicates that the cAMP–PKA signaling pathway remains intact in the prohypertensive animals.

Discussion

The novel findings in this study are as follows. The enhanced whole-cell Ca2+ current from the prohypertensive SHR PGSNs are predominately mediated by activation of the N-type Ca2+ channel. Elevating cGMP restored the SHR Ca2+ current to levels seen in normal neurons. Control neurons were unaffected by raised cGMP. Elevated cGMP also decreased cAMP levels and PKA activity in diseased neurons. In contrast, cAMP–PKA activity was increased in normal neurons, suggesting differential switching in PDE activity. PDE2A inhibition enhanced the Ca2+ current in normal neurons to a similar conductance to that seen in SHR neurons, whereas the inhibitor slightly decreased the current in diseased neurons. Global cAMP–PKA levels were not different between neurons when activated by an adenylyl cyclase activator. However, pharmacological evidence suggests a switching from cGMP acting via PDE3 in the control neurons to PDE2A in the SHR neurons in the modulation of the N-type Ca2+ current.

Channel dysfunction in cardiovascular diseases

The importance of the N-type Ca2+ channel function in relation to the release of noradrenaline (NA) in the sympathetic nervous system has long been known (Molderings et al., 2000; Uhrnholt and Nedergaard, 2005), but its relevance to hypertension came from the observation that N-type channel blockade causes hypotension (Pruneau and Béliechard, 1992). Moreover, the Cav2.2 mutant mouse has significant problems with blood pressure control that is linked to reduced baroreflex control of heart rate (Ino et al., 2001; Mori et al., 2002). Recently, it was shown that stellate neurons from rats with heart failure have markedly increased N-type channel currents (Tu et al., 2014). Interestingly, the increased current was not correlated with channel expression, suggesting a role for posttranslational regulation of channel activity. Although the N-type channel is the major contributor of Ca2+ entry coupled to sympathetic transmission (Uhrnholt and Nedergaard, 2003), it is possible that other Ca2+ channels are also involved and could account for the residual response that we observed after ω-conotoxin GVIA treatment. Although ω-conotoxin GVIA has high affinity for the N-type channel (Olivera et al., 1987), in rat sympathetic neurons, this drug has also been shown to block L-type currents (McCleskey et al., 1986). It is therefore possible that the effect that we observed could be attributed partially to overactivity of this channel in the SHR (Heaton...
et al., 2006) because its inhibition on the mesenteric artery significantly reduces NA release in response to neuronal stimulation (Tsuda et al., 2001). Another possibility is that alterations in cell excitability could be linked to N-type Ca\(^{2+}\)/H11001 channel activity. Therefore, we cannot rule out that such changes in threshold or action potential frequency might also be linked with the N-type Ca\(^{2+}\)/H11001 channel phenotype. This could conceivably contribute to the overall modulation of channel excitability and enhanced neurotransmission.

**CN regulation of Ca\(^{2+}\)/H11001 channels**

Ca\(^{2+}\)/H11001 channels are regulated by CNs via PKA- and protein kinase G (PKG)-dependent phosphorylation. CAM and cGMP act via PKA and PKG to cause activation and inhibition of the channel current, respectively (Bannister et al., 2005; Catterall, 2011; Zamponi et al., 2015). This occurs either via direct phosphorylation of the channels (Tohse and Sperelakis, 1991; Jiang et al., 2000) or through cross-play between the two CN pathways via PDEs (Conti and Beavo, 2007; Zocco and Movsesian, 2007; Stangherlin et al., 2011; Stangherlin and Zaccolo, 2012a). CNs also act to terminate their own signals by activating protein phosphatases that dephosphorylate downstream targets and PDEs that break down cAMP and cGMP (Francis et al., 2010, 2011). The larger Ca\(^{2+}\) currents observed in the SHR neurons could be due to reduced PKG-mediated inhibition and/or increased PKA-mediated activation of the current. Because \(\omega\)-conotoxin GVIA reduced the currents of both models down to similar levels, this suggests that the differences seen are due to modulation of the N-type Ca\(^{2+}\)/H11001 channel. Moreover, we observed that currents were normalized after a 4-minute perfusion of cGMP, suggesting that changes in channel regulation rather than expression are likely to account for the differences reported here.

**NO–cGMP pathway in sympathetic responsiveness**

SHR sympathetic neurons have lower bioavailability of NO and reduced expression of the \(\beta_1\) subunit of sGC (Li et al., 2013), resulting in lower levels of cGMP (Li et al., 2007). This is associated with increased Ca\(^{2+}\) transients (Li et al., 2012) and enhanced neurotransmission (Herring et al., 2011; Li et al., 2012; Shanks et al., 2013c) compared with control neurons. Gene transfer of nNOS (Li et al., 2013) or its adaptor protein, CAPON (Lu et al., 2015), rescues this sympathetic phenotype. However, we cannot rule out that a part of the inhibitory effect of NO modulation on Cav\(_{2.2}\) is via non-GMP-mediated S-nitrosylation of the channel protein itself. Zhou et al. (2015) reported in HEK 293 cells that the NO donor SNAP can generate S-nitrosothiols that block the N type conductance, an effect reversed by methanethiosulfonate ethylammonium. Moreover, they identified the consensus motifs for channel inhibition that involved several intracellular cysteine residues. In particular, site-directed mutagenesis
of cysteine 1045 in the II–III loop significantly reduced NO sensitivity of Cav2.2. Whether this pathway is functionally activated within the range of physiological concentrations of NO remains to be established.

Nevertheless, our results provide direct evidence in sympathetic neurons that cGMP causes attenuation of the Ca2+ channel current. Indeed, previous studies have demonstrated that the NO–cGMP pathway can regulate Ca2+ currents via PKG activation, but also through cross-talk with PDEs (Méry et al., 1991; Fischmeister and Méry, 1996). Studies have reported that application of 8b-cGMP leads to a direct phosphorylation of Ca2+ channels via PKG (Jiang et al., 2000). This resulted in smaller currents, but no change in the voltage dependency (Tohse and Sperelakis, 1991), of the channel, which is analogous to what we describe here.

cAMP-cGMP cross-talk via PDEs

In addition to its direct effects on ion channels, cGMP can act via cross-talk with the cAMP pathway via its activation/inhibition of cellular PDEs (Zaccolo and Movsesian, 2007; Francis et al., 2010; Stangherlin et al., 2011). cGMP and cAMP compete with each other for the catalytic sites on the PDEs with varying affinities (Conti and Beavo, 2007; Francis et al., 2010), resulting in inhibition and/or activation of these enzymes. PDE2A and PDE3 have dual specificity for cAMP and cGMP (Conti and Beavo, 2007; Francis et al., 2010). cGMP activates PDE2A, leading to the breakdown of cAMP (and thus reduction in PKA-mediated phosphorylation of downstream targets including Ca2+ channels), whereas it can inhibit PDE3A with the opposite result. Although a cell is likely to express several PDEs that regulate the efficacy of CNs, PDE2A and PDE3A have been localized in cardiac myocytes (Stangherlin and Zaccolo, 2012a; Maass et al., 2015; Zoccarato et al., 2015), where they are critically involved in cardiac function (Stangherlin and Zaccolo, 2012a). PDE2A has been reported recently in sympathetic stellate neurons with high activity in SHR neurons (Li et al., 2015), whereas there is no direct evidence of PDE3 expression within sympathetic stellate neurons.

To test whether the cGMP dysregulation seen in the SHR was affecting cAMP signaling, we elevated cGMP levels pharmacologically and used FRET to record changes in cAMP levels. We investigated its functional relevance by assessing PKA activity. Application of 8b-cGMP led to a reduction of cAMP in SHR neurons, with no activation of PKA, suggesting that cAMP hydrolysis via cGMP stimulation of PDE2A is occurring. The cGMP-dependent reduction in the Ca2+ channel current after 8b-cGMP is consistent with the notion of PDE2A dominance in diseased neurons affecting the efficacy of cGMP, because overexpressing PDE2A decreases cGMP and increases intracellular Ca2+ transients and neurotransmission (Li et al., 2015; Seifert, 2015). Conversely, in the control neurons, even though 8b-cGMP significantly increased cAMP levels and PKA activity (suggesting the involvement of other PDEs inhibited by cGMP), this did not translate into a change in Ca2+ channel current because activation of the inhibitory cGMP-PKG pathway most likely counterbalanced changes in the stimulatory cAMP–PKA pathway. However, slowing cGMP hydrolysis with PDE2A inhibition markedly increased the Ca2+ current in normal neurons, probably as a consequence of cGMP acting to inhibit PDE3 leading to greater levels of cAMP–PKA activity (Figure 5).

Evidence of altered PDE2A/PDE3A balance in sympathetic neurons

Mutations in PDE3A have been implicated in human hypertension (Houslay, 2015; Maass et al., 2015). Given the observed increase in cAMP after administration of 8b-cGMP in control neurons, we hypothesized that PDE3 may be functionally expressed within stellate neurons, as has been demonstrated in the superior cervical ganglions (Nunes et al., 2012). The selective PDE3 inhibitor milrinone led to similar increases in cAMP levels within both SHR and control neurons. Comparable results were also observed using the clinically approved PDE3 inhibitor cilostamide. The notion of differential PDE2/3 involvement in control and diseased neurons helps to clarify the effects that PDE2A inhibition has in the regulation of the Ca2+ currents (Figure 5). In control neurons, this leads to significantly increased currents, possibly due to the direct elevation of cAMP causing inhibition of cAMP hydrolysis, but also via the indirect elevation of cAMP mediated by cGMP inhibition of PDE3. This in turn may result in increased PKA-mediated phosphorylation of Ca2+ channel currents. Conversely, in the SHR, the net result of PDE2A inhibition is a decrease in channel currents, possibly due to cGMP-mediated channel inhibition. This is consistent with the evidence that PDE2A has a higher affinity for hydrolysis of cGMP over cAMP (Xu et al., 2013; Li et al., 2015). Altered PDE2A/PDE3 balance could therefore be linked to the dysautonomia associated with hypertension (Figure 8).

Limitations

CNs (Mongillo et al., 2006; Stangherlin et al., 2011; Stangherlin and Zaccolo, 2012b; Lomas and Zaccolo, 2014) and PDEs reside in distinct subcellular compartments (Lefkimmiatis and Zaccolo, 2014). Specifically, in the rat, there are three splice variants of PDE2A that are localized in the cytosol (PDE2A1) or membrane (PDE2A2 and 3; Bender and Beavo, 2006), although their exact roles remain to be established. Similarly, PKA/PKG regulation of the Ca2+ channels also occurs in distinct signalosomes near the channels themselves, conferring site-specific regulation of Ca2+ entry coupled to neurotransmission (Toda and Okamura, 2003; Garthwaite, 2016). Furthermore, the rate of PDE hydrolysis is critically dependent on the concentration of both cAMP and cGMP that is reported to be different between cell types (Francis et al., 2010; Zhao et al., 2016). Although the global cAMP responses after PDE2A and PDE3 inhibition were similar between control and diseased neurons, this does not in itself establish that localized control within specifically regulated microdomains is normal in the diseased neuron.

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