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Voltage- and receptor-mediated activation of a non-selective cation channel in rat carotid body glomus cells

Jiaju Wang, James O. Hogan, and Donghee Kim

Department of Physiology and Biophysics, Chicago Medical School, Rosalind Franklin University of Medicine and Science, 3333 Green Bay Road, North Chicago, IL 60064, USA

Abstract

Recent study showed that hypoxia activates a Ca^{2+} -sensitive, Na^{+} -permeable non-selective cation channel (NSC) in carotid body glomus cells. We studied the effects of mitochondrial inhibitors that increase Ca^{2+} influx via Ca^{2+} channel (Ca_v), and receptor agonists that release Ca^{2+} from endoplasmic reticulum (ER) on NSC. Mitochondrial inhibitors (NaCN, FCCP, H_2S , NO) elevated $[\text{Ca}^{2+}]_i$ and activated NSC. Angiotensin II and acetylcholine that elevate $[\text{Ca}^{2+}]_i$ via the Gq-IP_3 pathway activated NSC. However, endothelin-1 (Gq) and 5-HT (Gq) showed little or no effect on $[\text{Ca}^{2+}]_i$ and did not activate NSC. Adenosine (Gs) caused a weak rise in $[\text{Ca}^{2+}]_i$ but did not activate NSC. Dopamine (Gs) and γ -aminobutyric acid (Gi) were ineffective in raising $[\text{Ca}^{2+}]_i$ and failed to activate NSC. Store-operated Ca^{2+} entry (SOCE) produced by depletion of Ca^{2+} stores with cyclopiazonic acid activated NSC. Our results show that Ca^{2+} entry via Ca_v , ER Ca^{2+} release and SOCE can activate NSC. Thus, NSC contributes to both voltage- and receptor-mediated excitation of glomus cells.

Keywords

Carotid body; Hypoxia; Mitochondria; Receptors; G proteins; Calcium

1. Introduction

Hypoxia depolarizes carotid body glomus cells by inhibiting the outward K^{+} current (Buckler, 2015; Lopez-Barneo et al., 2016). The depolarization causes opening of the voltage-dependent Ca^{2+} channel (Ca_v) and elevation of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Elevation of $[\text{Ca}^{2+}]_i$ stimulates the secretion of neurotransmitters and neuropeptides, some of which bind to receptors at the afferent carotid sinus nerve terminals to elicit an increase in the ventilatory response to hypoxia (Lopez-Barneo et al., 2009; Pardal et al., 2000). Neurotransmitters and neuropeptides secreted by glomus cells also act on

Corresponding author: Donghee Kim, PhD. Department of Physiology and Biophysics, Chicago Medical School, Rosalind Franklin University of Medicine and Science, 3333 Green Bay Road, North Chicago, IL 60064, USA, Tel 847-578-8356; donghee.kim@rosalindfranklin.edu.

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autoreceptors expressed in glomus cells and presumably modulate the basal level of excitability and the chemosensitivity of glomus cells (Nurse, 2010; Zhang et al., 2000).

In a recent study, we found that hypoxia not only inhibited the outward background K^+ channel (TASK) but also activated a non-selective cation channel (NSC) that was highly permeable to Na^+ (Kang et al., 2014). Depolarizing stimuli such as high extracellular KCl that open Ca_v also activated NSC (Kang et al., 2014). Ca^{2+} channel agonist such as FPL64176 was also effective in activating NSC. Thus, our study indicated that activation of NSC was coupled to Ca^{2+} influx via Ca_v . Because the reversal potential of NSC is ~ 35 mV more positive than the resting E_m (~ -60 mV), activation of NSC is expected to increase Na^+ influx and help to promote and sustain the depolarization of glomus cells in response to hypoxia. Activation of NSC is also expected to enhance the sensitivity of glomus cells to other depolarizing stimuli.

In addition to Ca^{2+} influx via Ca_v , elevation of $[Ca^{2+}]_i$ can occur via Ca^{2+} release from intracellular stores by the action of receptor agonists. Do neurotransmitters and neuropeptides known to elicit Ca^{2+} release from internal stores activate NSC? Activation of NSC by neurotransmitters and neuropeptides would show that the mechanism of their excitatory effects on glomus cells involves Na^+ influx via NSC. The subcellular localization of Ca^{2+} generated by Ca^{2+} release from internal stores (i.e. Ca^{2+} microdomains) can be different from that produced by Ca^{2+} entry via Ca_v . Ca^{2+} microdomains within the cell have been well described whereby the elevated level of $[Ca^{2+}]_i$ is localized near the source of Ca^{2+} generation such as Ca_v at the plasma membrane or the endoplasmic reticulum (ER) at the intracellular compartment of the cell (Berridge, 2006; Parekh, 2008). Such Ca^{2+} microdomains are thought to serve an important role in Ca^{2+} signaling, and provide specificity of Ca^{2+} effects within the cell. In this study, we further investigated the $[Ca^{2+}]_i$ -NSC relationship in glomus cells by recording NSC in response to mitochondrial oxidative phosphorylation inhibitors that activate Ca_v and to receptor agonists that are known to cause Ca^{2+} release from internal stores.

2. Methods

2.1. Cell Isolation

The Animal Care and Use Committee of Rosalind Franklin University approved the protocol used in this study. Rats (Sprague-Dawley; postnatal days 18–24) were anaesthetized by inhalation of isoflurane until cessation of breathing. Carotid bodies were removed and placed in ice-cold phosphate buffered saline (low Ca^{2+}/Mg^{2+} PBS: 137 mM NaCl, 2.8 mM KCl, 2 mM KH_2PO_4 , 0.07 mM $CaCl_2$, 0.05 mM $MgCl_2$, pH 7.4). Each carotid body was cut into several pieces and placed in a solution containing trypsin (0.4 mg/ml) and collagenase (0.4 mg/ml) in low Ca^{2+}/Mg^{2+} PBS and incubated at $37^\circ C$ for ~ 25 minutes. Following trituration, the dispersed cells were resuspended in carotid body growth medium (Ham's F-12, 10% fetal bovine serum, 23 mM glucose, 4 mM Glutamax-1 (L-alanyl glutamine), 10 kU penicillin/streptomycin and 300 $\mu g/ml$ insulin) for 2 hours at $37^\circ C$ in a humidified atmosphere of 95% air/5% CO_2 . Cells were used within 8 hours after plating.

2.2. Electrophysiology

Electrophysiological recording was performed using a patch clamp amplifier (Axopatch 200B, Molecular Devices, Sunnyvale, CA, USA). Cell-attached patches were formed with gentle suction with sylgard-coated borosilicate glass pipettes with tip resistance of ~2–3 megaohms. Channel current was filtered at 2 kHz using an eight-pole Bessel filter (–3 dB; Frequency Devices, Ottawa, IL, USA) and transferred to a computer using the Digidata 1320 interface at a sampling rate of 20 kHz. Single-channel currents were analyzed with the pCLAMP program (version 10). Channel openings were analyzed to obtain channel activity (NP_o , where N is the number of channels in the patch, and P_o is the probability of a channel being open). NP_o was determined from 20–30 sec of current recording. Data were obtained from 3–5 independent cell preparations. In experiments using cell-attached patches, pipette solution contained (mM): 140 KCl, 1 MgCl₂, 5 EGTA, 11 glucose and 10 HEPES (pH 7.3). Extracellular bath (physiological) solution contained (mM): 117 NaCl, 5 KCl, 23 NaHCO₃, 1 MgCl₂, 1 CaCl₂, 10 HEPES and 11 glucose. pH of the solution was adjusted to pH 7.3 with NaOH. Recording temperature was 34–35°C.

2.3. Hypoxia Studies

Cell-attached patches were perfused with a bath solution bubbled with 5% CO₂/21% O₂/balanced N₂ gas mixture (normoxia). After a steady-state channel current was obtained, the perfusion solution was switched to solution bubbled vigorously with 5% CO₂/95% N₂ gas mixture (labeled as 0% O₂). The temperature of the perfusion solutions was 37°C, and the rate of perfusion was 2.4 ml/min. Stainless steel tubing was used for perfusion lines to prevent gas diffusion. O₂ content of the solutions was checked using an oxygen meter (ISO2, World Precision Instruments, Sarasota, FL, USA) that was calibrated to 0% with solution gassed with pure nitrogen for 60 min and to 21% with solution gassed with air for 60 min at 37°C.

2.4. [Ca²⁺]_i measurement

Isolated cells plated on glass coverslips were incubated with 2 μM fura-2 acetoxymethyl ester (fura-2 AM) for 30 min at 37 °C in culture medium, and mounted in a recording chamber positioned on the stage of an inverted microscope (IX71; Olympus America Inc., Center Valley, PA, USA). Fura-2 was alternately excited at 340 nm and 380 nm, and the emitted fluorescence was filtered at 510 nm and recorded using a charge-coupled device (CCD)-based imaging system running SimplePCI software (Hamamatsu Corp.). The cells in the recording chamber were continuously perfused with physiological solution containing (mM): 117 NaCl, 5 KCl, 23 NaHCO₃, 1 MgCl₂, 1 CaCl₂, 10 HEPES and 11 glucose (pH 7.3). Ratiometric data were calibrated by applying experimentally determined constants to the equation: $[Ca^{2+}]_i = K_d \times \beta \times (R - R_{min}) / (R_{max} - R)$ (Grynkiewicz et al. 1985). Values for R_{max} (12.0), R_{min} (0.1) and β (11.6) were determined *in vitro*, and a K_d value of 300 nM for fura-2 was assumed. Each experiment was performed on 3–5 cell preparations. Data obtained from each cell preparation consisting of >10 cells were averaged and used to calculate the mean ± standard deviation of the averaged means.

2.5. Data analysis and Materials

Student's *t*-test (for comparison of two sets of data) and one-way analysis of variance (comparison of three or more sets of data) were used. Data were analyzed using PRISM software and represented as mean±standard deviation. Post hoc testing was based on unpaired *t*-test with Bonferroni correction. Significance level was set at $p<0.05$.

Caffeine, sodium cyanide (NaCN), carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), sodium hydrogen sulfide (NaHS), *S*-Nitroso-*N*-acetylpenicillamine (SNAP), bromo-cDP-ribose (Br-cADPR-ribose), cyclopiazonic acid (CPA) were purchased from Sigma Aldrich Co. BTP-2 (also named YM 58483) and all receptor agonists were purchased from Tocris (Ellisville, MO, USA). Fura-2 AM was purchased from TEF Labs.

3. Results

3.1. Activation of NSC by hypoxia

We showed previously that hypoxia activates a 20-pS NSC in cell-attached patches of glomus cells, and characterized the single channel properties of NSC (Kang et al., 2014). We have continued our study of NSC in glomus cells and present additional observations on the properties of NSC. In cell-attached patches with pipette solution containing 140 mM KCl, pipette potential set at 0 mV, and perfused with physiological solution containing 5 mM KCl, the 34-pS TASK was active in all cells we chose and hypoxia (0% O₂) strongly inhibited TASK activity (Fig. 1A; current tracings a and b). In this study, ~30% of patches showed mainly TASK, and this allowed us to observe a clear effect of hypoxia on TASK. Hypoxia activated NSC in ~70% of patches (52 of 75 cells), and the number of NSC activated ranged from one to six channels. Activation of one to three NSC was most commonly observed as indicated by the arrow (Fig. 1B; tracings c and d), but activation of four to six NSC was also observed in a few patches (Fig. 1C; tracings e and f). The recovery from activation upon perfusion with normoxic solution occurred within a few seconds of washout, and the hypoxic effect could be repeated several times in the same patch. For example, Fig. 1C shows the effect of hypoxia on NSC in the same patch at the pipette potential of 0 mV, 30 mV and 60 mV. With these basic observations, we further studied the role of Ca²⁺ influx via Ca_v and Ca²⁺ release from ER in NSC activation.

3.2. Ca²⁺ influx via Ca_v and NSC

Our recent study showed that cell depolarization produced by hypoxia and high [KCl]_o activated NSC by causing Ca²⁺ influx via Ca_v (Kang et al., 2014). Therefore, we tested the prediction that other depolarizing stimuli also activate NSC by a similar mechanism. We first tested the effect of a mitochondrial inhibitor (NaCN) and an uncoupler (FCCP) that also depolarize glomus cells (Fernandez-Aguera et al., 2015; Wyatt and Buckler, 2004). As predicted, NaCN elicited a concentration-dependent elevation of [Ca²⁺]_i in all KCl-sensitive glomus cells (Fig. 2A; average of 33 cells). In cell-attached patches with 140 mM KCl in the pipette, pipette potential set at 0 mV and perfused with a physiological bath solution (5 mM KCl), TASK was open at rest (Fig. 2B; tracing a). NaCN (0.1 mM) caused activation of NSC in 7 of 10 cells (tracing b), and washout of NaCN closed NSC with a full recovery of TASK activity. FCCP also produced a concentration-dependent elevation of [Ca²⁺]_i in all KCl-

sensitive glomus cells, and also activated NSC in 7 of 11 cells (Fig. 2C and 2D). NSC activation by NaCN (n=5) or FCCP (n=5) did not occur in Ca^{2+} -free perfusion solution in all cell-attached patches. The opposite effects of NaCN and FCCP on TASK and NSC calculated from 5 patches are shown in Fig. 2E.

Recent studies have suggested that H_2S is a signal that mediates the hypoxia-induced excitation of glomus cells, although this idea is still debated (Haouzi et al., 2011; Kim et al., 2015; Peng et al., 2010; Yuan et al., 2015). Because H_2S is also a potent mitochondrial inhibitor that depolarizes glomus cells and elevates $[\text{Ca}^{2+}]_i$ (Buckler, 2012; Kim et al., 2015), we tested the effect of H_2S on NSC. NaHS (50 μM), a H_2S donor, elicited a strong increase in $[\text{Ca}^{2+}]_i$ in all KCl-responding cells (Fig. 3A; average of 27 cells). In all 6 cell-attached patches, NaHS produced a reversible activation of NSC (Fig. 3B). Activation of NSC by NaHS was not observed in Ca^{2+} -free extracellular solution (n=4) or in the presence of 2 μM nifedipine (n=4). Nitric oxide (NO) is another inhibitor of mitochondrial respiratory complex. Application of SNAP (1 mM), an S-nitrosothiol which serves as a NO donor, also elicited a strong rise in $[\text{Ca}^{2+}]_i$ (Fig. 3A) and activated NSC in 5 of 7 cell-attached patches (Fig. 3C). These findings show that inhibitors of mitochondrial oxidative phosphorylation activate NSC, and show that increased Ca^{2+} entry via Ca_v is well coupled to NSC activation.

3.3. Ca^{2+} release from internal stores and NSC

Another source of Ca^{2+} is ER where IP_3 and ryanodine act on different receptors to elicit Ca^{2+} release. We used angiotensin II (AngII) to release Ca^{2+} from the IP_3 -sensitive pool, and caffeine to release Ca^{2+} from the ryanodine-sensitive pool. In ~50% of glomus cells (range, 22–82%; 6 separate cell preparations), AngII (0.2 μM) produced either a sustained $[\text{Ca}^{2+}]_i$ increase or $[\text{Ca}^{2+}]_i$ oscillations (Fig. 4A; top two figures; average of 22–28 cells). In other glomus cells, AngII failed to elicit a rise in $[\text{Ca}^{2+}]_i$, despite the positive $[\text{Ca}^{2+}]_i$ response to 20 mM KCl, suggesting that the expression of AngII receptor in some glomus cells is low or absent. AngII failed to elevate $[\text{Ca}^{2+}]_i$ in 100% of glomus cells preincubated in Ca^{2+} -free solution containing cyclopiazonic acid (10 μM , 10 min), a sarcoplasmic-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) inhibitor that depletes Ca^{2+} content of the ER. cADP-ribose is an intracellular signaling molecule that elicits Ca^{2+} release from ER in certain cell types (Lee, 2012). The rise in $[\text{Ca}^{2+}]_i$ elicited by AngII was not blocked by Br-cADP ribose (100 μM , 30 min pre-incubation), an antagonist of cADP ribose (Fig. 4A; bottom figure). In cell-attached patches, AngII (0.2 μM) activated NSC in 6 of 13 patches tested, and an example of such activation is shown in Fig. 4B. We speculate that in those cells in which AngII did not activate NSC, the $[\text{Ca}^{2+}]_i$ responses were weak or absent. Although we were unable to record changes in NSC and $[\text{Ca}^{2+}]_i$ in the same cell at the same time, the results show that AngII can activate NSC.

Caffeine produced a small transient increase in $[\text{Ca}^{2+}]_i$ in all KCl-sensitive cells from 3 separate cell preparations (Fig. 4C; n=42), confirming that a ryanodine-sensitive Ca^{2+} pool is present in glomus cells as reported earlier (Mokashi et al., 2001). In cell-attached patches, caffeine applied to the bath perfusion solution briefly activated one NSC in 4 of 13 cells, presumably due to the relatively low elevation of $[\text{Ca}^{2+}]_i$ (Fig. 4D). As with AngII, caffeine failed to elicit an increase in $[\text{Ca}^{2+}]_i$ in all 5 cells that were pre-incubated in Ca^{2+} -free

solution containing cyclopiazonic acid (10 μ M, 10 min). Thus, these results indicate that Ca^{2+} released from ER via IP_3 and ryanodine receptor pathways can activate NSC.

We also tested the ability of other neurotransmitters and neuropeptides that bind to G protein-coupled receptors on $[\text{Ca}^{2+}]_i$ and NSC. Among receptor agonists tested (Fig. 5), ACh (10 μ M, 24 of 35 cells), muscarine (10 μ M, 25 of 32 cells), pituitary adenylate cyclase-activating polypeptide (PACAP, 200 nM, 52 of 65 cells) and adenosine (100 μ M, 53 of 122 cells) produced measurable increases in $[\text{Ca}^{2+}]_i$. Among these agonists, adenosine produced the weakest response, elevating mean $[\text{Ca}^{2+}]_i$ from ~100 nM to ~170 nM. Endothelin-1 (ET-1, 10 nM) transiently elevated $[\text{Ca}^{2+}]_i$ by ~10 nM only in a small fraction of cells (3/36 cells; Fig. 5A). These effects of receptor agonists on $[\text{Ca}^{2+}]_i$ are qualitatively similar to those reported earlier (Chen et al., 2000; Dasso et al., 1997; Fung et al., 2001; Xu et al., 2006). Other receptor agonists such as serotonin (100 μ M), γ -aminobutyric acid (GABA, 100 μ M) and dopamine (100 μ M) failed to elicit a detectable rise in $[\text{Ca}^{2+}]_i$ even at the high concentrations used here.

ACh activated NSC in 7 of 10 cell-attached patches and muscarine activated NSC in 6 of 10 patches (+ sign in the bar of Fig. 5B). PACAP (200 nM) activated NSC in only 3 of 10 patches, probably because the rise in $[\text{Ca}^{2+}]_i$ was not strong enough to activate NSC in many cells. Adenosine that elicited a small increase in $[\text{Ca}^{2+}]_i$ did not activate NSC in all 10 cells tested, probably because the rise in $[\text{Ca}^{2+}]_i$ did not reach the threshold level for activation of NSC. All other receptor agonists failed to activate NSC ($n=8$ for each agonist). These results show that receptor agonists that elevate $[\text{Ca}^{2+}]_i$ to levels above ~200 nM can activate NSC in glomus cells. None of these receptor agonists significantly altered TASK activity in the cell-attached patches when the agonist was applied to the bath solution ($p>0.05$). This is consistent with the observation that TASK is unaffected by changes in $[\text{Ca}^{2+}]_i$ (Kim et al., 2009).

Although neurotransmitters and neuropeptides may not elevate the basal $[\text{Ca}^{2+}]_i$, they could potentially affect the hypoxia-induced activation of NSC if they inhibit hypoxia-induced signals such as Ca^{2+} influx via the Ca^{2+} channel. Hypoxia causes robust secretion of dopamine from the carotid body (Fishman et al., 1985; Gonzalez et al., 1994) and dopamine has been shown to inhibit the Ca^{2+} current in rabbit glomus cells (Benot and Lopez-Barneo, 1990). Therefore, we studied the effect of dopamine (100 μ M) on hypoxia-induced rise in $[\text{Ca}^{2+}]_i$ and activation of NSC. The effect of hypoxia on $[\text{Ca}^{2+}]_i$ was assessed in the presence and absence of 100 μ M dopamine. Contrary to our prediction, dopamine did not affect the hypoxia-induced rise in $[\text{Ca}^{2+}]_i$ ($n=8$ from 3 separate preparations; Fig. 5C and 5E). In cell-attached patches, hypoxia caused activation of NSC as predicted. After recovery in normoxic solution, dopamine was added to the normoxic perfusion solution for ~1 min and then the effect of hypoxia tested again in the presence of dopamine, as illustrated in Fig. 5D. Hypoxia-induced activation of NSC was not affected by dopamine in all nine cell-attached patches (Fig. 5E). Together, these results show that the autocrine action of dopamine is too weak to affect hypoxia-induced elevation of $[\text{Ca}^{2+}]_i$ and activation of NSC in rat glomus cells.

3.4. SOCE and NSC

Depletion of $[Ca^{2+}]_i$ in the ER is well known to cause opening of Ca^{2+} -permeable channels (also known as CRAC channels) in the plasma membrane by a STIM-ORAI type of interaction, which is referred as SOCE (Cahalan, 2009; Smyth et al., 2010).

Neurotransmitters and neuropeptides that cause release of Ca^{2+} from ER therefore stimulates SOCE that contributes to the net rise in $[Ca^{2+}]_i$. SOCE pathway is present in many cell types and has been identified by measuring $[Ca^{2+}]_i$ rise in response to re-addition of extracellular Ca^{2+} after a brief period of depletion of Ca^{2+} from ER in Ca^{2+} -free solution. To show the presence of SOCE in glomus cells, we perfused the cells with Ca^{2+} -free solution containing cyclopiazonic acid (10 μ M; 10 min). This produced a slow decline in basal cytosolic $[Ca^{2+}]_i$ due to the continued expulsion of cytosolic $[Ca^{2+}]_i$ without replenishment (Fig. 6A; average of 26 cells). When 1 mM $[Ca^{2+}]_i$ was added back to the perfusion solution, a rapid increase in $[Ca^{2+}]_i$ was followed by a slow decline (Fig. 6A). BTP-2 (10 μ M), a specific inhibitor of SOCE, reduced the elevation of $[Ca^{2+}]_i$ and the duration of $[Ca^{2+}]_i$ rise, indicating the presence of a functional SOCE in glomus cells (Fig. 6B; average of 12 cells). The inhibitory effect of BTP-2 on the $[Ca^{2+}]_i$ transient plotted in Fig. 6C (average from 3 cell preparations).

Can SOCE activate NSC? To answer this question, cell-attached patches were formed and perfused with Ca^{2+} -free solution containing cyclopiazonic acid for ~10 min, and then 1 mM Ca^{2+} added to the perfusion solution. TASK was active during perfusion with Ca^{2+} -free solution and was not affected by cyclopiazonic acid. Addition of 1 mM Ca^{2+} caused activation of the 20-pS channel in all six patches tested (Fig. 6D). Removal of Ca^{2+} from the perfusion solution closed NSC. These results show that the SOCE mechanism is present in glomus cells and presumably helps in the activation of NSC in response to receptor agonists such as AngII and ACh.

4. Discussion

Glomus cells express a Ca^{2+} -sensitive, non-selective cation channel (NSC) that is activated by hypoxia via increased Ca^{2+} influx via Ca_v (Kang et al., 2014). As Ca^{2+} microdomains have been identified in various cell types and shown to provide specificity and localization of Ca^{2+} signaling (Berridge, 2006; Parekh, 2008), we asked whether the activation of NSC is specific to a depolarizing stimulus such as hypoxia or whether the $[Ca^{2+}]_i$ rise elicited by other mechanisms are also able to activate NSC. Glomus cells secrete neurotransmitters and neuropeptides that bind to receptors coupled to Ca^{2+} -mobilizing processes such as SOCE and Ca^{2+} release from internal stores. These receptor-mediated processes could potentially affect the function of NSC and modulate the basal cell excitability as well as the sensitivity to hypoxia. Using stimuli other than hypoxia, we further examined the effects of voltage- and receptor-mediated changes in Ca^{2+} signaling on NSC activity.

4.1. Mitochondrial inhibitors and NSC

We showed earlier that hypoxia and high $[KCl]_o$ activate NSC by opening of Ca_v and increasing Ca^{2+} influx (Kang et al., 2014). Inhibition of mitochondrial oxidative phosphorylation is thought to mediate the hypoxia-induced depolarization and excitation of

glomus cells. Therefore, we predicted that agents that interfere with the mitochondrial function would also activate NSC, as they depolarize glomus cells and open Ca_v . Our finding that NaCN, FCCP, NaHS and SNAP all activate NSC further demonstrates that Ca^{2+} influx via Ca_v is coupled to activation of NSC.

H_2S has recently been claimed as an intracellular gaseous signal that mediates the hypoxia-induced excitation of glomus cells (Peng et al., 2010; Prabhakar, 2012). A complex signaling pathway was recently described in which the hypoxia-induced decrease in the level of carbon monoxide (CO) relieved the activity of cystathionine- γ -lyase via protein kinase G to increase $[\text{H}_2\text{S}]$ (Yuan et al., 2015). A recent study using H_2S -sensitive fluorescent dye (SF-7) showed that hypoxia (0% O_2) weakly elevated $[\text{H}_2\text{S}]$ in glomus cells, but this elevation was not sufficiently high to cause inhibition of TASK or elevation of $[\text{Ca}^{2+}]_i$ (Kim et al., 2015). Furthermore, inhibition of cystathionine- γ -lyase and cystathionine- β -synthase that synthesize H_2S had no effect on hypoxia-induced inhibition of TASK and elevation of $[\text{Ca}^{2+}]_i$ (Kim et al., 2015). As discussed in detail by Buckler (Buckler, 2012), H_2S at high concentrations elevates $[\text{Ca}^{2+}]_i$ by inhibiting the mitochondrial cytochrome oxidase, and is unlikely to be the physiological signal in hypoxia-induced excitation of glomus cells. We suspect that the activation of NSC by H_2S is therefore simply due to elevation of $[\text{Ca}^{2+}]_i$ caused by inhibition of mitochondrial oxidative phosphorylation, similar to those produced by NaCN.

4.2. Ca^{2+} release from ER and NSC

As NSC is sensitive to $[\text{Ca}^{2+}]_i$, we hypothesized that neurotransmitters and neuropeptides that cause Ca^{2+} release from ER would activate NSC if the rise in $[\text{Ca}^{2+}]_i$ were sufficiently high. In our experiments, only those agonists that elevated the basal $[\text{Ca}^{2+}]_i$ to levels above ~200 nM were effective in activating NSC. This is consistent with the earlier finding that the threshold level of $[\text{Ca}^{2+}]_i$ for activation of NSC is ~200 nM (Kang et al., 2014). AngII and ACh were most effective in elevating $[\text{Ca}^{2+}]_i$ and this was associated with their ability to activate NSC consistently. PACAP is a Gq- and Gs-coupled receptor agonist and the rise in $[\text{Ca}^{2+}]_i$ is probably mediated via the Gq-PLC pathway (Roy et al., 2013; Xu et al., 2007). The relatively weak rise in $[\text{Ca}^{2+}]_i$ by PACAP is also consistent with the low percentage of cells that showed NSC activation. Interestingly, 5-HT and ET-1 that bind to receptors coupled to Gq and are expected to cause Ca^{2+} release from ER produced either no and very weak increase in $[\text{Ca}^{2+}]_i$. This probably explains why 5-HT and ET-1 did not activate NSC. These results suggest that 5-HT and ET-1 play a minor role in glomus cell excitability under normal conditions. It has been reported that the 5-HT and ET-1 signaling is greatly augmented during chronic hypoxia and chronic intermittent hypoxia (Peng et al., 2009; Rey et al., 2006). For example, ET-1 receptor and ET-1 peptide expression levels were elevated by chronic hypoxia conditions and this augmented the chemosensitivity of glomus cells. Therefore, 5-HT and ET-1 may have a stronger effect on $[\text{Ca}^{2+}]_i$ in chronic hypoxia conditions and activate NSC to augment the chemosensitivity of glomus cells. Similar effects may occur in models of heart failure where the AngII and endothelin signaling is augmented, causing an enhanced CB chemoreflex (Schultz et al., 2015).

In mammalian expression systems, receptor agonists linked to Gq has been shown to inhibit TASK via several different intracellular signaling pathways involving diacylglycerol, PKC or Gq α subunit (Chen et al., 2006; Kang et al., 2006; Wilke et al., 2014). We therefore suspected that agonists such as AngII, muscarine and endothelin-1 that bind receptors coupled to Gq would inhibit TASK in glomus cells via one of these mechanisms. However, we found no significant effect of these agonists on TASK activity. Therefore, any effect of these agonists on glomus cell excitability is likely to be due to ER Ca²⁺ release and subsequent intracellular signals, without involving TASK. An earlier study reported that adenosine, an excitatory agonist, inhibited TASK via protein kinase A and depolarized glomus cells, causing a mild increase in [Ca²⁺]_i (Xu et al., 2006). Adenosine was also found to inhibit a 4-AP-sensitive current without affecting the resting Em (~ -55mV) (Vandier et al., 1999). In our experiments, adenosine induced a rise in [Ca²⁺]_i but the level of rise was not high enough to activate NSC, and also did not affect TASK activity under the conditions of our experiments.

We have studied the response of glomus cells to an agonist concentration of 100 μ M, except for AngII, ET-1 and PACAP where lower concentrations were used. Many earlier studies have used 100 μ M or less to elicit a near-maximal response for an agonist (Nurse, 2014). We also assume that an agonist concentration of 100 μ M is very high because many physiological responses to a receptor agonist occur at submicromolar concentrations. In glomus cells, ACh and muscarine elicited maximal [Ca²⁺]_i responses at 100 μ M (Dasso et al., 1997). Dopamine at 1 μ M was found to cause a 40% decrease in Ca²⁺ current in rabbit carotid body glomus cells (Benot and Lopez-Barneo, 1990). Adenosine or its analogue (N-ethylcarboxamido-adenosine) was used at 100 μ M to elicit strong increases in catecholamine secretion and elevation of [Ca²⁺]_i (Conde et al., 2008; Xu et al., 2006). For 5-HT, 10 μ M very mildly enhanced hypoxia-induced increase in [Ca²⁺]_i (Yokoyama et al., 2015). A much lower concentration of 5-HT (100–300 nM) elicited increased CB sensory activity (Peng et al., 2006). For ET-1, 200 pM produced a maximal effect on CB sensory discharge (Rey et al., 2006) and 100 nM only doubled the hypoxia-induced elevation of [Ca²⁺]_i (Chen et al., 2000). Although the *in vivo* levels of agonists near the receptors are not known, these findings indicate that 100 μ M of an agonist is a relatively high concentration that elicits near-maximal responses.

Although an agonist may not elicit a rise in [Ca²⁺]_i, it may affect the hypoxia-induced excitation of glomus cells by altering the hypoxia signaling pathway. For example, dopamine has been reported to inhibit Ca²⁺ current in rabbit glomus cells (Benot and Lopez-Barneo, 1990). Inhibitory effects of dopamine on cat and rabbit carotid body chemosensory responses have also been observed as reviewed recently (Iturriaga and Alcayaga, 2004; Nurse and Piskuric, 2013). In rat glomus cells, dopamine (100 μ M) inhibited Na₂S₂O₄-induced increase in [Ca²⁺]_i in a small fraction of cells (Jiang and Eyzaguirre, 2004), and tyramine, used as a dopamine agonist, showed little or no effect on hypoxia-induced changes in [Ca²⁺]_i (Yoshizaki et al., 2000). These studies in rat glomus cells do not directly test the effect of dopamine itself on hypoxia-induced changes in [Ca²⁺]_i. In the rat, recording of the single fiber afferent nerve activity was independent of catecholamine secretion, indicating that these transmitters do not significantly affect the chemosensory response in this species (Donnelly, 1996). Our findings that dopamine does not inhibit hypoxia-induced increase in

$[Ca^{2+}]_i$ and hypoxia-induced activation of NSC are in keeping with the lack of effect of dopamine on the chemosensory response in the rat. It is possible that dopamine does not inhibit the Ca^{2+} current sufficiently in rat glomus cells to modulate the hypoxic response. The studies reported so far suggest that the dopamine effect on the chemosensory response is species-dependent. For other neurotransmitters and neuropeptides, it would be necessary to study in detail the effect of each agonist on $[Ca^{2+}]_i$ and NSC using a range of hypoxia to be certain of their effects on the O_2 sensitivity of rat glomus cells.

4.3. SOCE and NSC

SOCE is a mechanism for refilling the ER with Ca^{2+} following IP_3 -induced depletion of Ca^{2+} in the ER. SOCE is observed in many types of cells and involves an interaction between STIM expressed in the ER and a Ca^{2+} -permeable channel (such as Orai and TRPC) in the plasma membrane (Cahalan, 2009). Because agonists (such as muscarine and AngII) that bind receptors coupled to Gq- IP_3 pathway elicit Ca^{2+} release from ER and deplete ER Ca^{2+} , we hypothesized that glomus cells also possess functional SOCE to help refill ER Ca^{2+} store. To identify SOCE, we used a well-established protocol, i.e., depleting ER Ca^{2+} with SERCA inhibitor in Ca^{2+} -free bath solution and recording changes in $[Ca^{2+}]_i$ upon addition of Ca^{2+} back to the bath solution. An elevation of $[Ca^{2+}]_i$ observed with this protocol and its reduction by BTP-2, a specific blocker of SOCE, indicated the presence of the SOCE mechanism in glomus cells. We have not determined the molecular identity of the channel that mediates SOCE in glomus cells, but the channel is most likely an Orai/TRPC type, similar to those found in many other cell types.

4.4. Unknown molecular identity of NSC

A robust activation of NSC by hypoxia and some receptor agonists shows that this Na^+ -permeable channel is an important signaling component of excitation of glomus cells evoked by voltage- and receptor-mediated pathways. Although NSC is not very active at rest, its activation as $[Ca^{2+}]_i$ begins to rise in response to a depolarizing stimulus is expected to contribute to the depolarization and rise in $[Ca^{2+}]_i$. To determine the role of NSC in glomus cell excitability, we need to identify the gene that encodes NSC to delete or silence it. A recent single glomus cell transcriptome analysis indicated that TRPC3, TRPC5 and TRPM7 were most highly expressed TRP ion channels (Zhou et al., 2016). However, the single channel and pharmacological properties of these TRP channels do not match those of NSC, as most TRP ion channel have high single channel conductance (>40 -pS) and the modulation by pharmacological agents such as 2-APB are clearly different (Kang et al., 2014). Thus, the molecular identity of NSC is still unknown.

4.5. Limitations of the study

(1) Our finding is based on the use of isolated glomus cells in short-term culture. The use of isolated glomus cells is always a potential limitation, because the sensitivity of glomus cells *in vivo* to various stimuli may be slightly different from that *in vitro*. (2) Unlike $[Ca^{2+}]_i$ recording where many cells can be studied at one time, single channel recording is studied in a limited number of cells (10–20 cells from several cell preparations). Therefore, the data from $[Ca^{2+}]_i$ recording is more robust than those from single channel recordings. (3) We do not have a specific inhibitor for NSC and we are unable to manipulate its expression because

we do not know its molecular identity. The proposed role of NSC in glomus cell excitability is based on our single channel recording.

4.6. Conclusions

We have studied the role of different sources of Ca^{2+} in their ability to activate NSC. We confirmed, using various inhibitors of mitochondrial oxidative phosphorylation, that opening of Ca_v leads to activation of NSC. We showed that neurotransmitters and neuropeptides that elevate $[\text{Ca}^{2+}]_i$ in glomus cells via Ca^{2+} release from ER can activate NSC. We have also demonstrated the presence of a SOCE mechanism in glomus cells, which probably assists in the activation of NSC in response to a receptor agonist. Therefore, NSC is likely to contribute to the increased excitability of glomus cells produced not only by hypoxia via Ca_v , but also by neurotransmitters and neuropeptides via ER Ca^{2+} release and SOCE.

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Highlights

- Opening of Ca_v by inhibitors of mitochondrial oxidative phosphorylation activates NSC.
- Receptor agonists that elevate $[\text{Ca}^{2+}]_i$ via Ca^{2+} release from ER activates NSC.
- SOCE is involved in the activation of NSC by receptor agonists.
- Activation of NSC contributes to glomus cell excitation by various stimuli.

Figure 1. Inhibition of TASK and activation of NSC by hypoxia

A. Cell-attached patch shows TASK openings in normoxia. Hypoxia reversibly inhibits TASK. Expanded current tracings (a, b) shows TASK openings during normoxia and hypoxia.

B. Hypoxia inhibits TASK and activates two NSC channels (current tracings c and d). Arrow indicates open NSC.

C. Hypoxia reversibly and reproducibly activates 5 NSC channels (current tracings e and f). Effect of hypoxia is shown at three different pipette potentials. Arrow indicates NSC opening.

Figure 2. Effects of NaCN and FCCP on $[Ca^{2+}]_i$, TASK and NSC

A. Changes in $[Ca^{2+}]_i$ in response to 20 mM KCl and a range of [NaCN] are shown.

B. Cell-attached patch shows the effect of NaCN on single channel currents. Expanded current tracings show channel openings before (tracing a) and during (tracing b) perfusion with NaCN. Note the activation of NSC in tracing b.

C. Changes in $[Ca^{2+}]_i$ are recorded in response to 20 mM KCl and FCCP.

D. FCCP activates NSC in a cell-attached patch.

E. Summary of the effects of NaCN and FCCP on TASK and NSC. Each bar is the mean $\pm$ SD of 5 patches. *Significantly different from the control value ($p < 0.05$).

Figure 3. Effects of NaHS and SNAP on $[Ca^{2+}]_i$ and NSC

A. Changes in $[Ca^{2+}]_i$ in response to 20 mM KCl, 50 μ M NaHS and 1 mM SNAP are shown.

B. Activation of NSC in a cell-attached patch by NaHS is shown. Expanded current tracings show channel openings before (tracing a) and during (tracing b) perfusion with NaHS. Note the activation of NSC in tracing b.

C. Cell-attached patch shows activation of NSC by 1 mM SNAP.

Figure 4. Effects of AngII and caffeine on $[Ca^{2+}]_i$ and NSC

A. Changes in $[Ca^{2+}]_i$ in response to 20 mM KCl, 0.2 μ M AngII and 0.1 mM NaCN are shown (top and middle figures). In one set of experiments (bottom figure), cells were pre-incubated with 100 μ M Br-cADP-ribose for 30 min at 37°C and then experiment performed in the continuous presence of Br-cADP-ribose. Averaged responses from 22–28 cells are shown.

B. AngII activates NSC in cell-attached patch. Expanded current tracing shows the opening of 20-pS channel.

C. Changes in $[Ca^{2+}]_i$ in response to 20 mM KCl and 20 mM caffeine are shown.

D. Caffeine (20 mM) activates NSC in cell-attached patches (4/13 cells).

Figure 5. Effects of neurotransmitters and neuropeptides on $[Ca^{2+}]_i$ and NSC

A. Changes in $[Ca^{2+}]_i$ in response to 20 mM KCl, PACAP (0.2 μ M), dopamine (DA, 100 μ M), 5-HT (100 μ M), GABA (100 μ M), ACh (100 μ M), adenosine (ADO, 100 μ M) and ET-1 (10 nM) are shown.

B. Graphs shows the summary effect of receptor agonists on $[Ca^{2+}]_i$ elevation. (+) indicates activation of NSC in cell-attached patches. The percentage of cells whose NSC is activated by agonists is stated in the text.

C. Changes in $[Ca^{2+}]_i$ in response to hypoxia in the presence and absence of DA (100 μ M) is shown. Eight experiments from three separate cell preparations were done.

D. Current tracing shows activation of NSC by hypoxia and the effect of dopamine in a cell-attached patch.

E. Summary of results on the effect of dopamine on $[Ca^{2+}]_i$ and NSC activity are shown. Each bar is the mean $\pm$ SD from 8–9 experiments from 3 separate cell preparations. No significant differences were present ($p>0.05$).

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Figure 6. SOCE activates NSC

A. Changes in $[Ca^{2+}]_i$ in response to 20 mM KCl, and 10 μ M CPA (with and without 1 mM Ca^{2+}) are shown.

B. Changes in $[Ca^{2+}]_i$ in response to 10 μ M CPA (with and without 1 mM Ca^{2+}) in the presence of 10 μ M BTP-2 are shown.

C. A plot of $[Ca^{2+}]_i$ vs. time with and without BTP-2. Each point is the mean $\pm$ SD from 3 separate cell preparations. All data points are significantly different from the corresponding control value ($p < 0.05$). Time zero represents $[Ca^{2+}]_i$ at the peak.

D. In cell-attached patches perfused with Ca^{2+} -free solution containing CPA for ~ 10 min, adding 1 mM Ca^{2+} to the patch activated NSC.