Spermine attenuates carotid body glomus cell oxygen sensing by inhibiting L-type Ca\(^{2+}\) channels

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**Abstract**

An increase in intracellular Ca\(^{2+}\) is crucial to O\(_2\) sensing by the carotid body. Polymamines have been reported to modulate both the extracellular Ca\(^{2+}\)-sensing receptor (CaR) and voltage-gated Ca\(^{2+}\) channels in a number of cell types. Using RT-PCR and immunohistochemistry, the predominant voltage-gated Ca\(^{2+}\) channels expressed in the adult rat carotid body were L (Ca\(_V\).1.2) and N (Ca\(_V\).2.2)-type. CaR mRNA could not be amplified from carotid bodies, but the protein was expressed in the nerve endings. Spermine inhibited the hypoxia-evoked catecholamine release from isolated carotid bodies and attenuated the depolarization- and hypoxia-evoked Ca\(^{2+}\) influx into isolated glomus cells. In agreement with data from spermine body, recombinant Ca\(_V\).1.2 was also inhibited by spermine. In contrast, the positive allosteric modulator of CaR, R-568, was without effect on hypoxia-induced catecholamine release from carotid bodies and depolarization-evoked Ca\(^{2+}\) influx into glomus cells. These data show that spermine exerts a negative influence on carotid body O\(_2\) sensing by inhibiting L-type Ca\(^{2+}\) channels.

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**1. Introduction**

The naturally occurring polyamines, putrescine, spermidine and spermine, are small organic molecules which are normally present in plasma at micromolar concentrations (Chaisiri et al., 1979). Whilst necessary for optimal cell growth at these physiological concentrations (Tabor and Tabor, 1984), polyamines increase both as a consequence of normal hormonal variations (Gilad et al., 2002) and during pathologies, such as malignant transformation (Casero and Marton, 2007), brain injury (Dogan et al., 1999a,b), or neuronal ischemia (Li et al., 2007). Moreover, synaptic vesicles contain spermine at concentrations as high as 2 mM (Masuko et al., 2003), suggesting that their co-release with classical neurotransmitters will result in very large concentrations being present in the synaptic cleft during neuronal activity. Polyamines are positively charged at physiological pH and this polycationic nature endows them with greater affinities than other mono- and di-valent cations for acidic components of proteins. This makes them ideal candidates for regulators of ion channels and receptors involved in cellular Ca\(^{2+}\) homeostasis (Li et al., 2007). Indeed, there is already good evidence that polyamines inhibit voltage-gated Ca\(^{2+}\) channels (Gomez and Hellstrand, 1995; Lasater and Solessio, 2002) and activate the G protein coupled, extracellular Ca\(^{2+}\)-sensing receptor, CaR (Quinn et al., 1997).

The principal mammalian arterial chemoreceptor is the carotid body (Gonzalez et al., 1994). During periods of reduced O\(_2\) availability, the type I (or glomus) cells of the carotid body release neurotransmitters onto the terminals of the carotid sinus nerve, evoking increased afferent traffic to the brain stem from the carotid sinus nerve which results in a reflex hyperventilatory response (Gonzalez et al., 1992). Whilst a wide variety of molecular mechanisms have been proposed to underlie the response of glomus cells to hypoxia (Kemp and Peers, 2007, 2009), they all converge onto an increase in intracellular Ca\(^{2+}\) concentration via activation of voltage-dependent Ca\(^{2+}\) channels (Buckler and Vaughan-Jones, 1994), a prerequisite for neurotransmitter release (Conde et al., 2007; Lopez-Barneo et al., 2001). Since polyamines have been shown to be stored at high concentration in neurotransmitter vesicles (Masuko et al., 2003), and Ca\(^{2+}\) influx (rather than release of Ca\(^{2+}\) from intracellular stores (Gonzalez et al., 1993; Vicario et al., 2000)) is crucial to the O\(_2\) sensing pathway of the carotid body, we postulated that extracellular polyamines (in particular, spermine) may play a role in the modulation of the response of carotid body glomus to hypoxia. Therefore, the first aim of this study was to determine the expression profiles of known targets for spermine action, namely voltage-gated Ca\(^{2+}\) channels (Gomez and Hellstrand, 1995; Lasater and Solessio, 2002) and CaR (Chattopadhyay et al., 1997; Wang et al., 2003), in the adult rat carotid body. Secondly, in order to examine the possibility that spermine might modulate carotid body function, the ability of spermine to regulate hypoxia- and depolarization-evoked catecholamine release from isolated carotid bodies was tested. Finally, the effects of spermine...
on recombinant L-type Ca\textsuperscript{2+} channel activity and hypoxia-induced Ca\textsuperscript{2+} influx into isolated glomus cells were investigated in order to determine the molecular mechanisms responsible for extracellular spermine regulation of carotid body function. Some of these data were presented in preliminary form at the International Society for Arterial Chemoreceptors, Valladolid, Spain in July 2008 (Cayzac et al., 2009).

2. Materials and methods

2.1. Experiments employing adult rat carotid body

Male Wistar rats (100–350 g) were deeply anaesthetized with sodium pentobarbitone (60 mg kg\textsuperscript{-1}; I.P.), according to U.K. Home Office regulations, and were then subjected to the following experimental procedures: (i) reverse transcription followed by PCR (RT-PCR) of mRNA isolated from whole carotid bodies; (ii) immunohistochemistry on thin carotid body sections; (iii) neurotransmitter release from intact carotid bodies; (iv) Ca\textsuperscript{2+} imaging in isolated glomus cells.

2.1.1. RT-PCR of mRNA isolated from whole carotid bodies

The carotid bifurcations were resected, the carotid bodies were identified, cleaned of surrounding connective tissue under a dissecting microscope, and total RNA was extracted from six carotid bodies using RNeasy Micro kit (Qiagen, Crawley, Surry, U.K.) according to the manufacturer’s instructions. As positive controls for calcium channel amplification, total RNA was extracted from six carotid bodies using RNeasy Micro kit (Qiagen, Crawley, Surry, U.K.) according to the manufacturer’s instructions. 1 µg of total RNA was reverse-transcribed at 50°C for 30 min using Superscript III (Invitrogen, Paisley, Strathclyde, U.K.) according to the manufacturer’s instructions. 2 µl of cDNA were used for each PCR reaction using the Premix Ex-taq polymerase kit (Lonza, Basel, Switzerland) with intron-spanning primers (4 µM) designed to amplify Ca\textsubscript{V}1.2–1.3–1.4 (L-type), Ca\textsubscript{V}2.1 (P/Q-type), Ca\textsubscript{V}2.2 (N-type), Ca\textsubscript{V}2.3 (R-type), Ca\textsubscript{V}3.1–3.2–3.3 (T-type) and CaR; see Table 1. PCR was carried out for 35 cycles of 1 min denaturation at 95°C, 1 min at the appropriate annealing temperature (see Table 1) and 1 min extension at 72°C. All PCR products which were amplified from carotid body were fully sequenced to demonstrate the presence of bona fide transcripts for the genes of interest.

2.1.2. Immunohistochemistry on thin carotid body sections

After opening of the thoracic cavity, the tissue was cleared of blood and fixed in situ by transcardial perfusion through the left ventricle, using a peristaltic pump, sequentially with 50 ml of heparinized (10 units ml\textsuperscript{-1}) phosphate-buffered saline (PBS) and at a rate of 8 ml min\textsuperscript{-1} and 100 ml of ice-cold fixative solution consisting of 4% (w/v) paraformaldehyde (Sigma–Aldrich, Poole, Dorset, U.K.) in PBS at a rate of 20 ml min\textsuperscript{-1}. The carotid bifurcations were then removed and placed in a 30% (w/v in PBS) sucrose solution containing (in mM): 140 NaCl, 5 KCl, 2 CaCl\textsubscript{2}, 1.1 MgCl\textsubscript{2}, 5 glucose, 10 HEPES, pH 7.4. Unincorporated [\textsuperscript{3}H]tyrosine was removed by rinsing the carotid bodies in the same solution for 1 h. Carotid bodies were individually incubated at 37°C in bicarbonate-buffered solution containing (in mM): 116 NaCl, 24 NaHCO\textsubscript{3}, 5 KCl, 2 CaCl\textsubscript{2}, 1.1 MgCl\textsubscript{2}, 5 glucose. 10 HEPES, pH 7.4, equilibrated with 20% O\textsubscript{2}, 5% CO\textsubscript{2}, 75% N\textsubscript{2}. The solutions used were bubbled either with 20% O\textsubscript{2}, 5% CO\textsubscript{2} and 75% N\textsubscript{2} (normoxia); or 7% O\textsubscript{2}, 5% CO\textsubscript{2} and 88% N\textsubscript{2} (hypoxia). Where a “high potassium” solution was employed, NaCl and KCl concentrations were 86 and 35 mM, respectively. Spermine (500 µM) and R-568 (100 nM), the latter prepared as a 10 mM stock solution in dimethyl sulfoxide, were diluted in the bicarbonate-buffered solution. Every 10 min, the bathing solution was collected and replaced by fresh solution. A solution containing ascorbic and acetic acid was added to each collected sample before [\textsuperscript{3}H]-catecholamine was quantified by liquid scintillation counting. Quantitation of the effect of spermine on the evoked release was achieved by calculating the ratio of the second hypoxic response (HOX2) to the first hypoxic response (HOX1) for each individual carotid body preparation (N), and these data were plotted as the mean ±S.E.M.) of HOX2/HOX1 values (see, for example, Conde et al., 2007).

2.1.3. Neurotransmitter release from intact carotid bodies

This technique has been extensively described elsewhere (e.g. Conde et al., 2007; Rocher et al., 2009). Briefly, the catecholamine store of intact carotid bodies was radiolabelled by a 2 h incubation with 30 µM [\textsuperscript{3}H]tyrosine (48 Ci mmol\textsuperscript{-1}, 100 µM of 6-methyl-tetrahydropteridine and 1 mM of ascorbic acid dissolved in a solution containing (in mM): 140 NaCl, 5 KCl, 2 CaCl\textsubscript{2}, 1.1 MgCl\textsubscript{2}, 5 glucose, 10 HEPES, pH 7.4. Unincorporated [\textsuperscript{3}H]tyrosine was removed by rinsing the carotid bodies in the same solution for 1 h. Carotid bodies were individually incubated at 37°C in bicarbonate-buffered solution containing (in mM): 116 NaCl, 24 NaHCO\textsubscript{3}, 5 KCl, 2 CaCl\textsubscript{2}, 1.1 MgCl\textsubscript{2}, 5 glucose. 10 HEPES, pH 7.4, equilibrated with 20% O\textsubscript{2}, 5% CO\textsubscript{2} and 75% N\textsubscript{2} (normoxia); or 7% O\textsubscript{2}, 5% CO\textsubscript{2} and 88% N\textsubscript{2} (hypoxia). Where a “high potassium” solution was employed, NaCl and KCl concentrations were 86 and 35 mM, respectively. Spermine (500 µM) and R-568 (100 nM), the latter prepared as a 10 mM stock solution in dimethyl sulfoxide, were diluted in the bicarbonate-buffered solution. Every 10 min, the bathing solution was collected and replaced by fresh solution. A solution containing ascorbic and acetic acid was added to each collected sample before [\textsuperscript{3}H]-catecholamines were absorbed with alumina. The alumina was then washed extensively and eluted with 1 N HCl before the [\textsuperscript{3}H]-catecholamine was quantified by liquid scintillation counting. Quantitation of the effect of spermine on the evoked release was achieved by calculating the ratio of the second hypoxic response (HOX2) to the first hypoxic response (HOX1) for each individual carotid body preparation (N), and these data were plotted as the mean ±S.E.M.) of HOX2/HOX1 values (see, for example, Conde et al., 2007).

2.1.4. Ca\textsuperscript{2+} imaging in isolated glomus cells

The carotid artery bifurcations were excised, carotid bodies dissected and placed in ice-cold solution containing (in mM): 143 NaCl, 2 KCl, 2 CaCl\textsubscript{2}, 1.1 MgCl\textsubscript{2}, 5.5 glucose and 10 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), pH 7.4, bubbled with 100% O\textsubscript{2}. Isolated carotid bodies were incubated for 15 min at 37°C in a solution (as above, but without Mg\textsuperscript{2+} and Ca\textsuperscript{2+}) containing type IV collagenase (2.5 mg ml\textsuperscript{-1})—Sigma–Aldrich, Poole, Dorset, U.K.) and bovine serum albumin (BSA; 6 mg ml\textsuperscript{-1})—Sigma–Aldrich, Poole, Dorset, U.K.). Following initial incubation, the carotid bodies were transferred to a Mg\textsuperscript{2+}- and Ca\textsuperscript{2+}-free solution containing trypsin (1 mg ml\textsuperscript{-1})—Sigma–Aldrich, Poole, Dorset, U.K.) and BSA (6 mg ml\textsuperscript{-1})—Sigma–Aldrich, Poole, Dorset, U.K.) and were incubated for a further 20 min at 37°C. The carotid bodies were then transferred to F-10/Dulbecco’s modified Eagle’s medium (F-10/DMEM) supplemented with 10% fetal calf serum, 1% (v/v) antibiotic (penicillin & streptomycin)/antimycotic, 2 mM L-glutamine (all from Gibco-BRL, Paisley, Strathclyde, U.K.), gently triturated and centrifuged at 150 × g for 4 min. The cell pellet was resuspended in 50 µl of supplemented F-10/DMEM, the cells seeded onto poly-L-lysine treated (0.1 mg ml\textsuperscript{-1}) glass coverslips and allowed to adhere for 50 min before the addition of 2 ml of
Table 1. Primers employed for the RT-PCR reactions.

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<th>Gene bank</th>
<th>mRNA</th>
<th>Primer sequences 5′–3′</th>
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<th>Tm/°C</th>
<th>Amplicon size, bp</th>
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<td>61.6</td>
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2.2. Experiments employing recombinant ion channels expressed in HEK 293 cells

2.2.1. HEK 293 cell culture

Wild type HEK 293 cells and HEK 293 cells that stably express either the human BKCa α subunit (KCNA1; accession number, NM_002247, see Telezhkin et al., 2010) or the human Cav1.2 (Accession number, AF465484, see Scrugg et al., 2008), were grown in Earle’s minimal essential medium containing 10% fetal calf serum, 100 μM L-ascorbic acid, 100 μg ml⁻¹ streptomycin, 1 μg ml⁻¹ glutamine, 1 mg ml⁻¹ G418 (all from Invitrogen, Paisley, Strathclyde, U.K.) in a humidified incubator gassed with 5% CO₂/95% air. HEK 293 cells were passaged every 5–7 days in a ratio 1:9 using Ca²⁺ and Mg²⁺-free PBS.

2.2.2. Whole-cell recordings of Cav1.2–HEK 293 cells

HEK 293 cells stably expressing L-type Cav1.2 Ca²⁺ channels were obtained from Prof. C. Peers (University of Leeds, West Yorkshire, U.K.); wild type HEK 293 cells do not express functional, voltage-activated L-type Ca²⁺ channels (Fearon et al., 1997). Cells attached to glass coverslips were placed in a continuously perfused solution recording chamber (volume ca. 200 μl) mounted on the stage of an inverted microscope equipped with phase-contrast optics. Bath solution contained (in mM): 135 NaCl, 5 KCl, 1.2 CaCl₂, 1.2 MgCl₂, 5 HEPES and 10 glucose, pH 7.4. Pipette solution contained (in mM): 120 CsCl₂, 20 TEA-Cl, 2 MgCl₂, 10 EGTA, 10 HEPES, 2 Na-ATP, pH 7.2 with CsOH. After the whole-cell configuration had been achieved, the bath solution was changed to a solution containing (in mM): 113 NaCl, 5 CsCl, 0.6 MgCl₂, 1.2 CaCl₂, 2 BaCl₂, 5 HEPES, 10 glucose and 20 TEA-Cl, pH 7.4. Cs⁺ and TEA⁺ were employed to block K⁺ channels and Ba²⁺ was employed as the charge carrier since it is more permeant than Ca²⁺ and its currents do not inactivate rapidly. Spermine, R-568 and nifedipine were diluted in this Ba²⁺-containing solution and applied extracellularly where indicated in text and figure legends. Cells were voltage-clamped at −70 mV and then ramped at 0.1 Hz from −100 mV to +100 mV for 200 ms before being stepped from −80 mV to +15 mV for 50 ms.

2.2.3. Whole-cell recordings of BKα HEK 293 cells

The bath solution contained (in mM): 135 NaCl, 5 KCl, 1.2 CaCl₂, 1.2 MgCl₂, 5 HEPES and 10 glucose, pH 7.4. The pipette solution was composed of (in mM): 10 NaCl, 117 KCl, 2 MgCl₂, 11 HEPES, pH 7.2; for the bath solution, free [Ca²⁺] was adjusted to 300 nM using ethylene–glycol–tetra–acetic acid (EGTA), as calculated by Maxchelator (http://maxchelator.stanford.edu). Cells were voltage-clamped at −70 mV and currents were evoked in the whole cell configuration by a 1 s voltage-ramp from 120 mV to +60 mV repeated at 0.05 Hz.

All electrophysiology experiments (K⁺ and Ca²⁺ currents) were conducted at ambient room temperature, and currents were leak-subtracted off-line. Current recordings were made using an Axopatch 200A amplifier and Digidata 1320 A/D interface (Axon Instruments, Forster City, CA, U.S.A.). All currents were digitized at 10 kHz and Bessel low-pass filtered at 5 kHz. Unless stated otherwise, all statistical comparisons were made using a Student’s paired t-test.

3. Results

Using RT-PCR, mRNAs encoding all of the voltage-activated Ca²⁺ channel α-subunits could be amplified from either adult rat brain or adult rat eye, as expected (Fig. 1). However, only Cav1.2 (α₁C L-type Ca²⁺ channel) and Cav2.2 (N-type Ca²⁺ channel) could be
Fig. 1. Expression profile of voltage-activated Ca\(^{2+}\) channels and CaR mRNAs in rat carotid body. RT-PCR of the voltage-activated Ca\(^{2+}\) channels reveals that only Cav1.2 (A) and Cav2.2 (E) mRNAs are expressed in the adult rat carotid body. Cav1.2 and Cav2.2 mRNAs were amplified from the carotid body and the brain (positive control) giving amplicons of the expected sizes (433 bp and 504 bp, respectively), which were confirmed by sequencing as rat Cav1.2 or Cav2.2 cDNA. All the other channels, namely Cav1.3 (B), Cav1.4 (C), Cav2.1 (D), Cav2.3 (F), Cav3.1 (G), Cav3.2 (H) and Cav3.3 (I), could not be detected in the carotid body whereas in the positive controls, brain or eye, amplicons of the expected size were amplified. In the case of Cav2.3, a splice-variant was detected in the brain and a non-specific product, identified by sequencing as the adenosine phosphorylase transferase-like mRNA, was amplified in the brain and carotid body (F). The CaR mRNA was not detected in the carotid body sample using two different sets of primers (J). The positive control, consisting of plasmid containing the human CaR, gave the correct size amplicons. The ladder shown on all gels provided markers at 100 bp intervals.

amplified from carotid body RNA (Fig. 1A and E, respectively). No other L-type (Fig. 1B and C), nor P/Q (Fig. 1D), R-type (Fig. 1F) and T-type (Fig. 1G–I) Ca\(^{2+}\) channels could be amplified from carotid body cDNA. Using two separate primer pairs, CaR signal could also not be detected in carotid body (Fig. 1J).

Consistent with the mRNA studies, immunohistochemistry demonstrated that both Cav1.2 (green—Fig. 2A) and Cav2.2 (green—Fig. 2D) were detected in type 1, tyrosine hydroxylase-positive (red—Fig. 2B and E) cells. In addition, Cav1.2 was also expressed in the nerve fibres (arrows—Fig. 2C). Although CaR could not be amplified by RT-PCR in the carotid body, immunohistochemistry revealed that the receptor protein was expressed in the nerve terminals (green—Fig. 2G and I). This pattern of CaR expression has also been observed elsewhere in the adult (Ruat et al., 1995) and developing (Vizard et al., 2008) brain. The lack of CaR PCR products from carotid body mRNA is consistent with the idea that receptor transcripts are predominantly expressed in the soma of the petrosal ganglion neurons (which are not present in mRNA isolated from the carotid body itself), whilst the receptor protein is also expressed in the nerve endings.
Measurement of neurotransmitter release from isolated carotid bodies in vitro showed that application of 500 µM spermine did not affect baseline catecholamine secretion (Fig. 3A), but dramatically inhibited the hypoxia-evoked release (HOX2 versus HOX1). Indeed, spermine significantly decreased the HOX2/HOX1 ratio by 55% ($p < 0.01$, $n = 6$—Fig. 3A and B). Moreover, spermine inhibited the depolarization-evoked (using 35 mM KCl) catecholamine release by 60% ($p < 0.01$, $n = 6$—not shown), indicating that the O2 sensing mechanism was not affected per se. In contrast to the inhibitory effect of spermine, 100 nM R-568 (a concentration of this CaR-positive allosteric modulator which is known to promote CaR activation (Finney et al., 2008)), was unable to effect significantly the hypoxia-evoked catecholamine secretion from the carotid body (Fig. 4A and B, $n = 6$).

As catecholamine secretion by the carotid body is mediated by, and strictly dependent upon, a rise in [Ca$^{2+}$] (Gonzalez et al., 1994), the effect of spermine on [Ca$^{2+}$] homeostasis was investigated directly in isolated, tyrosine hydroxylase-positive glomus cells (see Fig. 5F). Since spermine is a known activator of CaR (Chattopadhyay et al., 1997), the effect of selective activation of CaR was also tested. Spermine had no effect on baseline 340/380 values (Fig. 5A). However, it was a potent inhibitor of depolarization-evoked Ca$^{2+}$ influx (Fig. 5A and C); at 200 µM, spermine inhibited the Ca$^{2+}$ influx induced by high external K+ by 34% ($p < 0.01$, $N = 5$—Fig. 5A and C). The inhibition was concentration-dependent with a mean IC$_{50}$ value of 473 ± 70 µM and a mean Hill coefficient of 0.84 (Fig. 5E, $n = 17$, $N = 3$); these data were fitted with the maximal inhibition unfixed since spermine is not soluble at concentrations exceed-
Fig. 3. Inhibitory effect of spermine on hypoxia-evoked catecholamine secretion from isolated carotid bodies. Mean (±S.E.M.) time-courses of tritiated catecholamine (3H-CA) secretion induced by bubbling with 7% O₂ in the absence (squares) or presence (circles) of 500 μM spermine (A). The mean (±S.E.M) catecholamine secretion induced by 7% O₂ from 6 separate carotid body preparations in the presence of 500 μM spermine was significantly (**p < 0.01) different from that evoked by hypoxia in its absence (B).

ing those used in this study. The CaR positive allosteric modulator, R-568, did not induce an increase in [Ca²⁺]i alone (Fig. 5B), nor did it significantly affect Ca²⁺ influx induced by high K⁺ (p > 0.5, n = 4 —Fig. 5B and D). Together with the molecular observations described in Figs. 1 and 2, these experiments strongly suggest that the targets of the inhibitory action of spermine observed in Fig. 3 are voltage-dependent Ca²⁺ channels.

Since 60–80% of the hypoxia-evoked Ca²⁺ influx into glomus cells is mediated by L-type Ca²⁺ channels (Silva and Lewis, 1995), the effect of spermine on the only L-type channel which could be detected in glomus cells, namely Cav1.2 (Figs. 1 and 2), was determined directly using recombinant Cav1.2 heterologously expressed in HEK 293 cells. These recombinant Ca²⁺ currents did not “run-down” during the 5 min protocol (Fig. 6C). However, 300 μM of spermine inhibited strongly and significantly the Cav1.2 currents, by 53 ± 5% (Fig. 6A and C; n = 5, p < 0.01), but 100 nM R568 was without affect (Fig. 6B and C; n = 6). In contrast to the inhibitory action of spermine on Ca²⁺ currents, this polyamine was unable to modulate recombinant BKCa currents, at any test voltage (Fig. 6D, n = 6, p > 0.5).

With the knowledge that spermine inhibits recombinant Cav1.2 in physiological [Ca²⁺]i, the final series of experiments was designed to test whether spermine suppresses the response of the carotid body to low PO₂ by reducing hypoxia-evoked Ca²⁺ influx into glomus cells. Fig. 7A shows simultaneous recordings of fura-2 fluorescence ratio, temperature and PO₂. In the absence of an hypoxic stimulus, high K⁺ induced a robust Ca²⁺ influx. Reductions in bath PO₂ from 150 mmHg to ca. 15 mmHg evoked a rapid and reproducible Ca²⁺ influx. Importantly, bath co-application of 200 μM spermine with hypoxia resulted in a significant reduction in the hypoxia-evoked Ca²⁺ influx, which was fully reversible upon removal of spermine from the bath (Fig. 7A). Indeed, spermine significantly inhibited the hypoxia-evoked Ca²⁺ influx by 41 ± 12%, (ANOVA with Tukey post hoc test, p < 0.05, n ≥ 23, N ≥ 3, Fig. 7B) and the magnitude of this reduction was similar to that caused by spermine when Ca²⁺ influx was evoked by depolarization (see Fig. 5). To gain further insight into the effect of spermine on the chemoreception in type 1 cells, Fura-2 ratio was plotted against PO₂ and fitted to a single exponential (y = y₀ + Ae⁻Rx, Fig. 7C); PO₂ was measured on-line with the carbon fibre electrode, and Fig. 7C was constructed from data obtained during the transition from normoxia to full hypoxia. In absence and presence of 200 μM spermine, the data fits generated the following parameters: y = 0.72 ± 0.56 e⁻⁰.⁸₈x (Hypoxia) and y = 0.72 ± 0.18 e⁻⁰.⁷⁷x (Hypoxia plus spermine).

As predicted from earlier data, spermine did not affect the baseline [Ca²⁺]i (y₀ is constant, p > 0.05) or the sensitivity of the type 1 cells (R is constant, p > 0.05) but modified the amplitude of the response (A was significantly decreased upon application of spermine, p < 0.05, n = 16, N = 3). Taken together, these data show that spermine exerts an inhibitory influence on the carotid body’s response to hypoxia,
Fig. 5. Inhibitory effect of spermine and absence of effect of R-568 on Ca\textsuperscript{2+} influx induced by high K\. Application of 15 mM K\textsuperscript{+} invariably caused a robust rise in \([\text{Ca}^{2+}]_{\text{i}}\) (assayed as the change in fura-2 340/380 fluorescence ratio). Treatment with 200 \(\mu\text{M}\) spermine inhibited reversibly the Ca\textsuperscript{2+} influx (\(n = 22, N = 3, p < 0.01, \text{A and C}\)). The IC\textsubscript{50} was calculated at 473.52 ± 70.27 \(\mu\text{M}\) with a Hill coefficient of 0.84 ± 0.10 (\(n \geq 17, N \geq 3\) for each point, E). In contrast, application of the positive allosteric modulator of CaR, 100 nM R-568, did not affect the Ca\textsuperscript{2+} influx (\(n = 19, N = 4, \text{B and D}\)). At the end of each experiment, the identity of each glomus cell was confirmed by immunocytochemistry using the tyrosine hydroxylase antibody (F); the upper panel shows a typical bright field image and the lower panel shows the tyrosine hydroxylase staining (using an FITC-conjugated secondary antibody). Three clusters of type 1 cells can be seen (TH-positive) in addition to spindle shape type 2 cell (TH-negative, *). The scale bar is 10 \(\mu\text{m}\).

and that this effect is due, in the most part, to an inhibition of Ca\textsubscript{1.2} which is expressed in the glomus cells.

4. Discussion

Spermine is produced ubiquitously from \(\text{L-arginine}\) by the sequential actions of arginase, ornithine decarboxylase, spermidine synthase and spermine synthase (see (Persson, 2009)). Steady-state spermine concentration, which is a function of its rate of intracellular production and extracellular influx (Belting et al., 2003) has been shown to be 400 \(\mu\text{M}\) in smooth muscle cells (Sward et al., 1994). Within neurons, where it is co-packaged with neurotransmitters into vesicles, the synaptic vesicular spermine concentration may be as high as 2 mM (Masuko et al., 2003). The rate-limiting step in the polyamine synthesis cascade is the conversion of ornithine to putrescine in mitochondria by ornithine decarboxylase. Tight regulation of the expression of this enzyme, which has an extremely short half-life, is absolutely pivotal in controlling the intracellular concentrations of polyamines, including spermine (Persson, 2009). There are a number of physiological and pathological states which increase cellular spermine content.
via stimulation of ornithine decarboxylase expression, including malignant transformation (Casero and Marton, 2007; Chaisiri et al., 1979), brain injury (Dogan et al., 1999a; Dogan et al., 1999b), or neuronal ischemia (Li et al., 2007). Importantly, there is accumulating evidence that chronic hypoxia increases polyamine concentrations, by a combination of increased production and/or uptake, in several physiological systems including the lung (Babal et al., 2002; Ruchko et al., 2003), heart (Tantini et al., 2006) and brain (Longo et al., 1993). Conversely, it has been recently shown, by gene chip array, that chronic hypoxia causes a decrease in ornithine decarboxylase mRNA in the carotid body (Ganfornina et al., 2005), suggesting that spermine levels in this tissue may be reduced in this pathological situation.

The data presented herein show that the predominant voltage-gated Ca\(^{2+}\) channels which are expressed, at both the mRNA (Fig. 1) and the protein levels (Fig. 2), in the rat carotid body are of the L-type (Ca\(_{\alpha 1.2}\) or Ca\(_{\alpha 1.C}\)) and N-type (Ca\(_{\alpha 2.2}\)). These observations are broadly in line with other functional and molecular data obtained in this species (Caceres et al., 2009; Silva and Lewis, 1995), although T-type (Ca\(_{\alpha 3.1}\)) channel mRNA expression has been reported (Caceres et al., 2009). Interestingly, even though carotid body glomus cells are of neuroendocrine origin, they do not express the Ca\(_{\alpha 1.3}\) (or Ca\(_{\alpha 1.D}\)) channel type most usually observed in neurons. Spermine has been reported to inhibit voltage-dependent Ca\(^{2+}\) channels in several cell types, including intestinal smooth muscle (Gomez and Hellstrand, 1995), hippocampus (Eterovic et al., 1997) and retina. Here we demonstrate that human recombinant Ca\(_{\alpha 1.2}\) and Ca\(_{\alpha 1.2}\) natively expressed in rat carotid body glomus cells are inhibited by micromolar concentrations of spermine (Figs. 5 and 6). Furthermore, this inhibition leads to a spermine-dependent dampening of the carotid body’s response to hypoxia (Figs. 3 and 7). This reduction in the response to hypoxia occurs without a spermine-dependent decrease in hypoxic sensitivity per se, rather it is the result of an attenuated maximal Ca\(^{2+}\) influx, via blockade of L-type Ca\(^{2+}\) channels (Fig. 7).

The calcium-sensing receptor (CaR), a G protein-coupled receptor which is expressed widely in excitable and non-excitable cells (Riccardi et al., 2009), is exclusively expressed in the nerve endings arborising within the carotid body glomeruli (Fig. 2) and which originate from the petrosal ganglion. Since this structure is not present in either of the preparations (whole carotid bodies and isolated glomus cells), it is not surprising that neither the mRNA encoding the CaR (Fig. 1) nor any functional consequences, by monitoring catecholamine secretion (Fig. 4) and [Ca\(^{2+}\)]\(_i\) (Fig. 5), of its expression could be detected. This rules out the possibility that spermine might have been regulating the activity of the carotid body via activation of CaR on glomus cells. Furthermore, the idea that spermine exerts its effects on glomus cells and the whole carotid body via regulation of the L-type Ca\(^{2+}\) current is reinforced by the data in Fig. 6 which show that R-568 does not block Ca\(_{\alpha 1.2}\).

There are several physiological considerations that arise from our present findings. Spermine is stored at millimolar concentrations in neuronal synaptic vesicles (Masuko et al., 2003) and is co-secreted with neurotransmitters (Fage et al., 1992). Moreover, astrocytes have also been shown to contain high concentrations of polyamines and have been suggested as a major source of extracellular spermine in the brain (Laube and Veh, 1997). Although no data are currently available in the carotid body, it seems reasonable to assume that type 1 cells may well co-secrete spermine with neurotransmitters during stimulation. Such a notion is supported...
Fig. 7. Inhibitory effect of spermine on hypoxia-evoked Ca\(^{2+}\) influx. Exemplar, simultaneous, recordings from an isolated glomus cell exposed to either 15 mM K\(^+\) or to hypoxia in the absence and presence of 200 \(\mu\)M spermine (A), showing ratio bath PO\(_2\) (upper panel), fura-2 fluorescence (centre panel) and bath temperature (lower panel). The periods of spermine and high K\(^+\) applications are indicated by the shaded boxes and the text above each. Mean, hypoxia-evoked Ca\(^{2+}\) influx was 62 ± 15% of that triggered by 15 mM K\(^+\), and this was further reduced reversibly by spermine to 41 ± 12% (B, \(p < 0.05, n = 20, N = 4\)). Panel C shows the PO\(_2\) versus 340/380 ratio of an exemplar glomus cell during acute hypoxia, in the presence (red squares) and absence (black squares) of 200 \(\mu\)M spermine. The mean data fitted well to a single exponential: \(y = 0.72 + 0.56 e^{-0.08x}\) (Hypoxia) and \(y = 0.72 + 0.18 e^{-0.07x}\) (Hypoxia plus spermine). Spermine did not affect the baseline [Ca\(^{2+}\)] (\(y_b\) is constant, \(p > 0.05\)) or the sensitivity of the type 1 cells (\(R\) is constant, \(p > 0.05\)) but modified the amplitude of the response (A was significantly decreased upon application of spermine, \(p < 0.05, n = 16, N = 3\)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

5. Conclusions

These data show that an endogenously produced polyamine, spermine, exerts a negative influence on carotid body O\(_2\) sensing by inhibiting the natively expressed Ca\(^{2+}\) channels. In sum, they suggest that spermine should be considered as a new neuro-modulator present in chemoreceptor cells which might contribute to carotid body activity during both acute hypoxic stimulation as well as during chronic hypoxic acclimatisation, acting presynaptically at L-type channels on the chemoreceptor cells themselves.

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