Effects of the Polyamine Spermine on Arterial Chemoreception

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Abstract Polyamines modulate many biological functions. Here we report a novel inhibitory modulation by spermine of catecholamine release by the rat carotid body and have identified the molecular mechanism underpinning it. We used molecular (RT-PCR and confocal microscopy) and functional (i.e., neurotransmitter release, patch clamp recording and calcium imaging) approaches to test the involvement of: (i) voltage-dependent calcium channels, and; (ii) the extracellular calcium-sensing receptor, CaR, a G protein-coupled receptor which is also activated by polyamines. RT-PCR and immunohistochemistry of isolated carotid bodies revealed that only $Ca_v1.2$ and $Ca_v2.2$ were expressed in type 1 cells while $Ca_v1.3$, $Ca_v1.4$, $Ca_v2.1$, $Ca_v2.3$ and $Ca_v3.1$, $Ca_v3.2$ and $Ca_v3.3$, could not be detected. CaR expression was detected exclusively in the nerve endings. In isolated carotid bodies, the hypoxia-dependent (7% O_2 for 10 minutes) and depolarization-evoked catecholamine release were partially suppressed by pre- (and co)-incubation with 500 μM spermine. In dissociated type 1 glomus cells intracellular calcium concentration did not change following spermine treatment, but this polyamine did inhibit the depolarisation-evoked calcium influx. Whole-cell patch clamp recordings of HEK293 cells stably transfected with $Ca_v1.2$ demonstrated that spermine inhibits this calcium channel. Interestingly, this inhibition was not apparent if the extracellular solution contained a concentration of Ba^{2+} above 2 mM as the charge carrier. In conclusion, spermine attenuates catecholamine release by the carotid body principally via inhibition of $Ca_v1.2$. This mechanism may represent a negative feedback, which limits transmitter release during hypoxia.

Keywords Carotid body · Polyamine · Calcium sensing receptor · Spermine · Calcium channels · Catecholamine

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1 Introduction

Naturally occurring polyamines (putrescine, spermidine and spermine) are small organic molecules, which have been reported to modulate many cellular processes. Polyamines are necessary to maintain optimal cell growth (Tabor and Tabor, 1984) and are present in the plasma at micromolar concentrations (Chaisiri et al., 1979). However, tissue levels of polyamines are increased as a consequence of normal hormonal variations (Gilad et al., 2002), and also as during pathological states, such as malignant transformation (Casero and Marton, 2007), brain injury (Dogan et al., 1999) or neuronal ischemia (Li et al., 2007). Furthermore, synaptic vesicles contain spermine at concentrations as high as 2 mM (Masuko et al., 2003). Polyamines are charged at physiological pH (Heby, 1986) and, due to their polycationic nature, exhibit greater affinities than other mono- and di-valent cations for acidic components of proteins and have, therefore, been suggested to target ion channels and receptors involved in the trafficking of calcium ions (Li et al., 2007). Specifically, they can inhibit voltage-gated calcium channels (Gomez and Hellstrand, 1995; Lasater and Solessio, 2002) and activate the cell surface calcium-sensing receptor, CaR (Quinn et al., 1997).

The carotid body responds to a decrease in oxygen availability with a release of neurotransmitters by type 1 glomus cells. While a variety of molecular players have been postulated to form the basis of the oxygen sensing machinery (see, for instance, Kemp et al. (2006) for a recent review), all of these mechanisms converge onto an increase in intracellular calcium concentration, necessary for neurotransmitter release. This is achieved through activation of voltage-gated calcium channels (Conde et al., 2006a). Polyamines may be stored at high concentration in neurotransmitter vesicles, suggesting that they play some physiological role in the modulation of the carotid body response to hypoxia. The purpose of this study was to investigate the presence of known targets for spermine action, namely voltage-gated calcium channels (Gomez and Hellstrand, 1995; Lasater and Solessio, 2002) and CaR, in the carotid body, and to study the effects of spermine on catecholamine release by isolated carotid bodies. Polyamine effects on intracellular calcium homeostasis in dissociated type 1 cells and in recombinant systems overexpressing calcium channels were also investigated.

2 Methods

2.1 Surgery

Wistar rats (100–350 g) were anesthetized with sodium pentobarbital in accordance with U.K. Home Office regulations. The carotid artery bifurcations were excised, carotid bodies dissected and placed in ice-cold solution containing (in mM): 143 NaCl, 2 KCl, 2 $CaCl_2$, 1.1 $MgCl_2$, 5.5 glucose and 10 HEPES, pH 7.4, bubbled with 100% O_2.

2.2 RT-PCR and Immunohistochemistry

Total RNA was extracted from carotid bodies using RNeasy Micro kit (Qiagen, Crawley, U.K.) according to the manufacturer's instructions. As positive controls for calcium channel amplification, total RNA was extracted from eye and brain using Trizol (Invitrogen, Paisley, 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, Strathclyde, U.K.). 2 μl of cDNA were used for each PCR reaction using the Premix Ex-taq polymerase kit (Lonza, Basel, Switzerland) with published sequences of intron-spanning primers designed to amplify: $Ca_v1.2$-1.3-1.4 (L-type), $Ca_v2.1$ (P/Q-type), $Ca_v2.2$ (N-type), $Ca_v2.3$ (R-type), $Ca_v3.1$-3.2-3.3 (T-type), CaR, β-actin and tyrosine hydroxylase (TH). All PCR products amplified from carotid body were fully sequenced to demonstrate the presence of *bona fide* transcripts for the genes of interest.

For immunohistochemistry, rats were perfusion-fixed with 4% paraformaldehyde, the tissue dissected, embedded in OCT (Tissue Tek, Sakura Finetek, Torrance, USA) and 4 μm-thick cryosections were cut onto Superfrost slides. Primary antibodies were incubated over night at 4°C: $Ca_v1.2$ (1/200), $Ca_v1.3$ (1/200), $Ca_v2.1$ (1/200), $Ca_v2.2$ (1/100), $Ca_v2.3$ (1/200), TH (1/1000) and CaR (1/200). The Alexa 594- and FITC-conjugated secondary antibodies (1/1000) were applied for 1 h at room temperature.

2.3 Measurement of Neurotransmitter Release

This technique has been extensively described elsewhere (e.g. (Conde et al., 2006b)). Briefly, the catecholamine store of intact carotid bodies was radiolabelled by a 2 h incubation with 30 μM [^{3}H]tyrosine (48 Ci/mmol), 100 μM of 6-methyltetrahydropterine and 1 mM of ascorbic acid dissolved in a solution containing (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1.1 $MgCl_2$, 5 glucose, 10 HEPES, pH 7.4. Unincorporated [^{3}H]tyrosine was removed by rinsing the carotid bodies in the same solution for 1 h. Carotid bodies were individually incubated at 37 ± 1°C in bicarbonate-buffered solution containing (in mM): 116 NaCl, 24 $NaHCO_3$, 5 KCl, 2 $CaCl_2$, 1.1 $MgCl_2$, 5 glucose, 10 HEPES, pH 7.4, equilibrated with 20% O_2, 5% CO_2, 75% N_2. The solutions used were bubbled either with 20% O_2, 5% CO_2 and 75% N_2 (normoxia); or 7% O_2, 5% CO_2 and 88% N_2 (hypoxia). Where a "high potassium" solution was employed, NaCl and KCl concentrations were 86 and 35 mM, respectively. Spermine and R-568 (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 the [^{3}H]-catecholamines were absorbed with alumina. The alumina was then washed extensively and eluted with 1N HCl before the [^{3}H]-catecholamine was quantified by liquid scintillation counting.

2.4 Ca^{2+} Imaging in Type 1 Cells

Carotid bodies were dissociated with trypsin (1 mg/ml) and collagenase (2.5 mg/ml) and cultured for 24 h. carotid body cells were loaded with 4 μM fura-2 AM for 40 min in a HEPES-buffered solution containing (in mM): 125 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, 20 HEPES, 6 glucose (pH 7.4) supplemented with 0.1% (w/v) bovine serum albumin and then washed for 15 min in experimental HEPES-buffered physiological solution to be used for the experiments, which contained (in mM): 125 NaCl, 5 KCl, 0.5 $CaCl_2$, 0.5 $MgCl_2$, 5 HEPES and 10 glucose, pH 7.4. For the "high potassium solution", NaCl was lowered to 115 mM and KCl increased to 15 mM. All the recordings were performed at 26 ± 1°C. Individual cell types were identified by *post-hoc* immunostaining using a tyrosine hydroxylase antibody.

2.5 Whole-Cell Recordings of $Ca_v1.2$-HEK293 Cells

HEK293 stably expressing the human $Ca_v1.2$ ($Ca_v1.2$-HEK293) were a gift from Prof. C Peers (University of Leeds, U.K). Bath solution contained (in mM): 135 NaCl, 5 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES and 10 glucose, pH 7.4. Pipette solution contained (mM): 120 $CsCl_2$, 20 TEA-Cl, 2 $MgCl_2$, 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 $MgCl_2$, 2 $BaCl_2$, 5 HEPES, 10 glucose and 20 TEA-Cl, pH 7.4. Spermine, R-568 and nifedipine were diluted in the Ba^{2+} solution. Cells were voltage-clamped at −70 mV and then ramped at 0.1 Hz from −100 mV to +100 mV during 200 ms before being stepped from −80 mM to + 15 mV during 50 ms. Experiments were conducted at 21°C and all currents were leak-subtracted off-line.

3 Results and Discussion

RT-PCR showed that only $Ca_v1.2$ and $Ca_v2.2$ could be amplified from carotid body RNA (not shown). Consistent with these results, immunohistochemistry shows that both $Ca_v1.2$ (green – Fig. 1A) and $Ca_v2.2$ (green – Fig. 1D) were detected in type 1, tyrosine hydoxylase-positive cells (red - Fig. 1B and E). In addition, $Ca_v1.2$ was also expressed in the nerve fibres (arrows – Fig. 1C). These immunohistochemistry data were fully in agreement with the results obtained from RT-PCR amplifications of specific voltage-gated calcium channel mRNAs (not shown). In contrast, CaR could not be amplified by RT-PCR in the carotid body (data not shown) while immunostaining revealed the receptor to be present exclusively in the nerve terminals (green – Fig. 1G and I). This pattern of expression of CaR has also been observed in the adult (Ruat et al., 1995) and developing (Vizard et al., 2008) brain. The lack of CaR PCR products from carotid body RNA suggests that receptor

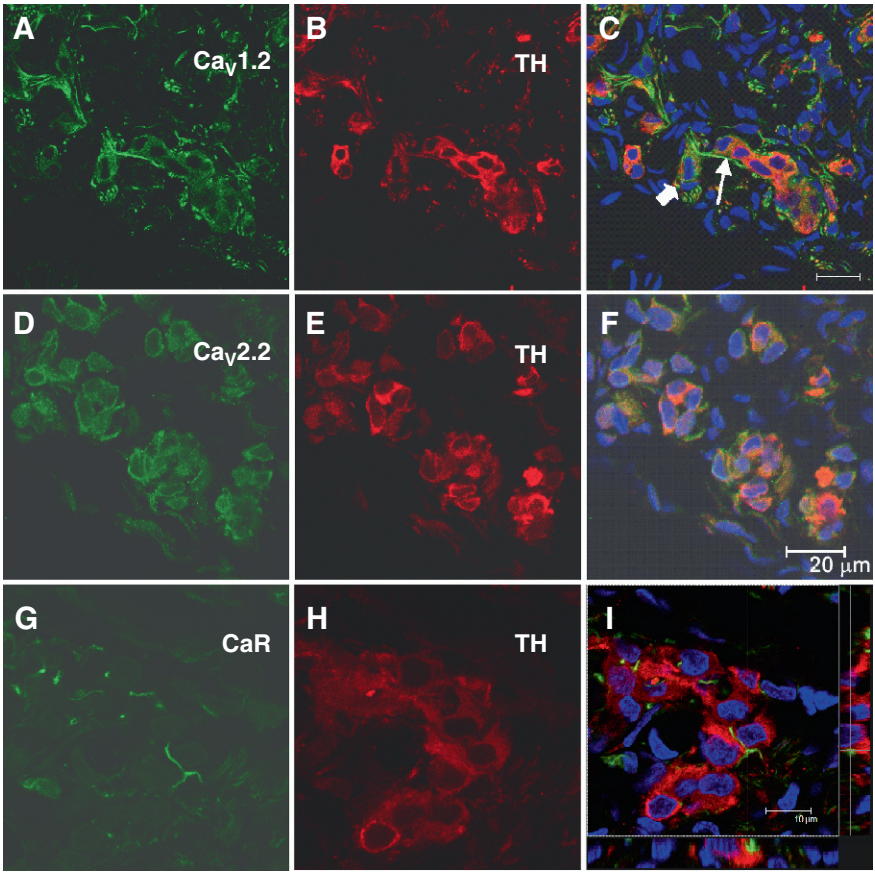

Fig. 1 Expression of voltage-gated calcium channels and CaR in the carotid body. Confocal images of $Ca_v1.2$ (**A**), $Ca_v2.2$ (**D**) and CaR (**G**) immunoreactivities in cryosections from rat carotid body. Type 1 cells are identified by tyrosine hydroxylase (TH, *red*) staining (**B**, **E** and **H**) and the overlay of the two images with the nuclear staining (DAPI, *blue*) are shown in panels C, F and I. $Ca_v1.2$-positive staining is found at the cell surface of type 1 cells (*block arrow*) and in the nerve terminals (*arrow*). $Ca_v2.2$ is found in type 1 cells while CaR immunoreactivity is selectively present in nerve terminals. Note that in panel I, the right and lower margins are z-stacks through the section along the white dotted lines, as indicated in the main panel. Scale bar = 20 μm (**C** and **F**) or 10 μm (**I**)

transcripts are predominantly expressed in the soma of neurons, presumably of the petrosal ganglion.

Measurement of neurotransmitter release from isolated carotid bodies in vitro showed that application of 500 μM spermine did not affect baseline catecholamine secretion but significantly inhibited the hypoxia-evoked release by 30% ($p < 0.05$, $n = 6$ – Fig. 2A) and the depolarization-evoked release by 60% ($p < 0.01$, $n = 6$ – Fig. 2B). That spermine inhibited the secretion evoked by depolarisation indicated that the O_2 sensing mechanism was not affected per se. As catecholamine secretion

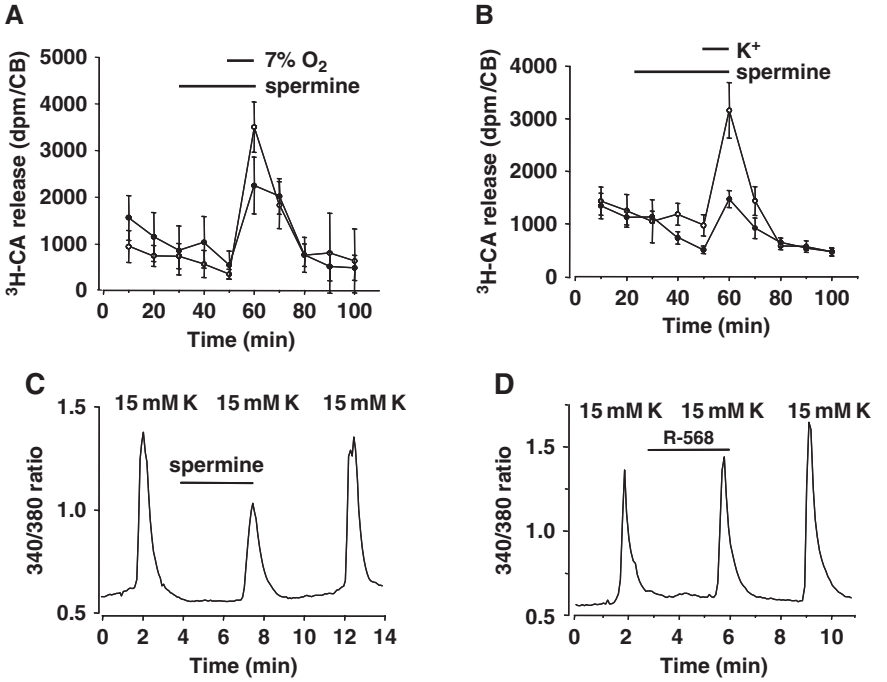

Fig. 2 Spermine inhibition of catecholamine secretion from isolated carotid bodies and $[Ca^{2+}]_i$ rises in type 1 cells. (**A**) Mean (±S.E.M.) tritiated catecholamine (^{3}H-CA) secretion induced by bubbling the tubes with 7% oxygen (hypoxia) in untreated (○) and spermine-treated (500 μM - ●); n = 6 carotid bodies. (**B**) Mean (±S.E.M.) tritiated catecholamine (^{3}H-CA) secretion induced by raising the extracellular potassium chloride concentration isoosmotically to 35 mM K^+ in untreated (○) and spermine-treated (500 μM - ●); n = 6 carotid bodies. (**C**) Exemplar recording of fura 2 fluorescence ratio from a cluster of carotid body type 1 cells. Voltage-dependent Ca^{2+} influx was stimulated by isoosmotic addition of 15 mM potassium chloride in the presence or absence of 200 μM spermine, as indicated (representative of 22 cell clusters from 5 isolations). (**D**) Typical recording of fura 2 fluorescence ratio from a cluster of carotid body type 1 cells. Voltage-dependent Ca^{2+} influx was stimulated by isoosmotic addition of 15 mM potassium chloride in the presence or absence of 100 nM of the CaR positive allosteric modulator, R-586, as indicated (representative of 19 cell clusters from 4 isolations)

is mediated by, and strictly depend upon, a rise in $Ca^{2+}{}_i$ (Gonzalez et al., 1994), the effect of spermine on $[Ca^{2+}]_i$ homeostasis was investigated in type 1 cells. 200 μM spermine inhibited the Ca^{2+} influx induced by high K^+ by 34% ($p < 0.01$, $N = 5$ – Fig. 2C). The CaR positive allosteric modulator, R-568, did not significantly affect Ca^{2+} influx induced by high K^+ ($p > 0.5$, $n = 4$ – Fig. 2D). Consistent with the molecular biology observations, these experiments strongly suggest that the target of the inhibitory action of spermine is the voltage-dependent Ca^{2+} channels.

Since 60 to 80% of the hypoxia-evoked Ca^{2+} influx is mediated by L-type Ca^{2+} channels (e Silva and Lewis, 1995), we investigated the effect of spermine on the only L-type channel which we could detect in type 1 cells, namely $Ca_v1.2$. 200 μM

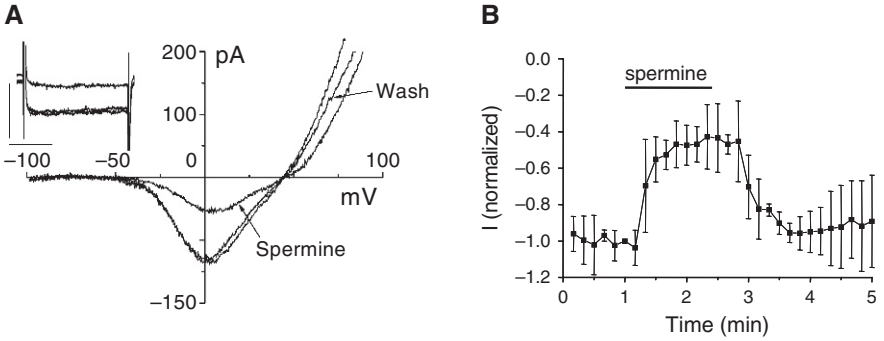

Fig. 3 Inhibitory effect of spermine on whole cell $Ca_v1.2$ currents. (**A**) Typical example of the effect of 200 μM spermine on $Ca_v1.2$ currents. The effect of spermine was reversible. The insert shows traces evocated by a + 15 mV step. Scale bars are 100 pA and 40 ms. (**B**) Mean (± S.E.M.) time-course of spermine inhibition of $Ca_v1.2$ currents evoked by repetitive steps to + 15 mV, n = 3

spermine inhibited whole cell currents from HEK293 cells stably expressing $Ca_v1.2$, by 53 ± 5% (n = 3; Fig. 3).

In conclusion, our data show that spermine exerts an inhibitory influence on carotid body chemosensitivity. This effect is due, at least in part, to an inhibition of the $Ca_v1.2$. The effect of spermine on $Ca_v2.2$, which is also expressed in carotid body glomus cells, is yet to be determined. Spermine is stored at millimolar concentrations in neuronal synaptic vesicles (Masuko et al., 2003) and is co-secreted with neurotransmitters (Fage et al., 1992). Although no data are currently available in the carotid body, it seems reasonable to assume that type 1 cells will also co-secrete spermine. If this is the case, spermine co-secretion during hypoxia may act to limit the effect of hypoxic depolarization by inhibiting L-type calcium channels. Such an inhibitory mechanism may work in parallel to that proposed for the effect of GABA where, through $GABA_B$ autoreceptors, background potassium currents are re-activated (Fearon et al., 2003). Such auto-feedback mechanisms may act to limit neurotransmitter release by type 1 cells during hypoxia.

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