Cysteine Residues in the C-terminal Tail of the Human $BK_{Ca}\alpha$ Subunit Are Important for Channel Sensitivity to Carbon Monoxide

S.P. Brazier, V. Telezhkin, R. Mears, C.T. Müller, D. Riccardi and P.J. Kemp

Abstract In the presence of oxygen (O_2), carbon monoxide (CO) is synthesised from heme by endogenous hemeoxygenases, and is a powerful activator of BK_{Ca} channels. This transduction pathway has been proposed to contribute to cellular O_2 sensing in rat carotid body. In the present study we have explored the role that four cysteine residues (C820, C911, C995 and C1028), located in the vicinity of the "calcium bowl" of C-terminal of human $BK_{Ca}\alpha$-subunit, have on channel CO sensitivity. Mutant $BK_{Ca}\alpha$-subunits were generated by site-directed mutagenesis (single, double and triple cysteine residue substitutions with glycine residues) and were transiently transfected into HEK 293 cells before subsequent analysis in inside-out membrane patches. Potassium cyanide (KCN) completely abolished activation of wild type BK_{Ca} channels by the CO donor, tricarbonyldichlororuthenium (II) dimer, at 100 μM. In the absence of KCN the CO donor increased wild-type channel activity in a concentration-dependent manner, with an EC_{50} of ca. 50 μM. Single cysteine point mutations of residues C820, C995 and C1028 affected neither channel characteristics nor CO EC_{50} values. In contrast, the CO sensitivity of the C911G mutation was significantly decreased (EC_{50} ca. 100 μM). Furthermore, all double and triple mutants which contained the C911G substitution exhibited reduced CO sensitivity, whilst those which did not contain this mutation displayed essentially unaltered CO EC_{50} values. These data highlight that a single cysteine residue is crucial to the activation of BK_{Ca} by CO. We suggest that CO may bind to this channel subunit in a manner similar to the transition metal-dependent co-ordination which is characteristic of several enzymes, such as CO dehydrogenase.

Keywords BK · Maxi K · Carbon monoxide · CO · Cysteine · Potassium channel · Mutagenesis · KCN

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C. Gonzalez et al. (eds.), *Arterial Chemoreceptors*, Advances in Experimental Medicine and Biology 648, DOI 10.1007/978-90-481-2259-2_5,

1 Introduction

Large conductance, calcium-dependent potassium channels (BK_{Ca}), expressed either natively in carotid body glomus cells (Riesco-Fagundo et al., 2001) and arterial smooth muscle (Wang et al., 1997), or heterologously in HEK 293 cells (Williams et al., 2004), are robustly activated by carbon monoxide (CO). Generation of cellular CO is largely due to the enzymatic catalysis of heme by endogenous hemeoxygenases (Tenhunen et al., 1968; Maines et al., 1986; McCoubrey et al., 1997). This process has a strict requirement for the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen (O_2), suggesting that this transduction pathway may contribute to cellular O_2 sensing in the carotid body. Indeed this hypothesis is strengthened by the observation that hemeoxygenase-2 is a protein partner closely associated with α-subunit of BK_{Ca} ($BK_{Ca}\alpha$) and that channel activation evoked by the co-application of NADPH, O_2 and heme is rapidly reversed upon removal of O_2 (Williams et al., 2004). Although the role that BK_{Ca} channels play in sensing acute changes in cellular O_2 levels has been extensively studied (see (Kemp and Peers, 2007) for recent review), the mechanism by which CO can modulate $BK_{Ca}\alpha$ activity is less clear. To date, two main theories have been proposed: (i) that CO activates $BK_{Ca}\alpha$ directly by binding to histidine residues within the channel protein (Wang and Wu, 1997; Hou et al., 2008) and; (ii) that CO activates the $BK_{Ca}\alpha$ indirectly via heme (Jaggar et al., 2005). However, our recent studies, and data presented herein, suggest that neither of these mechanism holds completely true and that cysteine residues within the C-terminal tail of $BK_{Ca}\alpha$ are central to the CO binding/channel modulation (Williams et al., 2008).

2 Materials and Methods

2.1 Cell Culture

HEK293 cells were maintained in Earle's minimal essential medium (containing L-glutamine) supplemented with 10% fetal calf serum, 1% antibiotic/antimycotic and 1% non-essential amino acids (Gibco BRL, Strathclyde, U.K.) in a humidified incubator gassed with 5% CO_2/95% air. Cells were transfected with $BK_{Ca}\alpha$ constructs using the Amaxa Nucleofector kit V, following the manufacturer's protocols (Amaxa Biosystems, Germany) and incubated for 24 hours before electrophysiological experiments were performed.

2.2 Site-Directed Mutagenesis

The Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to mutate four cysteine residues within the tail region of $BK_{Ca}\alpha$ (C820, C911, C995 and C1028). Double and triple mutant constructs were also generated by

performing sequential reactions with the same primer sets. The following sample reactions were set up in a final volume of 50 μl: 50ng of template DNA, 125 ng of each primer, 1 μl of dNTP mix, 5 μl of 10x reaction buffer and 2.5 Units of *Pfu-Turbo* DNA polymerase. Tubes were transferred to a thermal cycler and subjected to the following cycling parameters: 1 cycle at 95°C for 30 seconds followed by 18 cycles of 95°C 30 seconds, 55°C 1 minute and 68°C 7 minutes. Reactions were chilled on ice for 2 minutes before the addition of 10 units of *Dpn* I restriction enzyme, followed by incubation for 1 hour at 37°C. *Dpn* I treated DNA was used to transform XL-1 blue competent cells, single colonies were selected and plasmid DNA was isolated for sequencing to verify that individual cysteine residues were correctly modified. The chimeric human $BK_{Ca}\alpha$/mSlo3 channel was generated as previously reported (Williams et al., 2008).

2.3 Electrophysiological Recordings

BK_{Ca} channels were recorded from inside-out patches pulled from transiently transfected HEK293 cells. Pipette and bath solutions contained (in mM): 10 NaCl, 117 KCl, 2 $MgCl_2$, 11 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7.2) with $[Ca^{2+}]$ adjusted to quasi-physiological 300 nM using ethylene-glycol-tetra-acetic acid (EGTA). The CO donor molecule, tricarbonyldichlororuthenium (II) dimer ($[Ru(CO_3)Cl_2]_2$), was used to generate free CO in solution (Mann and Motterlini, 2007).

Current recordings were made at room temperature using an Axopatch 200A amplifier and Digidata 1320 A/D interface (Axon Instruments, Forster City, CA, USA). $BK_{Ca}\alpha$ single channel currents were studied using two protocols: (1) continuous gap-free recording at various holding potentials, and; (2) a 1s voltage ramp from −30 mV to +120 mV, repeated at 0.1 Hz. All voltages are reported with respect to the inner membrane leaflet. All recordings were filtered with a 8-pole Bessel filter at 5 kHz and digitized at 10 kHz.

3 Results and Discussion

Previous experiments have suggested that CO interacts with the $BK_{Ca}\alpha$ subunit either via interacting with histidine residues within the RCK1 domain (Hou et al., 2008) or via heme bound to $BK_{Ca}\alpha$ (Jaggar et al., 2005). However, we found that swapping the C-terminal domain of $BK_{Ca}\alpha$ with that of mSlo3, a mammalian homologue which shows no calcium sensitivity (Schreiber et al., 1998; Moss and Magleby, 2001), produced a construct which was insensitive to CO. Figure 1A shows that $BK_{Ca}\alpha$ channel activity was robustly and reversibly activated by 30 μM CO donor. 30 μM of the breakdown product of this compound, which does not release CO ($RuCl_2(DMSO)_4$), did not affect channel activity (data not shown). This suggests that CO was solely responsible for activating $BK_{Ca}\alpha$ channels, a notion

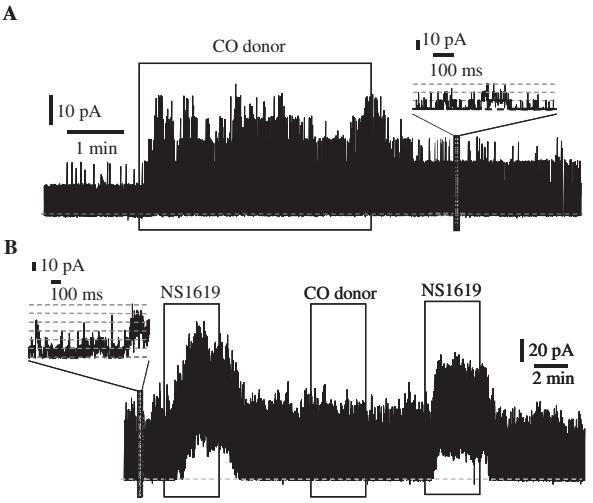

Fig. 1 Effect of CO donor on wild type $BK_{Ca}\alpha$ and $BK_{Ca}\alpha$/mSlo3 chimera channels. (**A**) Exemplar current recording from an inside-out patch excised from a HEK 293 cell transiently expressing wild type $BK_{Ca}\alpha$ channels. (**B**) Exemplar current recording from an inside-out patch excised from a HEK 293 cell transiently expressing $BK_{Ca}\alpha$/mSlo3 chimera channels. Periods of application of 30 μM CO donor (CO donor) and 10 μM of NS1619 (BK channel opener) are shown above each recording

supported by the observation that application of CO gas, dissolved in intracellular solution, was also able to activate the channel (data not shown). In contrast, application of the CO donor to patches containing the $BK_{Ca}\alpha$/mSlo3 chimera had no affect on channel activity, even though these channels were still functional as demonstrated by the application of the specific BK_{Ca} channel opener NS1619 (Fig. 1B). This suggests that modulation of the $BK_{Ca}\alpha$ channel is either due to CO binding directly to amino acid residues within this tail region or by a protein partner which associates with the $BK_{Ca}\alpha$ channel via the tail domain. As both the RCK1 and the heme binding domains are still present within the $BK_{Ca}\alpha$/mSlo3 chimera, our findings indicate that CO must be acting by a mechanism not yet fully defined.

Carbon monoxide is known to bind to a number of enzymes via metallocluster sites, which contain various metal ions (Iron, Nickel, Copper – (Lemon and Peters, 1999; Bennett et al., 2000; Drennan et al., 2001)). X-ray crystal structures of a number of these enzymes, such as carbon monoxide dehydogenase (Jeoung and Dobbek, 2007), suggest that both thiol groups of cysteine residues and imidazole side chains of histidine residues are essential for metal binding. CO can then interact with the enzymes via these bound transition metal ions.

To determine whether CO modulates channel activity by binding to a transition metal cluster within the tail domain, we have employed potassium cyanide as a substituent of the metal ion, as has been previously achieved at the active centre of carbon monoxide dehydrogenase (Ha et al., 2007). In the absence of KCN, the CO donor dramatically increased patch activity and caused a shift of the activation curve to the left (Va_{50} decreased from 75.3 ± 0.1 to 51.3 ± 0.1 mV; $n = 5$ – Fig. 2A). In a concentration-dependent manner, KCN attenuated the ability of 30μM CO donor either to activate the current (KCN $IC_{50} = 32.0 \pm 3.2$ μM; $n = 5$) or shift the Va_{50} value (KCN $IC_{50} = 20.1 \pm 4.2$ μM; n = 5 – Fig. 2A). 100 μM KCN completely

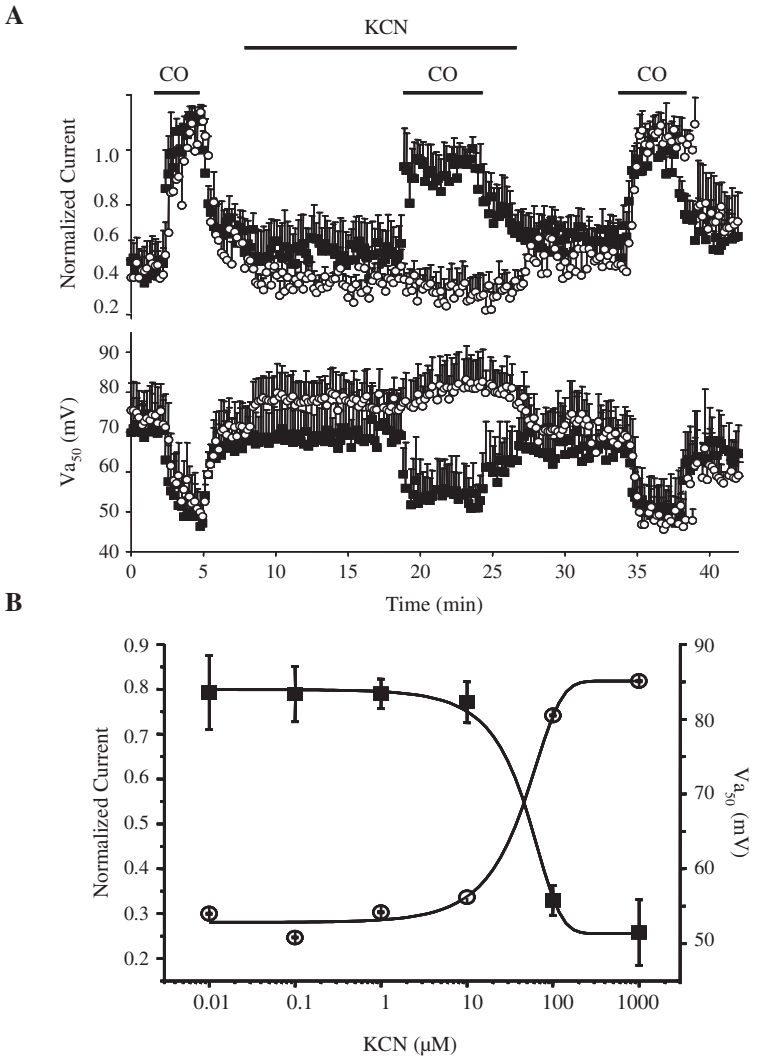

Fig. 2 Effect of KCN on activation of wild type $BK_{Ca}\alpha$ channels by the CO donor. (**A**) Mean (± S.E.M) normalized current time course showing the inhibitory actions of KCN on current activation (upper $-Vp = +60$ mV) and Va_{50} (lower) evoked by 30 μM CO donor. Time courses for 10 μM KCN (■) and 1mM KCN (○) are shown. Addition of CO donor and KCN are indicated by the solid lines and labels thereon. (**B**) Mean (± S.E.M) concentration–response plot showing the effects of KCN on normalized current at a holding voltage of +60 mV (■) and Va_{50} (○) in the presence of 30 μM CO donor

abolished activation by 30 μM CO donor (Fig. 2A). Although CN – can cleave disulphide bridges, such a reaction is not freely reversible in the absence of an oxidizing agent. Therefore, our data suggest that the mode of action for KCN is to compete for CO at the potential metal cluster of the channel.

Alignment of the amino acid sequences of the C-terminal region of human $BK_{Ca}\alpha$ and mSlo3 indicated that cysteine residues are largely conserved between these two homologues. However, four cysteine residues were identified as not being conserved between these two channels, namely residues at C820, C911, C995 and C1028. Thus, we engineered site-specific mutations, with replacement of cysteine residues for glycine residues at these amino acid positions. In a concentration-dependent manner, the CO donor increased open state probability (NPo) of wild type $BK_{Ca}\alpha$ channels with an EC_{50} of 49.5 $\pm$ 8.4 μM (n = 17; Fig. 3A and B). The single point mutations, C820G, C995G and C1028G, did not significantly change EC_{50}

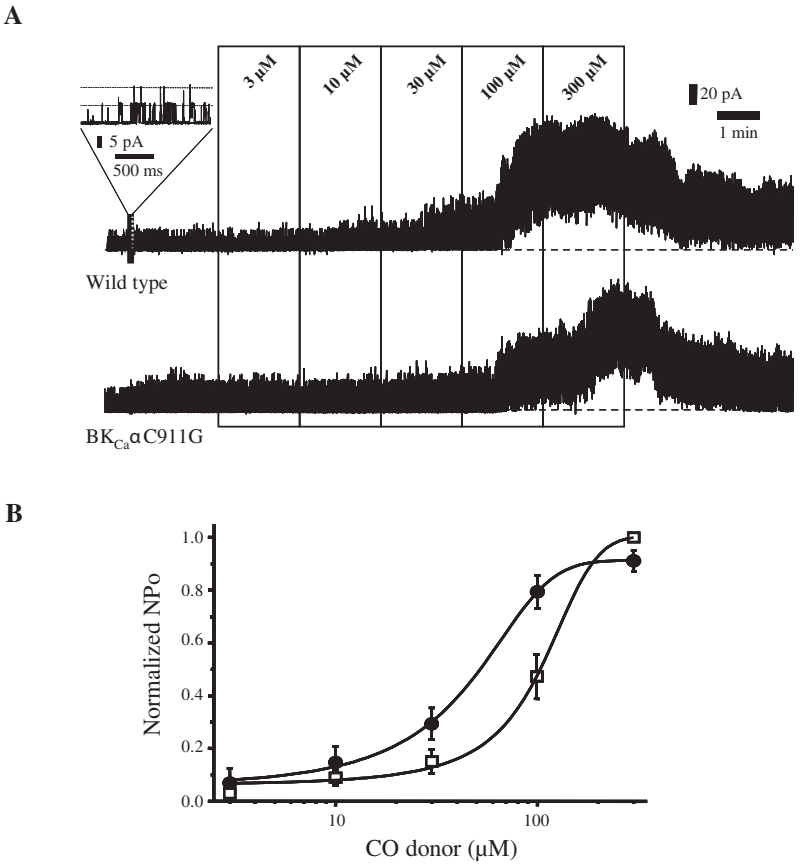

Fig. 3 Effect of a single cysteine mutation (C911G) upon channel activation by the CO donor. (**A**) Exemplar current recordings showing effect of sequential addition of increasing concentration of CO donor (3–300 μM) on wild type $BK_{Ca}\alpha$ channels (*upper*) and $BK_{Ca}\alpha$ C911G mutant channels (*lower*) at a holding potential of +40 mV; black dashed line marks the zero current level. (**B**) Mean ($\pm$ S.E.M) concentration–response plots displaying the relationship between normalized NPo of wild type $BK_{Ca}\alpha$ channels (●) and $BK_{Ca}\alpha$ C911G mutant channels (□) as a function of CO donor concentration

values for activation of $BK_{Ca}\alpha$ channels by the CO donor (data not shown, $n > 10$ for each). In contrast, the CO sensitivity of the C911G mutant was significantly decreased ($EC_{50} = 103.8 \pm 8.1\,\mu M$; $n = 12$, $P < 0.05$ – Fig. 3A and B). The increase in NPo of wild type $BK_{Ca}\alpha$ channels occurred at low concentrations of CO donor, became significant at $10\,\mu M$ and plateaued at $100\,\mu M$ and above (Fig. 3A). In contrast, the C911G mutant was not significantly activated until the CO donor was raised to $30\,\mu M$ and did not reach a plateau at CO donor concentrations up to $300\,\mu M$ (Fig. 3A and B), above which the CO donor was no longer soluble. All double and triple cysteine $BK_{Ca}\alpha$ mutant constructs which contained the mutation C911G exhibited reduced CO sensitivity, with EC_{50} values shifted towards $100\,\mu M$, whilst those of $BK_{Ca}\alpha$ channel mutants which did not contain mutation at C911G displayed essentially unaltered CO donor EC_{50} values (data not shown).

These data highlight that a single cysteine residue (C911) located in the C-terminal domain of human $BK_{Ca}\alpha$ is crucial to the activation of these channels by CO. We suggest that CO may bind to this channel subunit in a manner similar to the transition metal-dependent co-ordination which is characteristic of several enzymes, such as CO dehydrogenase.

Acknowledgments This work was supported by a grant from the British Heart Foundation (RG/03/001).

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