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## The effects of hypoxia on the activity of neuronal large conductance calcium-activated potassium ($BK_{Ca}$) channels expressed in AtT20 and HEK 293 cell lines

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Neuronal large conductance calcium-activated potassium ($BK_{Ca}$) channels act as coincidence detectors. A single (*Slo*) gene encodes all four heterogeneous $\alpha$-subunits that shape the channel pore. Alternative exon splicing of *Slo* mRNA provides the variation in characteristics required for $BK_{Ca}$ to show alternative activity in different cellular environments (Shipston, 2001). It has been suggested that $BK_{Ca}$ channel activity could be regulated by changes in oxygen tension (Lewis *et al.* 2002). The aim of this research was to determine whether hypoxia (20 mmHg) regulates the activity of alternative splice variants (mbr5 and STREX) of $BK_{Ca}$ channel $\alpha$-subunits using the single channel excised inside-out patch clamp recording technique.

All data are expressed as means ± S.E.M. and ANOVA or Student's unpaired *t* test was used to examine statistical differences, and $P < 0.05$ was considered significant.

In patch recordings from mbr5-expressing HEK-293 cells, hypoxia did not cause a significant change in single channel amplitude or channel open probability ($P_o$) in the presence of 0.1, 1 or 10 $\mu$M free calcium. In experiments using the STREX splice variant expressed in the HEK-293 cell line, changes in channel activity in response to hypoxia were observed. For example in 1 mM free calcium at +60 mV, $P_o$ was reduced from control values of 0.57 ± 0.04 ($n = 12$) in normoxic solutions to 0.24 ± 0.06 ($n = 15$) after 8 min exposure to hypoxic solution (Student's paired *t* test, $P < 0.05$). The inhibition of channel activity was reversed upon washout. Similar responses were observed in 0.1 mM free calcium. No change in $P_o$ was observed in response to hypoxia in the presence of 10 $\mu$M free calcium ($n = 8$).

In the STREX-expressing corticotroph cell line AtT20, endogenous $BK_{Ca}$channel activity was also inhibited upon application of hypoxic solutions. In 1 $\mu$M free calcium at +20 mV, $P_o$ was reduced from control values of 0.56 ± 0.03 ($n = 10$) to 0.11 ± 0.02 ($n = 10$) (Student's paired *t* test, $P < 0.05$). Similar reductions in $P_o$ were observed in 0.1 $\mu$M free calcium. This reduction was not observed in 10 $\mu$M free calcium ($n = 8$).

In conclusion, these data indicate that the mbr5 and STREX splice variants exhibit differential sensitivity to changes in oxygen tension. The inhibition of STREX variant activity by hypoxic solutions appears to be calcium dependent and further experiments will be required to elucidate the site and mechanism of channel modulation.

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C41

## Role of mitochondria in hypoxic inhibition of Kv3.1b channels expressed in HEK293 cells

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Kv3.1 potassium channels are expressed in neurons that fire action potentials at high frequency (Perney *et al.* 1992), and also in PC12 (Conforti & Millhorn, 1997) and pulmonary artery smooth muscle cells (Osipenko *et al.* 2000), both of which display an oxygen-sensitive $K^+$ current. Recent studies showed that in an *in vitro* expression system, hypoxia inhibits whole-cell Kv3.1b current and single-channel activity (Osipenko *et al.* 2000). This study investigated the underlying role of mitochondria in the modulation of Kv3.1b channels by hypoxia.

HEK293 cells stably expressing mouse Kv3.1b (mKv3.1b) DNA were used to record whole-cell currents. Bath and internal (pipette) solutions were based on Osipenko *et al.* (2000). Hypoxic solutions were prepared by bubbling with 100 % $N_2$, giving giving 7.1 ± 0.8 % $O_2$ (mean ± S.E.M., $n = 7$) (control = 20.3 ± 0.5 %, $n = 7$). A mitochondrial DNA (mtDNA)-depleted cell line ($\rho^0$) was generated from the transfected HEK293 cells ($\rho^+$) by culturing for 3–6 weeks in the presence of 50 ng ml$^{-1}$ ethidium bromide in medium supplemented with uridine and pyruvate (King & Attardi, 1989). Single-cell PCR demonstrated that mtDNA had been depleted in the cells used for current recording. Mitochondrial membrane potential was detected by confocal microscopy of cells incubated with 100 nM tetramethylrhodamine ethyl ester (TMRE). Statistical comparisons employed Student's two-tailed, unpaired *t* test, $P < 0.05$ being considered significant.

The mKv3.1b current displayed an activation threshold between −20 and −30 mV ($n = 31$) and amplitude of 157 ± 12 pA pF$^{-1}$ at 20 mV. When exposed to hypoxia for 10 min the current at 20 mV was reduced by 24 ± 2 % ($n = 17$). Mitochondrial inhibition caused by either rotenone (1 $\mu$M, $n = 7$), an electron transport chain inhibitor, or carbonyl cyanide *p*-(trifluomethoxy) phenylhydrazone (FCCP, 1 $\mu$M, $n = 7$), an uncoupler of oxidative phosphorylation, had no effect on Kv3.1b current under control conditions, but abolished the hypoxic inhibition. The effect of hypoxia on Kv3.1b current was also essentially abolished in $\rho^0$ cells, with a reduction of only 2.5 ± 0.4 % ($n = 8$, $P < 0.05$ compared with the control cells) at 20 mV. Measurements of TMRE fluorescence revealed a lower intensity in $\rho^0$ cells (117 ± 9 units, $n = 9$) compared with $\rho^+$ cells (150 ± 10 units, $n = 9$, $P < 0.05$), although both displayed similar, punctate staining that was depleted by 1 $\mu$M FCCP. This suggests a reduced mitochondrial membrane potential in $\rho^0$ cells.

These results suggest that mitochondria play a vital role in mediating the inhibition of Kv3.1b channels by hypoxia.

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C42

## Use of mutant Kv3.2 channels to identify residues required for block by BDS (blood depressing substance)

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Previously (Yeung & Robertson, 2003) we showed that delayed rectifier-type Kv3 channels were effectively blocked by the peptidic toxin blood depressing substance (BDS). Based on the data of Swartz & MacKinnon (1997), who demonstrated that particular residues on the Kv2.1 channel, when mutated to alanine, reduced sensitivity to block by the gating modifier hanatoxin (HaTx), we sought to identify the residues on Kv3.2 that may confer sensitivity to BDS.

Four mutant Kv3.2 channels with multiple point mutations to alanine were generated by site-directed mutagenesis. The mutated sites on Kv3.2 (in the S3/S4 regions) were aligned to those on Kv2.1 that conferred HaTx sensitivity (Table 1).

| Mutant | Kv3.2 residues mutated | Corresponding residues on Kv2.1 |
|---|---|---|
| 1 | V330, G331, G334 | I273, F274, E277 |
| 2 | R348, R351 | V291, I294 |
| 3 | L347, V350, V353 | R290, Q293, R296 |
| 4 | R354, R357 | R299, R302 |

Table 1. Mutant Kv3.2 channels. Residues on Kv3.2 selected based primarily on aligned region with Kv2.1 were mutated to alanine. All Kv3.2 mutated residues fall in part of the S3 and S4 voltage-sensing domain.

Recordings were made from HEK293 cells using our previous methods (Yeung & Robertson, 2003).

Of the four mutant Kv3.2 channels tested, mutant 3 was of particular interest. Under control conditions, mutant 3 showed a more hyperpolarised threshold (~−50 mV, shift of ~−30 mV) and a more rapid rate of current activation (2-fold change) (Fig. 1). BDS-II (500 nM), which blocks wild-type channels by 48.8 ± 4.3 % ($n$ = 3) at +40 mV, now potentiated the current through mutant 3 channels (26.3 ± 5.3 % at +40 mV, $n$ = 6 (means ± S.E.M.)).

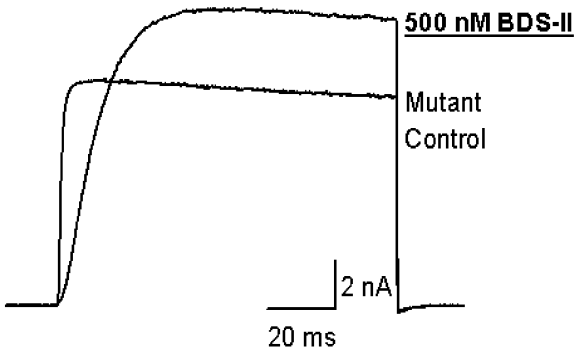


Figure 1. Mutant (3) Kv3.2 current recordings at a test potential of +40 mV under control conditions and when maximal BDS potentiation was observed.

Furthermore, consistent with data obtained from wild-type Kv3.2, 500 nM BDS-II produced a dramatic slowing in the rate of current activation at +40 mV with a depolarising shift in the gating (Fig. 2). Using Boltzmann fits, $V_{½}$ values for activation in BDS-II showed a shift of ~ +21 mV, which was greater than that for the wild-type channel (~ +11 mV). On washout, BDS effects were fully reversible.

These data indicate that the residues L347, V350 and V353 are involved in the gating process of the Kv3.2 channel and that these residues influence BDS block.

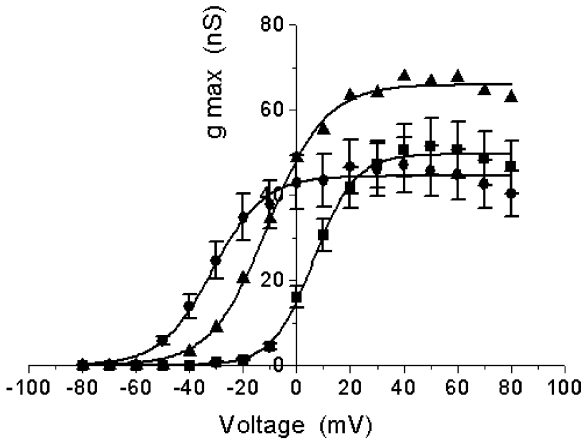


Figure 2. Conductance–voltage plots of wild-type Kv3.2b (■, $n$ = 21) under control conditions compared to mutant 3 in the absence (●, $n$ = 14) and presence (▲, $n$ = 5) of 500 nM BDS-II.

Swartz KJ & MacKinnon R (1997). *Neuron* **18**, 675–682.

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C43

## Interactions between rat SK channel subunits expressed in HEK 293 cells

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Small conductance $Ca^{2+}$-activated $K^+$ channels (SK) are widely distributed in both neuronal and non-neuronal tissues. Three genes, *SK1*, *2*, and *3*, code for mammalian SK channel subunits (Köhler *et al.* 1996). Heterologous expression of either the rat SK2 or SK3 genes (*rSK2* and *rSK3*) gives rise to functional homomeric channels, similar in pharmacology to native channels (e.g. Hosseini *et al.* 2001; Fanger *et al.* 2001). However, to date attempts to express the rat SK1 (*rSK1*) gene have not yielded detectable channel activity (Bowden *et al.* 2001). We have investigated the properties of *rSK1* in detail.

Using an antibody selective to the rSK1 subunit and a yellow fluorescent protein (YFP) tag we have found that the rSK1 protein is produced but it appears to be trapped in intracellular compartments. Similar results have been reported for known silent subunits (Post *et al.* 1996).

To test the idea that rSK1 is also a silent subunit we co-expressed rSK1 with rSK2 and found a 67 ± 22 % (mean ± S.E.M.) increase in $Ca^{2+}$-activated $K^+$ conductance compared with cells transfected with rSK2 alone. Moreover, in co-transfected cells the sensitivity of the channels to block by apamin and UCL 1848 was substantially reduced ($IC_{50}$ values for apamin and UCL 1848 were 95 ± 8 pM (mean ± S.D.) and 110 ± 26 pM, respectively, for inhibition of rSK2 alone compared with 1.4 ± 0.3 nM and 2.9 ± 0.3 nM for inhibition of rSK1/rSK2 currents). These findings indicate that rSK1 is able to reach the cell surface as an SK1/SK2 heteromer.

In contrast, co-transfection of rSK1 with rSK3 caused an 84 ± 4 % reduction in current level compared with cells expressing SK3 alone. By using a TEA-sensitive mutant of SK3 (SK3V515F), we were able to show that the TEA sensitivity of this residual current is similar to that of cells expressing SK3V515F alone, suggesting that there is little or no contribution from rSK1 and that most of the channels are probably homomeric rSK3 assemblies.

In conclusion our data support the idea that rSK1 can interact with both rSK2 and rSK3. The different interactions between rSK1 and the other subunits suggest that trafficking of the channels is influenced by subunit composition. Further, rSK1 appears to act as a silent subunit.

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## PC34

### Expression of N-type calcium channels is suppressed by the stargazin-related protein $\gamma$7

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We recently cloned and characterized a new putative $\gamma$ subunit for voltage-gated calcium channels called $\gamma$7. This stargazin-like protein exhibits 25 % homology to $\gamma$2 (Moss *et al.* 2002). N-type current through $Ca_V2.2$ is markedly decreased when it is transiently co-expressed with $\gamma$7 in tsA-201 cells from $-60.2 \pm 10.7$ pA pF$^{-1}$ (mean ± S.E.M., $n = 44$) to $-15.1 \pm 2.9$ pA pF$^{-1}$ ($n = 21$) at +5 mV (Student's unpaired $t$ test, $P < 0.01$) (Fig. 1*A*). This has also been shown in COS-7 and *Xenopus* oocytes (Moss *et al.* 2002). Furthermore, this inhibition is not due to an interference with normal trafficking of $Ca_V$ subunits but to a decrease of the level of expression of $Ca_V2.2$ protein (Fig. 1*B*).

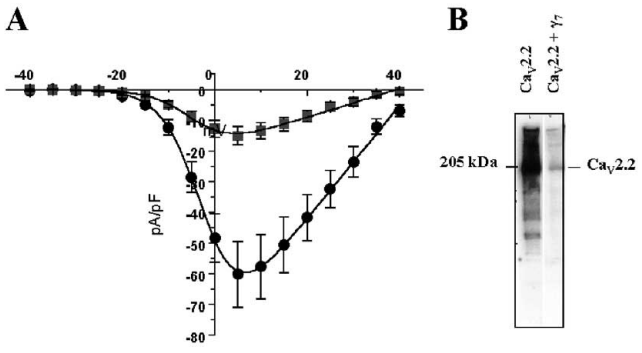


Figure 1. Suppression of expression of N-type calcium channel by $\gamma$7. *A*, mean current–voltage curves recorded in tsA-201 transfected with $Ca_V2.2$, $\beta_{1b}$, $\alpha_2\delta_2$, co-expressed without $\gamma$7 (circles) or with $\gamma$7 (squares). *B*, Western blot of total protein isolated from COS-7 cells transfected with either $Ca_V2.2$ alone or with $Ca_V2.2$ and $\gamma$7.

Over-expression of $\gamma$7 in sympathetic neurons does not affect native N-type currents, suggesting that it does not alter pre-existing functional calcium channels (Moss *et al.* 2002). Inhibition requires translation of $\gamma$7 as N-type currents are not significantly reduced ($-49.6 \pm 8.6$ pA pF$^{-1}$ at +5 mV, $n = 28$) when a stop codon is introduced two amino acids after the start codon of $\gamma$7. It was hypothesised that the last four amino acids of $\gamma$7 present in the C-terminus may be similar to the PDZ binding motif found to be important in other $\gamma$ subunits. However, the removal of these four amino acids does not prevent the inhibition. Co-expression with this truncated $\gamma$7 resulted in a reduction to 21.1 ± 9.5 % (mean ± S.E.M., $n = 16$) of the control currents in *Xenopus* oocytes. We have also examined the selectivity of the inhibition by determining the effect of $\gamma$7 on other calcium channels and on a potassium channel. There was no effect of $\gamma$7 on either $Ba^{2+}$ currents induced by expression of $Ca_V3.1$ ($-9.8 \pm 19.35$ % inhibition at −10 mV, $n = 17$) or $K^+$ currents induced by expression of $K_V3.1b$ ($11.3 \pm 11.6$ % inhibition at +60 mV, $n = 9$) when recorded in tsA-201 cells. It appears that $\gamma$7 inhibits the expression of high voltage activated calcium channels, particularly $Ca_V2.2$, at an early stage in synthesis. Inhibition requires translation of $\gamma$7 but is not dependent on the last four amino acids, hypothesised to be similar to a PDZ binding motif. Work is currently ongoing to elucidate the mechanism of inhibition.

Moss FJ *et al.* (2002). *EMBO Journal* **21**, 1514–1523.

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*All procedures accord with current UK legislation.*

## PC35

### Elevated $[K^+]_o$ enhances cultured adult rat cardiac myofibroblast contraction

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Cardiac fibroblasts comprise the largest group of cells within the mammalian heart. They reside primarily in the interstitium of the ventricular and atrial myocardium (Zak 1973). In health, fibroblasts are responsible for maintaining the extracellular matrix and synthesizing a number of autocrine and paracrine factors such as transforming growth factor $\beta$ (TGF$\beta$) (Eghbali 1989). Following myocardial infarction, fibroblasts are activated by the presence of necrotic muscle (Weber & Brilla 1992) and by TGF$\beta$ which is released by immune cells (de Almeida *et al.* 2002). Fibroblasts convert into myofibroblasts which lay down scar tissue (Hao *et al.* 1999). Despite these important roles of fibroblasts and myofibroblasts, little is known about how the membrane potential is controlled in cardiac fibroblasts and myofibroblasts, or how modulation of membrane potential may affect fibroblast or myofibroblast function.

We have recorded membrane currents in both freshly dissociated and primary cultures of adult rat cardiac fibroblasts and measured collagen I gel deformation due to myofibroblast contraction in the presence of different $[K^+]_o$ concentrations. Hearts were removed from adult Sprague-Dawley rats under isoflurane anaesthesia and were Langendorff perfused, then digested with collagenase II. Dispersed fibroblasts were kept at 4 °C and studied within 6 h or were separated from myocytes and other cell types and cultured in DMEM–10 % FCS.

Using whole-cell patch-clamp methods, $K^+$ currents in freshly dissociated and cultured cardiac fibroblasts were recorded. Inward currents in 10 mM $[K^+]_o$ Tyrode solution were blocked by 300 $\mu$M $Ba^{2+}$. At −120 mV, in freshly dissociated fibroblasts, the

$Ba^{2+}$-sensitive current measured $-13.8 \pm 6.7$ pA $pF^{-1}$ (means ± S.E.M.), ($n = 3$). In cultured fibroblasts, inward currents were recorded at −120 mV in 5.4 mM $[K^+]_o$ Tyrodes solution, and these were blocked by 1 mM $Ba^{2+}$ ($Ba^{2+}$-sensitive current = $-7.4 \pm 2.2$ pA $pF^{-1}$, $n = 20$). Time- and voltage-dependent outward currents ($51.2 \pm 14.3$ pA $pF^{-1}$, +40 mV, $n = 47$) were recorded from the majority of freshly dissociated fibroblasts. A component of this outward current was blocked by 1 mM TEA (TEA-sensitive current = $7.6 \pm 3.1$ pA $pF^{-1}$ at +40 mV, $n = 3$). Similar time- and voltage-dependent outward currents ($11.9 \pm 3.0$ pA $pF^{-1}$, +40 mV, $n = 20$) were recorded in cultured fibroblasts. The outward currents were also sensitive to 100 $\mu$M 4-aminopyridine (4-AP) in both freshly dissociated and cultured cardiac fibroblasts. The 4-AP-sensitive current measured $12.9 \pm 6.6$ pA $pF^{-1}$ at +40 mV in freshly dissociated fibroblasts ($n = 3$) and $6.0 \pm 1.8$ pA $pF^{-1}$ at +40 mV in cultured fibroblasts ($n = 5$).

In cultured fibroblasts, anti-$\alpha$ smooth muscle actin antibody staining was positive, indicating that when cultured, cardiac fibroblasts became activated and transformed into contractile myofibroblasts. Populations of myofibroblasts were seeded onto collagen I gels following first passage, and gel deformation due to myofibroblast contraction was compared when myofibroblasts were depolarised by exposure to 20 mM $[K^+]_o$ Tyrode solution. At 8 h, gel surface area was significantly reduced in 20 mM $[K^+]_o$ ($90 \pm 2$ $mm^2$ ($n = 8$) *vs.* $113 \pm 4$ $mm^2$ ($n = 15$) under control conditions, $P = 0.034$, Student's unpaired $t$ test). This suggests that modulation of membrane potential by modulating $K^+$ currents can affect myofibroblast function.

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Zak R (1973). *J Physiol* **31**, 211–219.

*All procedures accord with current National and local guidelines.*