Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 314 (2004) 63–68
BBRC
www.elsevier.com/locate/ybbrc

siRNA knock-down of γ-glutamyl transpeptidase does not affect hypoxic K^+ channel inhibition

Sandile E. Williams,[a,1] Phillippa Wootton,[a,1] Helen S. Mason,[a] David E. Iles,[b] C. Peers,[c] and Paul J. Kemp[a,*]

[a] School of Biomedical Sciences, University of Leeds, Leeds LS2 9JT, UK
[b] School of Biology, University of Leeds, Leeds LS2 9JT, UK
[c] School of Medicine, University of Leeds, Leeds LS2 9JT, UK

Received 9 December 2003

Abstract

Large conductance, Ca^{2+}-sensitive potassium (BK) channels are critical components of the O_2 signalling cascade in a number of cells, including the carotid body and central neurones. Although the nature of the BK channel O_2 sensor is still unknown, evidence suggests redox modulators might form part of the O_2 sensing channel complex. By metabolising glutathione, γ-glutamyl transpeptidase (γGT) could act as such an O_2 sensor. Western blotting and immunocytochemistry revealed high γGT expression in HEK293 cells expressing the α- and β-subunits of human recombinant BK and γGT co-immunoprecipitated with BKα. Acivicin blockade of γGT reversibly inhibited BK channels, suggesting that this BKα protein partner contributes to tonic channel activity. However, knock-out of γGT using siRNA had no effect on hypoxic BK channel inhibition. Together, these data indicate that γGT is a BKα protein partner, that its activity regulates BK channels but that it is not the BK O_2 sensor.

Keywords: Potassium channel; Hypoxia; Carotid body; γ-Glutamyl transpeptidase; Small interference RNA

Large conductance, Ca^{2+}-sensitive potassium (BK) channels are key initiators of the O_2-dependent cascade which links decreased O_2 availability to increased excitability in a number of cellular systems, including the carotid body glomus cell [1,2] and components of the central nervous system [3,4]. Although both native and recombinant BK channels are acutely and reversibly inhibited as O_2 supply becomes compromised [2–6] and, in the carotid body at least, the resulting cell depolarisation and voltage-gated Ca^{2+} influx induce hypoxia-dependent transmitter release [7], the molecular nature of the O_2 sensor in the arterial chemoreceptor is still unknown. Since regulation by O_2 of both native [2,4] and recombinant [6] BK channels is retained in channel recordings from whole-cell and excised, inside-out configurations of the patch clamp technique, it has been suggested by us and others that the O_2 sensor (and any potential substrates/reaction products) must be closely associated with the channel protein itself [4,6,7].

Quite recently, there have been reports that the membrane-bound ectoenzyme, γ-glutamyl transpeptidase (γGT), is intimately involved in O_2 sensing in the nucleus tractus solitarius [8] and some investigators in the field have speculated that this enzyme may act as an O_2 sensor in other cell types. Indeed, it is an attractive proposition that γGT, being so closely involved in setting cellular redox potential via glutathione metabolism and subsequent production of reactive O_2 species, might endow BK channels with their O_2 sensitivity.

To investigate this supposition, we have taken advantage of the easily amenable and well-established cellular model of BK channel acute O_2 sensing; HEK 293 cells stably expressing both the α- and β-subunits of the human BK channel [6,9,10]. Using this model we have employed a sequential approach of immunoprecipitation, immunocytochemistry, and Western blotting to establish γGT expression levels and protein localisation. Pharmacological and molecular intervention—using

* Corresponding author. Fax: +44-113-343-4228.
E-mail address: p.z.kemp@leeds.ac.uk (P.J. Kemp).
[1] These authors contributed equally to this work.

0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.12.052

siRNA—has then been used to probe the functional consequences of the protein partnership between γGT and the BK α-subunit.

Materials and methods

Materials

Unless otherwise stated, tissue culture reagents were purchased from Gibco-BRL, (Paisley, Strathclyde, UK), molecular biological reagents were purchased from Ambion (Austin, Texas, USA), and electrophysiological reagents were purchased from Sigma–Aldrich (Poole, Dorset, UK).

Methods

Cell culture. Human embryonic kidney 293 (HEK 293) cells expressing human α- and β-subunits of BK channels [6,10] were donated by Prof. M.L.J. Ashford (University of Dundee, UK). The cells were maintained in Earle's minimal essential medium (containing L-glutamine) supplemented with 10% fetal calf serum, 1% antibiotic–antimycotic, 1% non-essential amino acids, and 0.2% gentamicin in a humidified incubator gassed with 5% CO_2/95% air. Cells were passaged every 7 days in a ratio of 1:24 using Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). The co-expressed α- and β-subunits were KCNMA1 (GenBank Accession No. U11717) and KCNMB1 (GenBank Accession No. U42600), respectively.

Membrane preparation and immunoprecipitation. Approximately 10^8 HEK293 cells were pelletted at 1000*g* and then homogenised in 1 ml of ice-cold buffer A (50 mM Tris–HCl, pH 7.4, 140 mM KCl, 1 mM EGTA, 1 mM $MgCl_2$ containing 1 mM phenylmethanesulfonyl fluoride (PMSF), and 50 μg/ml aprotinin). Following centrifugation at 1000*g* for 5 min at 4 °C, the supernatants were re-centrifuged at 16,000*g* for 30 min at 4 °C and the pellets solubilised in 500 μl ice-cold buffer B (5 mM Tris–HCl, pH 7.4, 500 mM NaCl, 1.5% v/v Triton X-100 containing 1 mM PMSF, and 50 μg/ml aprotinin). Five micrograms of BKα antibody was added (4 °C for 1 h) before the addition of 50 μl Protein G beads (4 h at 4 °C with gentle rotation). The beads were then pelleted at 7000*g* for 5 min and washed 3 times in 1 ml ice-cold buffer C (5 mM Tris–HCl, pH 7.4, 20 mM NaCl, 0.5% v/v Triton X-100 containing 1 mM PMSF, and 50 μg/ml aprotinin).

Electrophoresis and protein visualisation. For SDS–PAGE, pelleted immunoprecipitates (or cell lysates) were suspended in 50 μl sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% v/v glycerol, 0.2% w/v SDS, 5% v/v β-mercaptoethanol, and 0.02% w/v bromophenol blue), heated to 100 °C for 3 min and loaded onto SDS–polyacrylamide gels comprising 4% stacking and 10% resolving gels (4 or 10% of 37.5:1 acrylamide/bisacrylamide in 125 mM Tris–HCl, pH 8.8, and 0.1% w/v SDS). Prestained molecular weight markers (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) were loaded next to the sample lanes. Electrophoresis was continued at 200 V until the 25 kDa marker had reached the bottom of the resolving gel. Gels were destained 3 times for 15 min in deionised H_2O.

Western blotting. Prior to transfer, filter papers, polyvinylidene difluoride (PVDF) membranes, and SDS–polyacrylamide gels were equilibrated in transfer buffer (25 mM Tris, 190 mM glycine, 0.0375% w/v SDS, and 20% v/v methanol). Electrophoretic transfer was undertaken using a Bio-Rad wet-transfer apparatus at 20 V overnight. After blotting, membranes were blocked in 5% w/v skimmed milk in PBS for 30–60 min at room temperature and transferred to anti-γGT antibody diluted 1:500 in blocking buffer. Blots were incubated at room temperature overnight at 4 °C. The blots were then washed 3 times with PBS, 0.05% v/v Tween 20, and then once in PBS before being incubated with the second layer antibody diluted 1:1000 in 5% w/v skimmed milk in PBS for 45 min and washed 3 times as described above. Enhanced chemoluminescent (ECL) detection of bound second layer antibody was carried out by mixing equal volumes of ECL Western blotting detection reagents 1 and 2 (Amersham–Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). The mixtures were added to the blots, which were then incubated for 1 min, wrapped in cling film, and then exposed to high performance chemoluminescent film from Amersham–Pharmacia.

Production of siRNA and lipofection of HEK 293 cells. Two short interfering (si)RNAs were designed to target bases 582–603 (γGT siRNA1) and 722–743 (γGT siRNA2) of the γGT coding sequence (Accession NM_013430). Using the siRNA Template Design Tool for the *Silencer* siRNA Construction Kit (Ambion), the following oligonucleotide templates were designed:

γGT siRNA1, antisense 5′- AACCTGACAACCATGTGTACAC CCTGTCTC -3′;
γGT siRNA1, sense 5′- AAGTGTACACATGGTTGTCAGG CCTGTCTC -3′.
γGT siRNA2, antisense 5′- AATGCCCACAGCATGGGCATCG CCTGTCTC -3′;
γGT siRNA2, sense 5′- AACGATGCCCATGCTGTGGGCA CCTGTCTC -3′.

Underlined bases correspond to γGT sequences. Oligonucleotides were purchased from Sigma Genosys (Poole, Dorset, UK) and diluted to 100 μM in nuclease-free water. Twenty microlitres aliquots were stored at −20 °C until use. The control siRNA (scrambled GAPDH sequence) was purchased from Ambion.

Template preparation, dsRNA synthesis, siRNA preparation, and purification were all carried out using the *Silencer* siRNA Labelling Kit—Cy3 according to the manufacturer's instructions. siRNAs were dissolved in nuclease-free H_2O and diluted 1:25 in TE buffer (10 mM Tris–HCl, pH 8, and 1 mM EDTA) for quantification at A_{260}. About 1.6 nmol duplex siRNA was taken for each Cy3-labelling reaction, which was carried out according to the manufacturer's instructions.

One day before transfection with Cy3-labelled siRNA, BK-expressing HEK293 cells were plated onto 22 × 22 mm coverslips in 6-well plates containing 2 ml of medium to ensure 30–50% confluence (~1×10^5 cells/well) at the time of transfection. In short, 10 pmol duplex, labelled siRNA was diluted into 194 μl OPTI-MEM (Gibco-BRL), and 6 μl siPORT Amine (Ambion). The mixture was vortexed and incubated at room temperature for 20 min, and then 800 μl of normal growth medium was added before the entire mixture was added to cells (which had previously had their medium removed). Cells were cultured at 37 °C in 5% CO_2 in air for 4 h before 3 ml of growth medium was added and the cells were cultured for a further 48 h.

Immunocytochemistry. Medium was removed from cells grown on coverslips and the cells were washed 2 times in 3 ml PBS. PBS was removed and cells were fixed with 3 ml of 10% v/v formalin (Sigma–Aldrich) at 37 °C for 5 min and the solution was discarded. About three milliliters of 0.5% v/v Triton X-100 in PBS was added to permeabilise the cells at room temperature for 15 min and the solution was removed. Cells were refixed in 3 ml of 10% formalin solution for 5 min at room temperature. The solution was removed and the cells were washed 3 times with 3 ml PBS. Cells were incubated in 3 ml PBS (containing 5% donkey serum and 1 mM NaN_3) at room temperature for 3 h. Thirty microlitres of γGT primary antibody was added to the medium and cells were incubated overnight at room temperature. The antibody solution was removed and cells were washed 8 times in 3 ml PBS. The FITC-labelled second layer antibody, diluted 1:100 in PBS containing 5% serum, was added and incubated for 3 h at room temperature. After removing the solution, the cells on coverslips were washed 8 times with 3 ml PBS. The coverslips were mounted with Vectashield onto slides and sealed with nail varnish. The cells were then visualised by confocal microscopy.

Patch-clamp recording. Cells were grown for 24 h on glass coverslips before being transferred to a continuously perfused (5 ml/min) recording chamber (volume ca., 200 μl) mounted on the stage of an

inverted microscope. For whole-cell patch clamp recordings, the standard pipette solution was composed of (in mM): 10 NaCl, 117 KCl, 2 $MgCl_2$, and 11 Hepes pH 7.2, with Ca^{2+} buffered to 300 nM using EGTA, and $CaCl_2$ in appropriate ratios. When filled with this solution, pipettes were of resistance 4–6 MΩ. The Na^+-rich bath solution was composed of (in mM): 135 NaCl, 5 KCl, 1.2 $MgCl_2$, 5 Hepes, 2.5 $CaCl_2$, and 10 D-glucose, and pH 7.4. Hypoxic solutions were bubbled with $N_{2(g)}$ for at least 30 min prior to perfusion of cells, which produced no shift in pH. pO_2 was measured (at the cell) using a polarised carbon fibre electrode [11] and was found to be between 30 and 40 mm Hg. Normoxic solutions were equilibrated with room air. All K^+ currents were recorded at a bath temperature of 22 ± 0.5 °C. Current recordings were made using an Axopatch 200 A amplifier and Digidata 1320 A/D interface (Axon Instruments, Forster City, California, USA). To evoke BK currents, a standard ramp protocol was employed: −70 mV holding potential, voltage ramped from −100 to +60 mV (2000 ms, 0.1 Hz. Wild type HEK293 cells do not express BK channel currents (see Fig. 1D and [6,9]). Data analysis was performed using the PClamp 8.0 suite of software (Axon Instruments). For the time-courses and mean current–density plots, the current during the final 5 ms of the ramp was employed (i.e., at +60 mV). Current density was calculated by dividing currents by the whole cell capacitance. Statistical comparisons were made using paired Student's *t* tests.

Results and discussion

HEK293 cells have been shown to express γGT enzyme activity [12]. Here, we extend that observation by employing Western blotting, immunoprecipitation, and immunocytochemistry (Fig. 1). Western blotting with an antibody raised against the human homologue of γGT revealed positive immunoreactive bands of the correct relative molecular mass in both wild type and BK expressing HEK 293 cells (Fig. 1A). Consistent with a previous report demonstrating both N- and O-linked glycosylation of the membrane-bound protein [13], γGT often appears in Western blot as a doublet. To probe the possible protein–protein interaction of γGT with the α-subunit of BK channel (BKα), immunoprecipitation of membrane proteins from wild type and BK-expressing HEK 293 cells was performed using a BKα-specific antibody. Subsequent Western blotting for γGT revealed a band of the appropriate mass only in BK-expressing cells, suggesting strongly that γGT and BKα are membrane protein partners (Fig. 1B). Interestingly, Western blot of BKα-immunoprecipitated membrane proteins resulted in a single band, as opposed to the usual doublet observed by Western blot of cell lysates, suggesting that BKα associates only with the mature, glycosylated protein. Confocal microscopy of BK-expressing HEK 293 cells demonstrated robust γGT immunoreactivity, localised at the plasma membrane and throughout the cytoplasm (Fig. 1C). This observation was paralleled in wild type cells (data not shown). Although functional γGT is a plasma membrane bound ectoenzyme, there is good evidence that cells which express similarly high γGT levels (often following stimulation) demonstrate significant cytoplasmic immunoreactivity (e.g., [14]). In many tissues, γGT activity is normally low but is inducible by numerous factors, including oxidative stress [14,15]. However, in HEK 293 cells, constitutive γGT activity is high [12]—an observation fully supported by the Western blot and immunocytochemical evidence presented here—and suggests that HEK 293 cells represent an excellent model in which to study the role of γGT in regulation of recombinant proteins.

Both native and recombinant BK currents are sensitive to acute changes in partial pressure of oxygen (pO_2). Thus, during hypoxia, BK activity of carotid body [1,2], central neurones [3,4], and BK expressing HEK 293 cells [6] is suppressed. γGT is crucial to glutathione metabolism and adaptation to oxidative stress, suggesting that it plays a key role in modulating the redox potential of the cell. Since many studies of O_2-sensitive ion channels in general, and BK channels in particular, have implicated redox couples in channel regulation (e.g., [16]), we postulated that activity and O_2 sensitivity of BK channels may be modulated by γGT. Such a suggestion seemed particularly exciting in light of the evidence in Fig. 1 which shows that BKα and γGT are protein partners in the plasma membrane.

Wild type HEK 293 cells expressed very little current and this current was not regulated by hypoxia (Fig. 1D). In agreement with our previous single channel studies [6], whole cell currents recorded from HEK 293 cells stably expressing both the α- and β-subunits of the human BK channel were inhibited when the bath perfusate pO_2 was reduced from 150 to between 30 and 40 mm Hg. This inhibition was dependent upon $[Ca^{2+}]_i$ and hypoxia was maximally effective when $[Ca^{2+}]_i$ was 300 nM (data not shown). Thus, within 60 s of bath hypoxia, mean BK current density was reduced significantly from 138 ± 25 to 91 ± 23 pA/pF ($n = 4$, $P < 0.05$). Re-oxygenation rapidly reversed the inhibitory effect of hypoxia such that mean current density returned to 121 ± 23 pA/pA, a value not significantly different from control (Fig. 1E).

In order to investigate a potential role for γGT in the regulation of BK channel activity by hypoxia, we employed siRNA as a strategy by which to knock down γGT expression in HEK 293 cells stably expressing the α- and β-BK subunits. In order to assess transfection efficiency, siRNA species were labelled with Cy3. The punctate, red fluorescence seen in Figs. 2A and B demonstrates that the use of siPort Amine as a transfection vehicle is extremely efficient as a means of delivering siRNA into HEK 293 cells. Forty-eight hours following transfection with the siRNA, Cy3-labelling was observed in the majority of cells. Transfection with the control siRNA (scrambled GAPDH siRNA) altered neither the pattern nor the degree of γGT immunofluorescence (Fig. 2A). In stark contrast, lipofection with γGT siRNA (γGT siRNA 1 and γGT siRNA 2) resulted in almost complete suppression of γGT immunofluorescence (Fig. 2B), indicating that this procedure

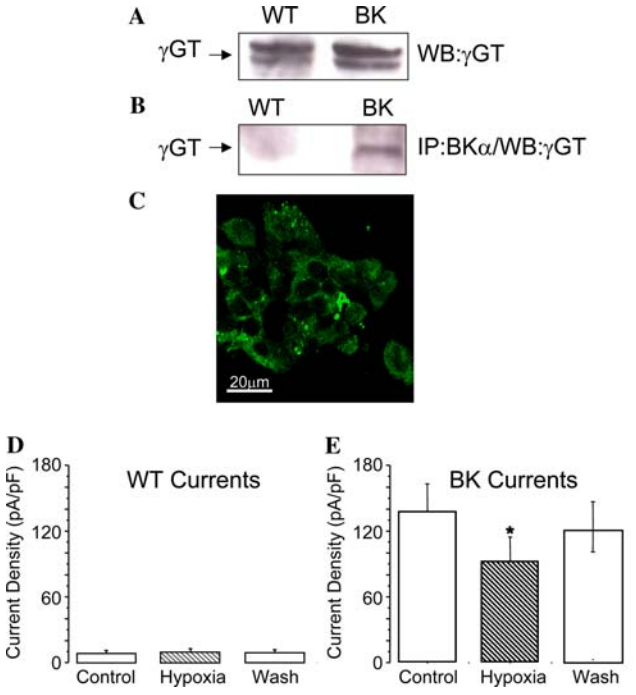

Fig. 1. Indentification and immunoprecipitation of γGT in oxygen-sensitive BK expressing HEK 293 cells. (A) Exemplar Western blot of cell lysates using and antibody raised against human γGT showing appropriately sized immunoreactive bands in both wild type (WT, left lane) and BK expressing (BK, right lane) HEK 293 cells. (B) Typical Western blot using the γGT antibody (WB: γGT) of membrane fractions following immunoprecipitation with an antibody raised against the α-subunit of human BK channels (IP: BKα) showing immunoreactivity at the appropriate size only in the βKα expressing cells (BK, right lane). (C) Representative confocal image (488 nm excitation) of BK expressing HEK 293 cells employing the γGT antibody and an FITC-labelled second layer antibody. (D) Mean (+SEM) current density bar chart of wild type HEK 293 cells in normoxia, hypoxia, and following wash-out into normoxia, as indicated below each bar ($n = 5$ cells). (E) Mean (+SEM) current density bar chart of BK expressing HEK 293 cells in normoxia, hypoxia, and following wash-out into normoxia ($n = 4$). Currents obtained for (D,E) were measured at + 60 mV in the whole-cell configuration with intracellular $[Ca^{2+}]_i$ buffered to 300 nM. * indicates significant difference from control ($P < 0.05$, Student's paired t test).

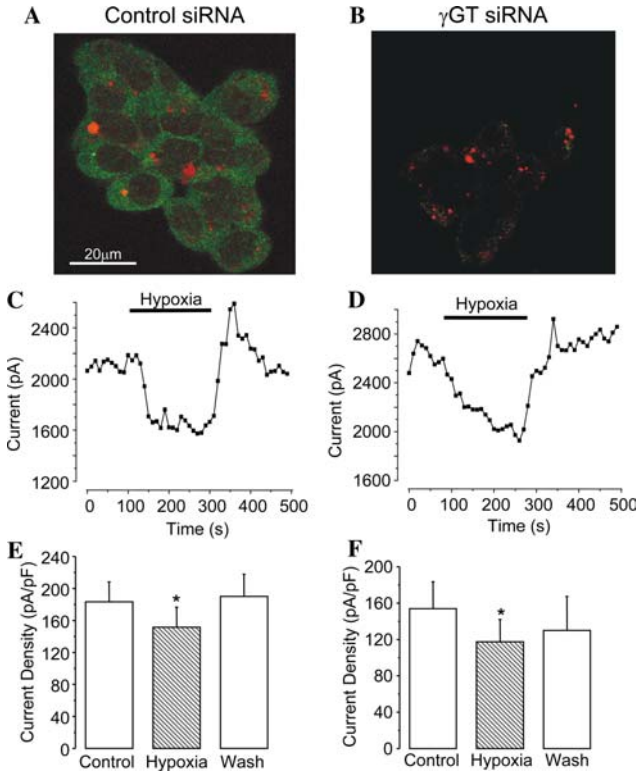

Fig. 2. Effect of transfection with siRNA on γGT protein levels and oxygen-sensitivity of BK currents. (A) Representative confocal image of γGT immunoreactivity (FITC-labelled second layer excited at 488 nm, green) and positive siRNA lipofection (Cy3-labelled siRNA excited at 543 nm, red) in exemplar BK expressing HEK293 cells 48 h post-transfection with scrambled GAPDH siRNA. (B) Exemplar confocal image of γGT immunoreactivity (green) and positive siRNA transfection (red) in exemplar BK expressing HEK293 cells 48 h post-transfection with γGT siRNA. (C) Representative current–time course to show the effect of hypoxia on whole-cell BK currents in cells 48 h of post-transfection with scrambled GAPDH siRNA during normoxia and hypoxia. Period of hypoxia indicated by horizontal line. (D) Typical current–time course to show the effect of hypoxia on whole-cell BK currents in cells 48 h of post-transfection with γGT siRNA during normoxia and hypoxia. Period of hypoxia indicated by horizontal line. (E) Mean (+SEM) whole cell current density bar chart of BK expressing HEK 293 cells 48 h post-lipofection with scrambled GAPDH siRNA in normoxia, hypoxia, and following wash-out into normoxia, as indicated ($n = 5$). (F) Mean (+SEM) whole cell current density bar chart of BK expressing HEK 293 cells 48 h post-transfection with γGT siRNA in normoxia, hypoxia, and following wash-out into normoxia, as indicated ($n = 5$). Currents obtained for (E,F) were measured at + 60 mV in the whole-cell configuration with intracellular $[Ca^{2+}]_i$ buffered to 300 nM. * indicates significant difference from control ($P < 0.05$, Student's paired t test).

produced HEK 293 cells in which γGT expression was essentially knocked out.

Employing a fluorescence microscope in the patch-clamp experiments, cells which were successfully transfected with Cy3-labelled siRNA could be positively identified. Single cells, which appeared red upon excitation at 453 nm, were patch-clamped using standard intracellular (300 nM $[Ca^{2+}]_i$) and extracellular solutions. Cells treated with scrambled GAPDH siRNA demonstrated robust inhibition when the bath solution was made hypoxic (Fig. 2C). Thus, mean, whole cell BK current density was significantly and reversibly reduced from 183 ± 25 to 151 ± 25 pA/pF (Fig. 2E; $n = 5$, $P < 0.05$). Importantly, treatment with γGT siRNA (which evokes complete knock-out of γGT protein) did not affect the hypoxic response (Fig. 2D). Mean current density was, again, reversibly and significantly reduced by hypoxia from 154 ± 30 to 117 ± 25 pA/pF (Fig. 2F; $n = 5$, $P < 0.05$). This 24% inhibition was not different from that observed in untreated cells (Fig. 1E) or cells treated with control siRNA (Fig. 2E).

Although γGT is clearly not involved in O_2-sensitivity of BK channels, the fact still remains that it is a closely associated protein. Such association would normally infer functional interaction and in order to address such

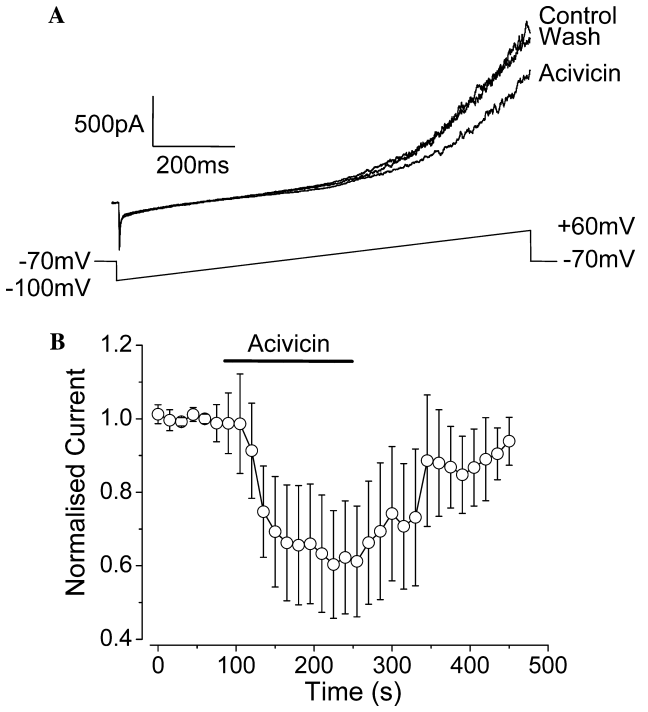

Fig. 3. Effect of 1 mM acivicin on whole-cell BK currents. (A) Typical currents from a representative HEK293 cell stably expressing BK channels evoked by the voltage ramp protocol before, during, and after the application of 1 mM acivicin. Currents were induced by a 1 s depolarisation from −100 to +60 mV (holding potential −70 mV, frequency 0.1 Hz), as shown. (B) Normalised, mean time–series plot of the effect of 1 mM acivicin on whole-cell maxi K current measured at +60 mV. The horizontal bar indicates the period of exposure to 1 mM acivicin. Each point represents the mean (±SEM) of 5 cells.

a proposal, we employed acivicin as a selective blocker of γGT activity. Addition of 1 mM acivicin to BK-expressing HEK 293 cells resulted in a significant and reversible reduction in mean current density from 105 ± 22 to 74 ± 23 pA/pF (Fig. 3; $n = 5$, $P < 0.01$). Thus, γGT activity is responsible for a degree of tonic BK channel activity when expressed in this recombinant system. The mechanism of such regulatory prowess is difficult to assess but redox activation by γGT-dependent production of superoxide and hydrogen peroxide seems consistent with observations made by other investigators of BK channel regulation (e.g., [16]).

In addition to catalysis of glutathione, γGT has also been shown to break down related compounds with a γ-glutamyl moiety, including low molecular weight *S*-nitrosothiols, such as nitrosoglutathione [17]. These compounds are released from haemoglobin in deoxygenated blood and when injected in the nucleus tractus solitarius of the rat brain, cause a reflex increase in ventilation rate [8]. This response was similar in magnitude to the ventilatory response of rats subjected to hypoxia. Furthermore, the normal hypoxic ventilatory response was much attenuated in γGT knock-out mice. However, although it is clear that *S*-nitrosothiols and γGT are of central importance in regulating whole-body responses to hypoxia, the cellular mechanisms underlying such observations are unknown. Since the hypoxic response of the carotid body and brain stem are likely linked to inhibition of K^+ channels coupled to the fact that BK channels are inhibited by acute hypoxia, it seemed reasonable to suggest that γGT might be involved in the BK channel modulation and the O_2 sensing process. Our immunoprecipitation data initially indicated this might be the case (Fig. 1B) since there is significant protein–protein interaction between BKα and γGT. Indeed, the use of a selective, non-competitive blocker of γGT activity, acivicin, showed very clearly that blockade of γGT resulted in BK channel inhibition of a comparable magnitude, and with a similar time course of action, to that observed with hypoxia (compare Figs. 2C and 3B). However, although γGT is involved in modulation of BK channels in normoxia, the siRNA data shown in Fig. 2 show clearly, and for the first time, that γGT is not involved in the inhibition of BK channels by hypoxia.

In conclusion, although γGT is a BK α-subunit protein partner whose turnover modulates BK channel activity, it is not involved in the signal transduction cascade which leads to BK channel inhibition during acute episodes of hypoxia.

Acknowledgments

The authors should like to thank the NIH, Wellcome Trust, and British Heart Foundation for financial support.

References

[1] C. Peers, Hypoxic suppression of K^+ currents in type-I carotid-body cells—selective effect on the Ca^{2+}-activated K^+ current, Neurosci. Lett. 119 (1990) 253–256.
[2] A.M. Riesco-Fagundo, M.T. Perez-Garcia, C. Gonzalez, J.R. Lopez-Lopez, O_2 modulates large-conductance Ca^{2+}-dependent K^+ channels of rat chemoreceptor cells by a membrane-restricted and CO-sensitive mechanism, Circ. Res. 89 (2001) 430–436.
[3] C. Jiang, G.G. Haddad, Oxygen deprivation inhibits a K^+ channel independently of cytosolic factors in rat central neurons, J. Physiol. 481 (1994) 15–26.
[4] C. Jiang, G.G. Haddad, A direct mechanism for sensing low oxygen levels by central neurons, Proc. Natl. Acad. Sci. USA 91 (1994) 7198–7201.
[5] C.N. Wyatt, C. Wright, D. Bee, C. Peers, O_2-sensitive K^+ currents in carotid-body chemoreceptor cells from normoxic and chronically hypoxic rats and their roles in hypoxic chemotransduction, Proc. Natl. Acad. Sci. USA 92 (1995) 295–299.
[6] A. Lewis, C. Peers, M.L.J. Ashford, P.J. Kemp, Hypoxia inhibits human recombinant maxi K^+ channels by a mechanism which is membrane delimited and Ca^{2+}-sensitive, J. Physiol. 540 (2002) 771–780.
[7] J. Lopez-Barneo, R. Pardal, P. Ortega-Saenz, Cellular mechanism of oxygen sensing, Ann. Rev. Physiol. 63 (2001) 259–287.

[8] A.J. Lipton, M.A. Johnson, T. Macdonald, M.W. Lieberman, D. Gozal, B. Gaston, *S*-nitrosothiols signal the ventilatory response to hypoxia, Nature 413 (2001) 171–174.
[9] M.E. Hartness, S.P. Brazier, C. Peers, A.N. Bateson, M.L. Ashford, P.J. Kemp, Post-transcriptional control of human maxiK potassium channel activity and acute oxygen sensitivity by chronic hypoxia, J. Biol. Chem. 278 (2003) 51422–51432.
[10] P.K. Ahring, D. Strobaek, P. Christophersen, S.P. Olesen, T.E. Johansen, Stable expression of the human large-conductance Ca^{2+}-activated K^+ channel alpha- and beta-subunits in HEK293 cells, FEBS Lett. 415 (1997) 67–70.
[11] M.H. Mojet, E. Mills, M.R. Duchen, Hypoxia-induced catecholamine secretion in isolated newborn rat adrenal chromaffin cells is mimicked by inhibition of mitochondrial respiration, J. Physiol. 504 (1997) 175–189.
[12] P.S. Robinson, C.F. Goochee, Kidney-specific enzyme expression by human kidney cell lines generated through oncogene transfection, J. Cell Physiol. 148 (1991) 54–59.
[13] G. Evjen, N.E. Huseby, Characterization of the carbohydrate moiety of human gamma-glutamyltransferases using lectin-blotting and glycosidase treatment, Clin. Chim. Acta 209 (1992) 27–34.
[14] K.E. Brown, M.T. Kinter, T.D. Oberley, M.L. Freeman, H.F. Frierson, L.A. Ridnour, Y. Tao, L.W. Oberley, D.R. Spitz, Enhanced gamma-glutamyl transpeptidase expression and selective loss of CuZn superoxide dismutase in hepatic iron overload, Free Radic. Biol. Med. 24 (1998) 545–555.
[15] A. Kugelman, H.A. Choy, R. Liu, M.M. Shi, E. Gozal, H.J. Forman, Gamma-Glutamyl transpeptidase is increased by oxidative stress in rat alveolar L2 epithelial cells, Am. J. Respir. Cell Mol. Biol. 11 (1994) 586–592.
[16] X.D. Tang, H. Daggett, M. Hanner, M.L. Garcia, O.B. McManus, N. Brot, H. Weissbach, S.H. Heinemann, T. Hoshi, Oxidative regulation of large conductance calcium-activated potassium channels, J. Gen. Physiol. 117 (2001) 253–274.
[17] N. Hogg, R.J. Singh, E. Konorev, J. Joseph, B. Kalyanaraman, *S*-Nitrosoglutathione as a substrate for gamma-glutamyl transpeptidase, Biochem. J. 323 (1997) 477–481.