Hypoxia inhibits human recombinant large conductance, Ca²⁺-activated K⁺ (maxi-K) channels by a mechanism which is membrane delimited and Ca²⁺ sensitive

A. Lewis *, C. Peers †, M. L. J. Ashford ‡ and P. J. Kemp *

** School of Biomedical Sciences and † Institute for Cardiovascular Research, Worsley Building, University of Leeds, Leeds LS2 9JT, UK
and ‡ Department of Pharmacology and Neuroscience, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, UK*

Large conductance, Ca²⁺-activated K⁺ (maxi-K) channel activity was recorded in excised, inside-out patches from HEK 293 cells stably co-expressing the α- and β-subunits of human brain maxi-K channels. At +50 mV, and in the presence of 300 nM Ca_i^{2+}, single channel activity was acutely and reversibly suppressed upon reducing P_{O_2} from 150 to > 40 mmHg by over 30 %. The hypoxia-evoked reduction in current was due predominantly to suppression in NP_o, although a minor component was attributable to reduced unitary conductance of 8–12 %. Hypoxia caused an approximate doubling of the time constant for activation but was without effect on deactivation. At lower levels of Ca_i^{2+} (30 and 100 nM), hypoxic inhibition did not reach significance. In contrast, 300 nM and 1 μM Ca_i^{2+} both sustained significant hypoxic suppression of activity over the entire activating voltage range. At these two Ca_i^{2+} levels, hypoxia evoked a positive shift in the activating voltage (by ~10 mV at 300 nM and ~25 mV at 1 μM). At saturating $[Ca^{2+}]_i$ (100 μM), hypoxic inhibition was absent. Distinguishing between hypoxia-evoked changes in voltage- and/or Ca_i^{2+}-sensitivity was achieved by evoking maximal channel activity using high depolarising potentials (up to +200 mV) in the presence of 300 nM or 100 μM Ca_i^{2+} or in its virtual absence (> 1 nM). Under these experimental conditions, hypoxia caused significant channel inhibition only in the presence of 300 nM Ca_i^{2+}. Thus, since regulation was observed in excised patches, maxi-K channel inhibition by hypoxia does not require soluble intracellular components and, mechanistically, is voltage independent and Ca_i^{2+} sensitive.

Acute modulation of ion channel activity by oxygen is crucial to the mechanisms which underlie chemosensing in carotid body (Peers, 1990), neuroepithelial body (Cutz & Jackson, 1999; Peers & Kemp, 2001) and vascular smooth muscle (Coppock *et al.* 2001) and is believed to play a significant role in modulation of excitability in several cellular components of the mammalian nervous system (Vergara *et al.* 1998). Crucial to these adaptive physiological and cellular responses is the acute inhibition of K⁺ channels by hypoxia. Although O₂-sensitive tissues express a wide variety of channel types, central to the cellular mechanism of O₂ sensing in many tissues is hypoxic suppression of large conductance Ca²⁺-activated K⁺ channels (maxi-K, BK_Ca or Slo channels). Thus, hypoxic inhibition of maxi-K channels has been demonstrated in carotid body (Peers *et al.* 1990; Riesco-Fagundo *et al.* 2001), pulmonary smooth muscle (Cornfield *et al.* 1996), chromaffin cells (Thompson & Nurse, 1998) and central neurones (Liu *et al.* 1999). Although their contribution to carotid body, chromaffin cell and central neuronal function is well supported, some

controversy still surrounds their involvement in pulmonary vasoconstriction (Coppock *et al.* 2001). Thus, there is good evidence for both delayed rectifier (Tristani-Firouzi *et al.* 1996) and tandem P domain K⁺ channels in the response (Gurney *et al.* 2002). The later observation is fully supported by our recent report of O₂ sensitivity of the tandem P domain channel, hTASK1, in a recombinant mammalian system similar to that employed in the present study (Lewis *et al.* 2001).

Although it has been suggested that the mechanism of hypoxic regulation of K⁺ channels in general, and maxi-K channels in particular, may involve the O₂-dependent modulation of cellular redox potential, the nature of the sensor is still unclear and the mechanism whereby decreased P_{O_2} evokes channel inhibition in different tissues remains controversial. Indeed, there are varying reports which have suggested involvement of either cytosolic factors (e.g. Jiang & Haddad, 1994; Wyatt & Peers, 1995) or more direct channel modulation (e.g. Liu *et al.* 1999; Riesco-Fagundo *et al.* 2001). In favour of potential regulation by cellular

redox state are recent data demonstrating activation by oxidising agents in a recombinant cellular system (Tang *et al.* 2001) and inhibition by reduced glutathione in rat hippocampal neurones (Soh *et al.* 2001) of these channels. However, to date, direct demonstration of hypoxia-induced inhibition of human recombinant maxi-K channels has been lacking.

In the present study, we have employed human embryonic kidney (HEK 293) cells stably expressing both the α- and β-subunits of human maxi-K and have examined the O_2 sensitivity of these recombinant K^+ channels at the single channel level. Our findings demonstrate directly that human brain $\alpha\beta$-maxi-K is an O_2-sensitive K^+ channel and support strongly the notion that hypoxic inhibition of this channel occurs primarily via a reduction in Ca^{2+}-sensitivity with an additional, minor reduction in unitary conductance.

METHODS

Cell culture

HEK 293 cells which express human brain $\alpha\beta$-maxi-K channels (Ahring *et al.* 1997) 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 (all purchased from Gibco BRL, Paisley, Strathclyde, UK) in a humidified incubator gassed with 5 % CO_2–95 % air. Cells were passaged every 7 days in a ratio of 1:25 using Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (Gibco BRL, Paisley, Strathclyde, UK). The co-expressed α- and β-subunits were KCNMA1 (Genbank accession number U11717) and KCNMB1 (Genbank accession number U42600), respectively.

Patch-clamp recording

Cells were grown for 24 h on glass coverslips before being transferred to a continuously perfused (5 ml min^{-1}) recording chamber (volume *ca* 200 μl) mounted on the stage of an inverted microscope. For inside-out, excised patch clamp recordings, the standard perfusate was composed of (mM): 10 NaCl, 117 KCl, 2 $MgCl_2$, 11 Hepes, (pH 7.2) with Ca^{2+} buffered to 30 nM, 100 nM,
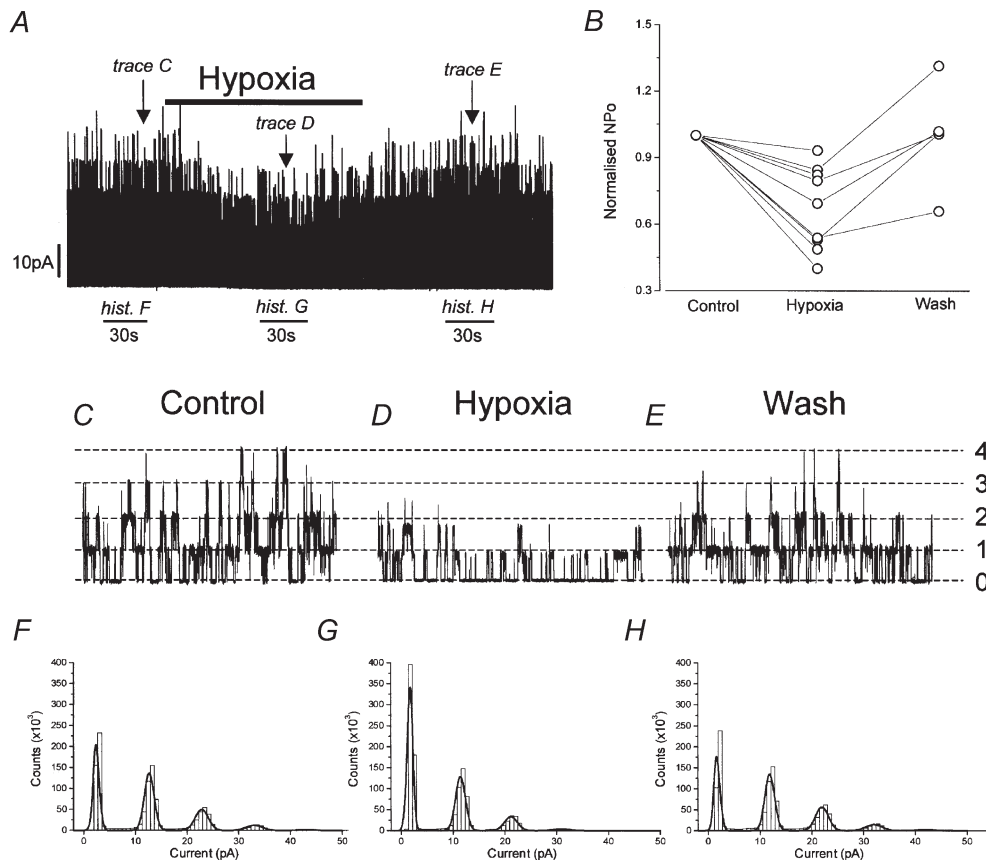

Figure 1. Hypoxic suppression of single channel activity

A, exemplar continuous current recording from an inside-out, excised membrane patch bathed in physiological solutions with $[Ca^{2+}]_i$ buffered to 300 nM and held at +50 mV. During the time indicated by the horizontal bar above the trace, perfusate P_{O_2} was reduced from 150 to 30–40 mmHg. *B*, normalised NP_o values from nine separate cells under the conditions described in *A* during normoxia (control), hypoxia and following re-establishment of normoxia (wash). *C, D* and *E,* single channel currents on an extended time-base (200 ms per trace) from the recording shown in *A* at the arrows in normoxia (*C*), hypoxia (*D*) and following re-introduction of normoxic perfusate (*E*). *F, G* and *H,* all-points histograms generated from 30 s segments (shown by horizontal bars below the recording of the trace shown in *A*) in normoxia (*F*), hypoxia (*G*) and following re-introduction of normoxic perfusate (*H*).

300 nm, 1 μm and 100 μm using EGTA and $CaCl_2$ in appropriate ratios. The Na^+-rich pipette solution was composed of (mm): 135 NaCl, 5 KCl, 1.2 $MgCl_2$, 5 Hepes, 2.5 $CaCl_2$, 10 D-glucose (pH 7.4). The K^+-rich pipette solution was composed of (mm): 10 NaCl, 117 KCl, 2 $MgCl_2$, 2.0 $CaCl_2$ (pH 7.2). When filled with these solutions, pipettes were of resistance 7–14 MΩ. Unless otherwise stated, all experiments were conducted in asymmetrical solutions. Hypoxic solutions were bubbled with N_2 (g) for at least 30 min prior to perfusion of cells, which produced no shift in pH. P_{O_2} was measured (at the cell) using a polarised carbon fibre electrode (Mojet *et al.* 1997) and found to be in the range 30–40 mmHg. Normoxic solutions were equilibrated with room air. All K^+ currents were recorded at a bath temperature of 22 $\pm$ 0.5 °C. Current recordings were made using an Axopatch 200A amplifier and Digidata 1320 A/D interface (Axon Instruments, Foster City, CA, USA). To evoke macro-patch K^+ currents, one of four protocols was employed. (1) *Standard ramp,* −70 mV holding potential, voltage ramps from −100 to +60 mV (2000 ms, 0.1 Hz). (2) *Extended ramp,* −70 mV holding potential, voltage ramps from −100 to +200 mV (2000 ms, 0.1 Hz). (3) *Activating step,* −70 mV holding potential, voltage steps from holding to between 0 and +50 mV in 10 mV increments (100 ms, 0.1 Hz). (4) *Deactivating step,* −70 mV holding potential, voltage step from holding to 130 mV (50 ms) followed by steps in the range −70 mV to −130 mV in 10 mV increments (1000 ms, 0.1 Hz)

All patches from stably expressing cells contained at least six maxi-K channels. Wild-type HEK 293 cells do not express maxi-K channels, see Fig. 4*E*, inset. Mean wild-type currents, recorded from inside-out patches with $[Ca^{2+}]_i$ buffered to 100 μm, are shown in Fig. 4*E*.

Data handling
Data analyses were performed using the pCLAMP 8.0 suite of software. The product of channel number and open state probability (NP_o) at any voltage was calculated from 30 s sections of current recording using the following equation, where I is the mean current and i is the single channel amplitude at any voltage (determined from all-points histograms):

$$I = NP_o\,i.$$

NP_o values were normalised to the values during the normoxic perfusion period (control) and NP_o data are reported as means $\pm$ s.e.m. values. Statistical comparisons were made using Student's paired or unpaired *t* tests, as appropriate. All-points histograms were generated using Fetchan 6.03 and single channel amplitudes were calculated using PStat 6.03 by iterative Gaussian fitting protocols. Means ($\pm$s.e.m.) and subtracted current–voltage relationships were generated using Microsoft Excel and are normalised to the current amplitude obtained at +50 mV in the presence of 300 nm $[Ca^{2+}]_i$. Activation/deactivation kinetics were determined from iterative fitting using a standard exponential routine (ClampFit 8.0.2). Concentration–response curves for Ca^{2+} were fitted to the Hill equation.

RESULTS

HEK 293 cells stably expressed recombinant human maxi-K channels. Figure 1 shows exemplar currents recorded at +50 mV in the presence of 300 nm $[Ca^{2+}]_i$ from an inside-out, excised patch containing at least six maxi-K channels (although transitions to the fifth and sixth levels represented, in this example, merely 0.44 % of the total observable events and so do not appear in either the expanded traces or the all-points histograms). The continuous current recording shown in Fig. 1*A* clearly demonstrates that hypoxia (30–40 mmHg) evoked a dramatic and reversible

Figure 2. Hypoxic modulation of single channel conductance

A, current–voltage relationships derived from single channels recorded in normoxia (control, filled symbols) and hypoxia (open symbols) with either Na^+-rich (circles, *n* = 6) or K^+-rich pipette solutions (squares, *n* = 4) with the intracellular membrane leaflet bathed in K^+-rich solution containing 300 nm $[Ca^{2+}]_i$. *B*, exemplar all-points histogram taken from a patch which was bathed in the asymmetrical solution conditions described above and held at +50 mV in normoxia (control, fine line) and hypoxia (bold line). $-V_p$ in this and subsequent figures refers to the potential with respect to inner membrane leaflet.
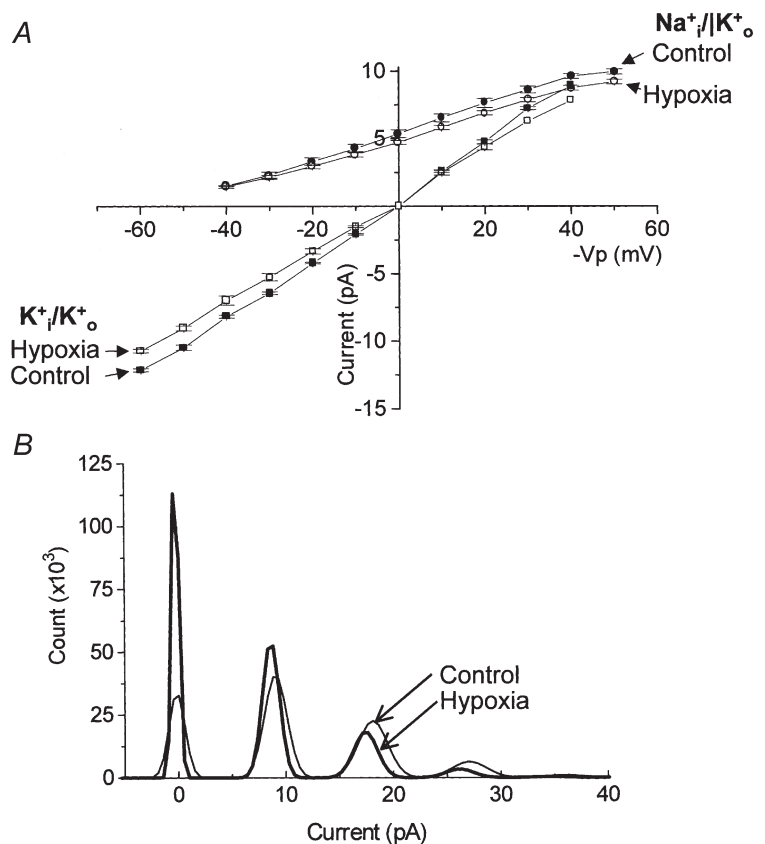
reduction in patch activity. All patches demonstrated similar hypoxic channel inhibition (Fig. 1*B*); mean normalised NP_o was reduced significantly ($P < 0.01$) by 32.0 ± 6.5 % in hypoxia ($n = 9$). Those patches which remained intact for sufficient time demonstrated recovery when returned to normoxia. Traces shown in Fig. 1*C*, *D* and *E* (200 ms, arrows on Fig. 1*A*) and the all-points histograms (derived from 30 s sections of trace, horizontal bars on Fig. 1*A*) exemplify the reduction in channel activity evoked by hypoxia. These data demonstrate clearly that hypoxic inhibition of maxi-K currents does not have an absolute requirement for soluble cytosolic factors.

In addition to the hypoxia-evoked reduction in patch current, inspection of Fig. 1 suggested that reduction in P_{O_2} also decreased single channel unitary current (see Fig. 1*C* *versus* Fig. 1*D*). This possibility was investigated and the data are presented in Fig. 2. In the presence of symmetrical K^+-rich solutions (Ca_i^{2+} 300 nM), hypoxia significantly ($P < 0.05$, $n = 4$) reduced single channel conductance by ~12 % from 215.4 ± 2.0 to 188.5 ± 10 pS (Fig. 2*A*). Similarly, in asymmetrical solutions, single channel currents at all activating test potentials (from -40 to $+60$ mV in 300 nM $[Ca^{2+}]_i$) were also reduced by hypoxia (Fig. 2*A*). These differences were significant ($P < 0.05$, $n = 5$) at test potentials positive to 0 mV. Single channel conductance

(measured between 0 and +40 mV) was mildly decreased by hypoxia from 106.0 ± 3.9 to 97.2 ± 3.4 pS ($n = 5$); a reduction of ~8 %. This observation of reduced unitary conductance is highlighted in Fig. 2*B* where the channel amplitudes, calculated from the all-points histograms of an exemplar patch in asymmetrical solutions and held at +50 mV, are seen to be reduced at all open levels.

Figure 3 shows the effects of hypoxia on channel activation and deactivation kinetics. In order to study activation/ deactivation at test potentials which were on the rising phase of the current–voltage relationship (i.e. before the apparent inactivation seen at high potentials; see Fig. 5), we employed inside-out patches in 1 μM Ca_i^{2+}. All activating and deactivating currents were best described by a single exponential function. Under these conditions, hypoxia decreased the rate of activation at all test potentials (Fig. 3*A* and *B*) in addition to reducing the steady-state current amplitude (by between 3.3 and 35.9 % at +50 mV, consistent with the reduced NP_o values shown in Fig. 1*B*). Proportionally, this decreased rate of activation was most pronounced at +50 mV. At +50 mV, the mean time constant was significantly ($P < 0.02$, $n = 10$) increased from 1.34 ± 0.15 ms (control) to 2.82 ± 0.64 ms (hypoxia). In contrast, deactivation was unaffected by hypoxia at all test potentials (Fig. 3*C* and *D*).
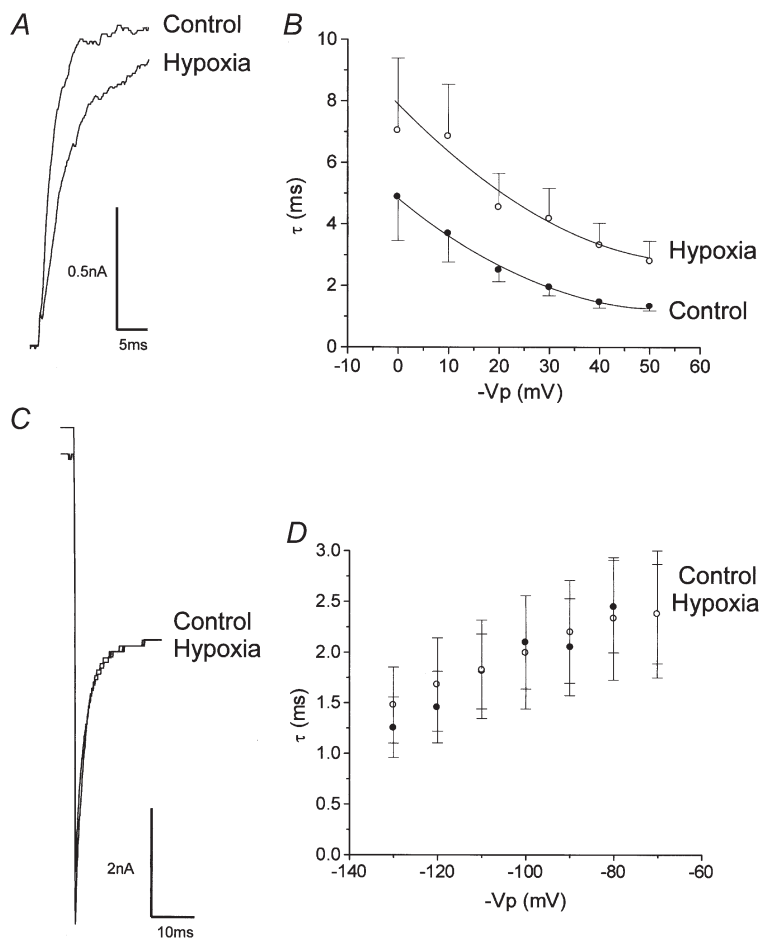

Figure 3. Activation and deactivation channel kinetics

A, exemplar activation current (from holding potential to +50 mV) in the presence (hypoxia) and absence (control) of hypoxia. *B*, voltage dependence of mean activation time constants in the presence (hypoxia, ○) and absence (control, ●) of hypoxia ($n = 10$). *C*, exemplar deactivating tail current (stepped from 130 mV to −130 mV) in the presence (hypoxia) and absence (control) of hypoxia. *D*, voltage dependence of mean deactivation time constants in the presence (hypoxia, ○) and absence (control, ●) of hypoxia ($n = 5$). All recordings were performed in asymmetrical solutions with 1 μM Ca_i^{2+}.

Figure 4 illustrates the dependence of hypoxic channel inhibition on $[Ca^{2+}]_i$. At 30 nM (Fig. 4A) and 100 nM $[Ca^{2+}]_i$ (Fig. 4B), hypoxia had no significant effect on channel activity. Normalised currents (see Methods, above), measured at +50 mV in 30 nM $[Ca^{2+}]_i$, were 0.059 ± 0.008 ($n = 4$) in control and 0.031 ± 0.004 ($n = 5$) in hypoxia ($P = 0.6$) whilst in 100 nM $[Ca^{2+}]_i$ they were 0.090 ± 0.011 ($n = 4$) in control and 0.084 ± 0.016 ($n = 5$) in hypoxia ($P = 0.9$). However, as $[Ca^{2+}]_i$ was raised to 300 nM (Fig. 4C) and 1 μM (Fig. 4D), channel inhibition became apparent at all activating voltages. Normalised currents measured at +50 mV in 300 nM $[Ca^{2+}]_i$ were 0.537 ± 0.024 ($n = 4$) in normoxia and 0.322 ± 0.019 ($n = 5$) in hypoxia ($P < 0.0001$) whilst in 1 μM $[Ca^{2+}]_i$ they were 3.88 ± 0.25 ($n = 4$) in normoxia and 2.13 ± 0.43 ($n = 5$) in hypoxia

($P < 0.0001$). Thus, in normoxia, increasing $[Ca^{2+}]_i$ caused the expected decrease in voltage-activation threshold but, importantly, this threshold was shifted to more positive potentials by reducing bath P_{O_2} (by ~10 mV at 300 nM and ~25 mV at 1 μM). Furthermore, increasing $[Ca^{2+}]_i$ to 100 μM resulted in a complete saturation of the hypoxic inhibition (Fig. 4E); mean normalised currents in normoxia and hypoxia were 6.91 ± 1.85 and 6.82 ± 1.73, respectively ($n = 4$). The Ca^{2+} dependence of hypoxic inhibition is quantified in Fig. 4F, which shows the normalised currents in normoxia and hypoxia plotted against $[Ca^{2+}]_i$; calculated EC_{50} values (employing the Hill equation) were 840 ± 15 nM in hypoxia *versus* 1805 ± 112 nM in hypoxia.
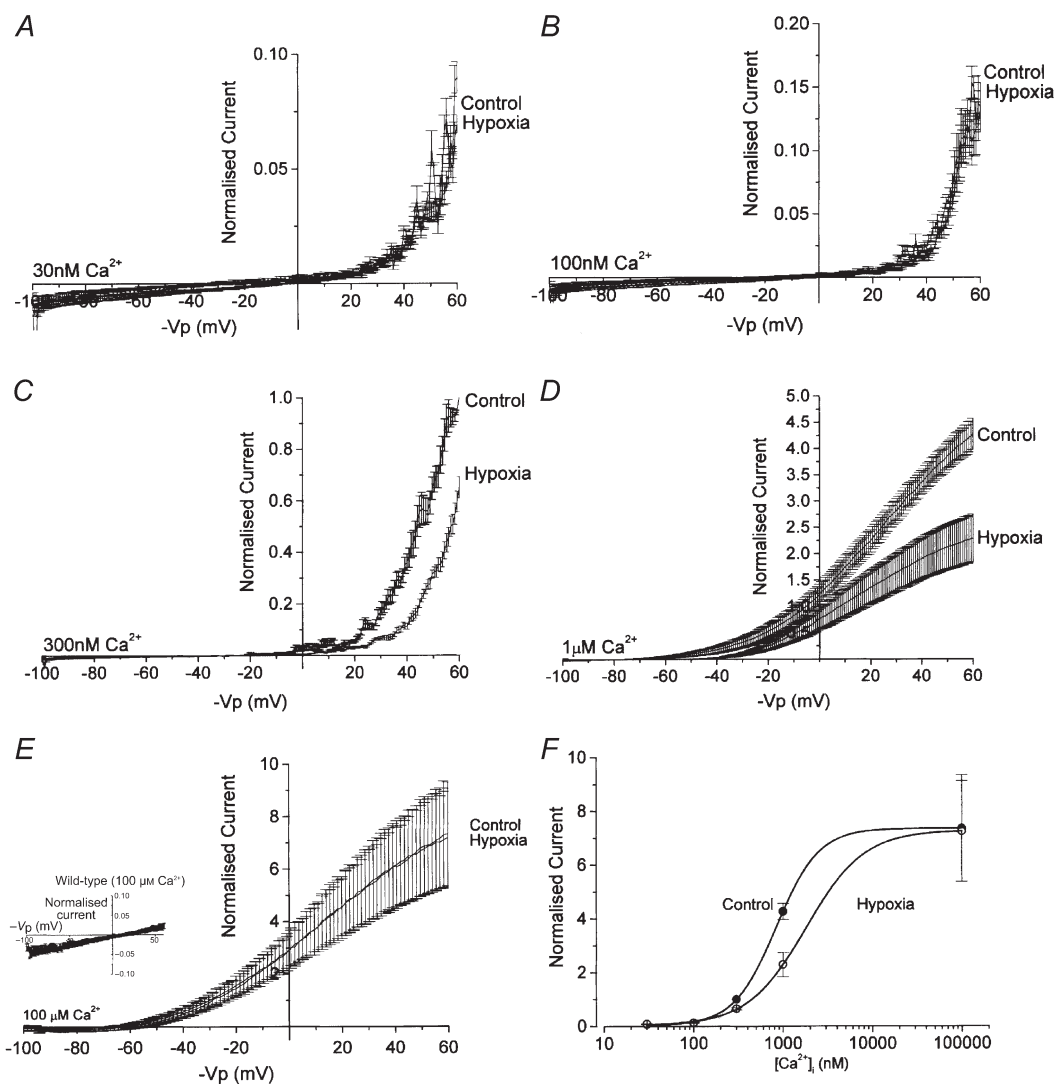

Figure 4. Ca^{2+}_i and voltage dependence of hypoxic channel inhibition

Mean ($\pm$ S.E.M) normalised, standard ramp-evoked currents in normoxia (control) and hypoxia at 30 nM(A), 100 nM (B), 300 nM (C), 1 μM (D) and 100 μM (E) $[Ca^{2+}]_i$. F, the $[Ca^{2+}]_i$ of hypoxia-evoked channel inhibition by plotting mean normalised currents in normoxia and hypoxia against $[Ca^{2+}]_i$. Also shown in E, inset, are mean ramp-evoked currents in wild-type HEK 293 cells in normoxia and at 100 μM $[Ca^{2+}]_i$; $n = 7$. Note the scale difference in the inset. F, dependence of currents (measured at +50 mV) on $[Ca^{2+}]_i$ in normoxia (●) and hypoxia (○), as indicated.

The data in Fig. 4 suggest that hypoxia evokes channel inhibition by either: (1) increasing the voltage threshold for channel activation; (2) decreasing Ca^{2+} sensitivity of the channels; or (3) a combination of the two. To distinguish between these possibilities, we employed a ramp protocol which was able to activate channels at different levels of $[Ca^{2+}]_i$. Interestingly, evoked currents displayed an apparent 'inactivation' at larger potentials of *ca* +70 mV to +120 mV. Since this effect was more striking with increasing Ca^{2+} levels, and was absent in Ca^{2+}-'free' solutions (Fig. 5B), we attribute this effect to Ca^{2+} blockade of maxi-K channels, although more detailed studies are required to resolve this issue. This protocol was able to evoke similar degrees of channel activity, albeit at different voltages, regardless of $[Ca^{2+}]_i$ (up to 100 μM). When $[Ca^{2+}]_i$ was buffered to less than 1 nM (zero added Ca^{2+} plus 11 mM EGTA), the large depolarising ramp was able to evoke activity which was not

significantly different from that in the presence of 300 nM or 1 μM $[Ca^{2+}]_i$ (Fig. 5B, control). Most importantly, in the presence of 300 nM $[Ca^{2+}]_i$, hypoxia caused a significant decrease in current at all activating potentials (Fig. 5B, hypoxia) whereas hypoxic current suppression was completely absent when $[Ca^{2+}]_i$ was buffered to below 1 nM; subtracted, hypoxia-sensitive currents in the 'absence' and 'presence' of $[Ca^{2+}]_i$ are plotted in Fig. 5C. These data suggest strongly that hypoxia inhibits maxi-K channels by reducing their Ca^{2+} sensitivity, and to confirm this possibility we employed a saturating concentration of Ca^{2+}_i in the extended ramp protocol. At 100 μM $[Ca^{2+}]_i$, hypoxia was ineffective at all voltages of the extended ramp protocol (Fig. 5D). This is shown clearly in Fig. 5E which plots the hypoxia-sensitive currents in an exemplar patch when $[Ca^{2+}]_i$ was buffered to either 300 nM or 100 μM.
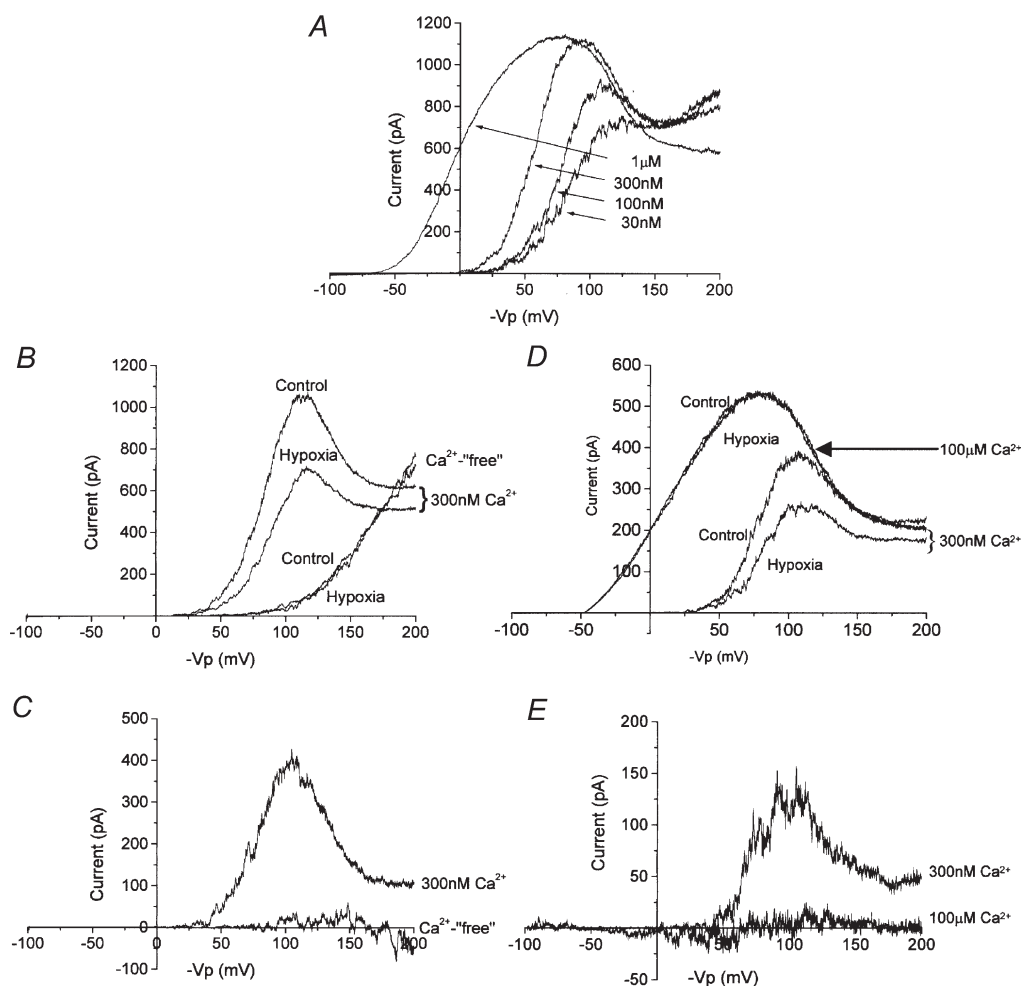

Figure 5. Dependence of hypoxic channel inhibition on $[Ca^{2+}]_i$

A, exemplar currents evoked using the extended-ramp protocol in the presence of varying $[Ca^{2+}]_i$ as indicated. *B*, effect of hypoxia in the extended voltage range in the presence (300 nM) and absence (Ca^{2+}-free) of $[Ca^{2+}]_i$. *C*, hypoxia-sensitive currents in the presence (300 nM) and absence (Ca^{2+}-free) $[Ca^{2+}]_i$. Data for *A–C* are representative of four such recordings. *D*, effect of hypoxia in the extended voltage range in the presence of 300 nM and 100 μM $[Ca^{2+}]_i$ as indicated. *E*, hypoxia-sensitive currents in the presence of 300 nM and 100 μM $[Ca^{2+}]_i$ as indicated. Data for *D* and *E* are representative of four further recordings.

DISCUSSION

Through the use of a recombinant expression system, our single channel analyses with inside-out membrane patches have demonstrated that hypoxia acutely inhibits maxi-K channels of known molecular identity. Furthermore, this work has revealed important new findings concerning the mechanism of maxi-K channel inhibition. Thus, hypoxia decreases channel sensitivity to Ca_i^{2+}, slows activation and reduces unitary conductance. Furthermore, hypoxic inhibition occurs independently of soluble cytosolic factors.

Maxi-K channels are widely distributed and, in excitable tissues are involved in a variety of physiological mechanisms including, controversially, control of vascular tone (Brenner *et al.* 2000; Pluger *et al.* 2000). Their involvement in neuronal excitability (Vergara *et al.* 1998) and chemo-sensing (Peers, 1997; Riesco-Fagundo *et al.* 2001) is less controversial and this channel type is clearly important to carotid body and central neuronal function (Lopez-Barneo *et al.* 2001). The α- and β-subunits examined in the present study were first cloned from human brain but are essentially ubiquitously expressed (Tseng-Crank *et al.* 1994, 1996). In chemosensory tissues, episodes of acute hypoxia result in modulation of ventilation and optimisation of ventilation–perfusion matching via the concerted actions of the carotid bodies, the neuroepithelial bodies of the lung and the pulmonary vasculature (Cutz & Jackson, 1999; Peers & Kemp, 2001; Lopez-Barneo *et al.* 2001; Coppock *et al.* 2001). Evidence supports the idea that hypoxic inhibition of maxi-K channels is central to some of these effects. There is clear evidence for divergent mechanisms underlying the initial steps in O_2 signal transduction in different chemosensory tissues (for recent review see Peers & Kemp, 2001). Thus, even though carotid body (Wyatt *et al.* 1995; Buckler *et al.* 2000), pulmonary vasculature (Weir & Archer, 1995) and airway O_2 chemosensory pathways (Wang *et al.* 1996; Fu *et al.* 2000; O'Kelly *et al.* 2000, 2001) contain different O_2 sensors, they each express specific K^+ channels which are inhibited by acute hypoxia. In the neuroepithelial body of the lung, and its immortal cellular counterpart the H146 cell line, there is accumulating evidence to suggest that central to cellular O_2 sensing is hypoxic inhibition of members of the K_{2P} class of K^+ channels (O'Kelly *et al.* 1999; Hartness *et al.* 2001), although the contribution of voltage-activated K^+ channels to the responses is still a matter of some debate (Wang *et al.* 1996). Similarly, in pulmonary smooth muscle, there is clear evidence for the involvement of maxi-K and other voltage-activated K^+ channels (Post *et al.* 1992; McCulloch *et al.* 1999) and emerging evidence to suggest a role for K_{2P} channels (Gurney *et al.* 2002). In the carotid body, there is also some controversy as to the identity of the O_2-sensitive channel, with both a K_{2P} channel (Buckler *et al.* 2000) and a maxi-K channel (Peers, 1990; Wyatt & Peers, 1995) suggested as potential targets for O_2 (Peers, 1990; Wyatt &

Peers, 1995; Pardal *et al.* 2000; Lopez-Barneo *et al.* 2001). However, the notion that there is large contribution to the physiological response in carotid body from maxi-K channel inhibition has recently been reinforced by the observation that glomus cells cultured in clusters, and slices of intact carotid body, release catacholamines both in hypoxia and following application of iberiotoxin (Jackson & Nurse, 1998; Pardal *et al.* 2000).

Central neurones also respond rapidly to reduced P_{O_2}, and such responses include inhibition of a variety of different K^+ channels. However, none of these have been identified at the molecular level, and different mechanisms have been proposed to account for hypoxic inhibition. For example iberiotoxin and charybdotoxin-insensitive maxi-K channels are inhibited by hypoxia via a mechanism which requires cytosolic factors (Liu *et al.* 1999). In contrast, other maxi-K-like channels, which are also regulated by ATP levels, are inhibited by hypoxia in a membrane-delimited manner (Jiang & Haddad, 1994).

A number of studies in both native tissues and recombinant systems have demonstrated that maxi-K channels are modulated by oxidising and reducing agents (Liu *et al.* 1999; Soh *et al.* 2001; Tang *et al.* 2001). This has led to the proposal that hypoxic inhibition is through reduction of the channel protein. Thus, where redox modulators evoke regulation of recombinant maxi-K channels in a manner which is consistent with this proposal (i.e. activation by oxidising agents and inhibition by reducing agents), there are clear differences in the mechanism of modulation, suggesting that these direct chemical interventions cannot explain the effect of environmental hypoxia which we report here. For example recombinant maxi-K channels are activated by specific oxidation of methionine residues with chloramine T (Tang *et al.* 2001). Such an increase in channel activity, which is observed in the absence of Ca^{2+}, is abrogated by channel blockade with tetraethyl-ammonium and accompanied by a shift in the voltage dependence and slowed deactivation without a change in unitary conductance. Reducing agents evoke the opposite effects and it seems likely, therefore, that the principal mechanism of regulation by such chemical interventions is covalent modification of a methionine in the channel pore (Tang *et al.* 2001). These effects are in marked contrast to the decreased unitary conductance, decreased activation (in the absence of modulation of deactivation) and Ca^{2+} dependence of hypoxic inhibition which we report.

That the use of such agents in attempting to define the mechanism of hypoxic inhibition is not entirely appropriate has been reinforced by observations of native maxi-K channels. Thus, neocortical neurones are activated by the reducing agents dithiothreitol and glutathione (Liu *et al.* 1999), whilst glutathione promotes maxi-K activity in rat hippocampal neurones (Soh *et al.* 2001). This issue is clouded by the lack of molecular identification of the

channel under investigation (indeed numerous splice variants of the maxi-K α-subunit have been identified, which may co-express with various β-subunits (Toro *et al.* 1998)), together with clearly different requirements for cytosolic factors and probable cell-specific responses. Indeed, a recent study of native maxi-K channels expressed in rat carotid body has shown membrane-restricted hypoxic inhibition of channel activity. However, this response was only investigated at $[Ca^{2+}]_i$ concentrations exceeding those seen physiologically and the molecular identity of the subunits constituting the functional channel was unknown (Riesco-Fagundo *et al.* 2001). Our data demonstrate that a brain-type maxi-K channel complex is sensitive to hypoxia at between 100 and 300 nM $[Ca^{2+}]_i$. In living cells, $[Ca^{2+}]_i$ is close to 100 nM which suggests that hypoxic inhibition of this channel type may be of physiological significance; further study at levels bracketed by those employed here should shed further light on such issues. Absolute $[Ca^{2+}]_i$ notwithstanding, our recombinant study significantly extends earlier studies in native tissues in that it employed clearly defined and identified channels in a membrane-delimited preparation. In so doing, it clarifies some of the issues described above and suggests that different isoforms of maxi-K channels may behave differently. Therefore, cell-specific expression of these isoforms will probably determine the response of an individual cell to hypoxia.

Since modulation by redox reagents appears to be inconsistent with the channel regulation which we report herein, it seems likely that changes in environmental P_{O_2} proceeds via a different mechanism. In support of this idea are data from native neocortex which show that hypoxia inhibits activity by decreasing Ca^{2+} sensitivity (Liu *et al.* 1999). However, in contrast to our findings, hypoxic inhibition of neocortical maxi-K channels is dependent upon cytosolic components. One explanation for this difference (which supports the notion of cell-specific channel expression detailed above) appears to be that the native neuronal channels are a different class (since they are charybdotoxin/iberiotoxin insensitive) from the recombinant channels which were used in the present study. Thus, the iberiotoxin-sensitive maxi-K channel itself, or a tightly associated membrane-delimited protein(s), is directly sensitive to the inhibitory actions of hypoxia. Whether the presence of an intact intracellular milieu will alter the mechanism and/or degree of hypoxic inhibition will be important to investigate before the full cell-specific physiological consequences of decreased P_{O_2} can be determined. However, this study shows that the presence of $\alpha\beta$-maxi-K channel protein within a biological membrane is sufficient for a hypoxic response. This notwithstanding, our data clearly demonstrate that hypoxic inhibition involves reduced Ca^{2+} sensitivity. Although we have no direct evidence for the mechanism of

this hypoxic modulation, we speculate that there are a number of possibilities worthy of consideration. Based on the observations that mitochondria-derived reactive oxygen species (ROS) have been implicated in hypoxic modulation in a number of tissues (Chandel & Schumacker, 2000; Leach *et al.* 2001), together with the possibility that mitochondria can be contained within excised patches (Levitan & Kramer, 1990) it is possible that local production of ROS may be responsible for this effect. Another possibility, which currently remains speculative yet attractive (since the probability of mitochondria being retained in excised-membrane patches is not consistent with the highly reproducible effects of hypoxia as shown here), is that hypoxia alters channel activity by modulating the direct interaction of an, as yet, unidentified sensor with the channel protein. Additionally, it may simply be that the amino acid composition of the subunit *per se* is the site of O_2 interaction (Lopez-Barneo, 1996) and this could be addressed by examining the actions of hypoxia on pure α-subunit protein in an artificial lipid bilayer system. It is also plausible that the hypoxic stimulus results in functional dissociation of the β-subunit from the α-subunit, resulting in slowing of activation and reduced Ca^{2+} sensitivity (Toro *et al.* 1998). Further alternatives include activation of protein kinases embedded in the isolated patch (Schubert & Nelson, 2001) or hypoxia-driven modulation of cytoskeletal structure (Friedman *et al.* 1998) which has been demonstrated previously to modify channel function (Mironov & Richter, 1999).

The consequences of inhibiting maxi-K channels via reduced Ca^{2+} sensitivity are probably both physiologically and pathologically important. In central neurones, the known pathological effect of prolonged hypoxia, which can lead to focal cell death during cerebral ischaemia, may be augmented by prevention of channel reactivation due to rises of $[Ca^{2+}]_i$. Physiologically, prevention of reactivation may contribute to the persistent excitation of the carotid body by hypoxia; initially, hypoxia inhibits maxi-K channels, thereby causing membrane depolarisation and voltage-gated Ca^{2+} entry. Without a decrease in the Ca^{2+} sensitivity of maxi-K channels this would lead to reactivation of these channels and consequently a transient hypoxic response in the tissue, but this has not been observed (e.g. Landauer *et al.* 1995).

REFERENCES

AHRING, P. K., STROBAEK, D., CHRISTOPHERSEN, P., OLESON, S. P. & JOHANSEN, T. E. (1997). Stable expression of the human large-conductance Ca^{2+}-activated K^+ channel alpha- and beta-subunits in HEK293 cells. *FEBS Letters* **415**, 67–70.

BRENNER, R., PEREZ, G. J., BONEV, A. D., ECKMAN, D. M., KOSEK, J. C., WILER, S. W., PATTERSON, A. J., NELSON, M. T. & ALDRICH, R. W. (2000). Vasoregulation by the β_1 subunit of the calcium-activated potassium channel. *Nature* **407**, 870–876.

Buckler, K. J., Williams, B. A. & Honore, E. (2000). An oxygen-, acid- and anaesthetic-sensitive TASK-like background potassium channel in rat arterial chemoreceptor cells. *Journal of Physiology* **525**, 135–142.

Chandel, N. S. & Schumacker, P. T. (2000). Cellular oxygen sensing by mitochondria: old questions, new insight. *Journal of Applied Physiology* **88**, 1880–1889.

Coppock, E. A., Martens, J. R. & Tamkun, M. M. (2001). Molecular basis of hypoxia-induced pulmonary vasoconstriction: role of voltage-gated K^+ channels. *American Journal of Physiology – Lung Cellular and Molecular Physiology* **281**, L1–8.

Cornfield, D. N., Reeve, H. L., Tolarova, S., Weir, E. K. & Archer, S. (1996). Oxygen causes fetal pulmonary vasodilation through activation of a calcium-dependent potassium channel. *Proceedings of the National Academy of Sciences of the USA* **93**, 8089–8094.

Cutz, E. & Jackson, A. (1999). Neuroepithelial bodies as airway oxygen sensors. *Respiration Physiology* **115**, 201–214.

Friedman, J. E., Chow, E. J. & Haddad, G. G. (1998). State of actin filaments is changed by anoxia in cultured rat neocortical neurons. *Neuroscience* **82**, 421–427.

Fu, X. W., Wang, D., Nurse, C., Dinauer, M. C. & Cutz, E. (2000). NADPH oxidase is an O_2 sensor in airway chemoreceptors: evidence from K^+ current modulation in wild type and oxidase-deficient mice. *Proceedings of the National Academy of Sciences of the USA* **97**, 4374–4379.

Gurney, A. M., Osipenko, O. N., MacMillan, D. & Kempsill, F. E. J. (2002). Potassium channels underlying the resting membrane potential of pulmonary artery smooth muscle cells. *Clinical and Experimental Pharmacology and Physiology* **29**, 330–333.

Hartness, M. E., Lewis, A., Searle, G. J., O'Kelly, I., Peers, C. & Kemp, P. J. (2001). Combined antisense and pharmacological approaches implicate hTASK as an airway O_2 sensing K^+ channel. *Journal of Biological Chemistry* **276**, 26499–26508.

Jackson, A. & Nurse, C. A. (1998). Role of acetylcholine receptors and dopamine transporter in regulation of extracellular dopamine in rat carotid body cultures grown in chronic hypoxia or nicotine. *Journal of Neurochemistry* **70**, 653–662.

Jiang, C. & Haddad, G. G. (1994). Oxygen deprivation inhibits a K^+ channel independently of cytosolic factors in rat central neurons. *Journal of Physiology* **481**, 15–26.

Landauer, R. C., Pepper, D. R. & Kumar, P. (1995). Effect of chronic hypoxaemia from birth upon chemosensitivity in the adult rat carotid body *in vitro*. *Journal of Physiology* **485**, 543–550.

Leach, R. M., Hill, H. M., Snetkov, V. A., Robertson, T. P. & Ward, J. P. (2001). Divergent roles of glycolysis and the mitochondrial electron transport chain in hypoxic pulmonary vasoconstriction of the rat: identity of the hypoxic sensor. *Journal of Physiology* **536**, 211–224.

Levitan, E. S. & Kramer, R. H. (1990). Neuropeptide modulation of single calcium and potassium channels detected with a new patch clamp configuration. *Nature* **348**, 545–547.

Lewis, A., Hartness, M. E., Chapman, C. G., Fearon, I. M., Meadows, H. J., Peers, C. & Kemp, P. J. (2001). Recombinant hTASK1 is an O_2-sensitive K^+ channel. *Biochemical and Biophysical Research Communications* **285**, 1290–1294.

Liu, H., Moczydlowski, E. & Haddad, G. G. (1999). O_2 deprivation inhibits Ca^{2+}-activated K^+ channels via cytosolic factors in mice neocortical neurons. *Journal of Clinical Investigation* **104**, 577–588.

Lopez-Barneo, J. (1996). Oxygen sensing by ion channels and the regulation of cellular function. *Trends in Neurosciences* **19**, 435–440.

Lopez-Barneo, J., Pardal, R. & Ortega-Saenz, P. (2001). Cellular mechanism of oxygen sensing. *Annual Review of Physiology* **63**, 259–287.

McCulloch, K. M., Osipenko, O. N. & Gurney, A. M. (1999). Oxygen-sensing potassium currents in pulmonary artery. *General Pharmacology* **32**, 403–411.

Mironov, S. L. & Richter, D. W. (1999). Cytoskeleton mediates inhibition of the fast Na^+ current in respiratory brainstem neurons during hypoxia. *Journal of Neuroscience* **11**, 1831–1834.

Mojet, M. H., Mills, E. & Duchen, M. R. (1997). Hypoxia-induced catecholamine secretion in isolated newborn rat adrenal chromaffin cells is mimicked by inhibition of mitochondrial respiration. *Journal of Physiology* **504**, 175–189.

O'Kelly, I., Lewis, A., Peers, C. & Kemp, P. J. (2000). O_2 sensing by airway chemoreceptor-derived cells: protein kinase C activation reveals functional evidence for involvement of NADPH oxidase. *Journal of Biological Chemistry* **275**, 7684–7692.

O'Kelly, I., Peers, C. & Kemp, P. J. (2001). NADPH oxidase does not account fully for O_2 sensing in model airway chemoreceptor cells. *Biochemical and Biophsyical Research Communications* **283**, 1131–1134.

O'Kelly, I., Stephens, R. H., Peers, C. & Kemp, P. J. (1999). Potential identification of the O_2-sensitive K^+ current in a human neuroepithelial body-derived cell line. *American Journal of Physiology* **276**, L96–104.

Pardal, R., Ludewig, U., Garcia-Hirschfeld, J. & Lopez-Barneo, J. (2000). Secretory responses of intact glomus cells in thin slices of rat carotid body to hypoxia and tetraethyl-ammonium. *Proceedings of the National Academy of Sciences of the USA* **97**, 2361–2366.

Peers, C. (1990). Hypoxic suppression of K^+ currents in type-I carotid-body cells – selective effect on the Ca^{2+}-activated K^+ current. *Neuroscience Letters* **119**, 253–256.

Peers, C. (1997). Oxygen-sensitive ion channels. *Trends in Pharmacological Sciences* **18**, 405–408.

Peers, C. & Kemp, P. J. (2001). Acute oxygen sensing: diverse but convergent mechanisms in airway and arterial chemoreceptors. *Respiration Research* **2**, 145–149.

Pluger, S., Faulhaber, J., Furstenau, M., Lohn, M., Waldschutz, R., Gollasch, M., Haller, H., Luft, F. C., Ehmke, H. & Pongs, O. (2000). Mice with disrupted BK channel beta1 subunit gene feature abnormal Ca^{2+} spark/STOC coupling and elevated blood pressure. *Circulation Research* **87**, E53–60.

Post, J. M., Hume, J. R., Archer, S. L. & Weir, E. K. (1992). Direct role for potassium channel inhibition in hypoxic pulmonary vaso-constriction. *American Journal of Physiology* **262**, C882–890.

Riesco-Fagundo, A. M., Perez-Garcia, M. T., Gonzalez, C. & Lopez-Lopez, J. R. (2001). O_2 modulates large-conductance Ca^{2+}-dependent K^+ channels of rat chemoreceptor cells by a membrane-restricted and CO-sensitive mechanism. *Circulation Research* **89**, 430–436.

Schubert, R. & Nelson, M. T. (2001). Protein kinases: tuners of the BKCa channel in smooth muscle. *Trends in Pharmacological Sciences* **22**, 505–512.

Soh, H., Jung, W., Uhm, D. Y. & Chung, S. (2001). Modulation of large conductance calcium-activated potassium channels from rat hippocampal neurons by glutathione. *Neuroscience Letters* **298**, 115–118.

Tang, X. D., Daggett, H., Hanner, M., Garcia, M. L., McManus, O. B., Brot, N., Weissbach, H., Heinemann, S. H. & Hoshi, T. (2001). Oxidative regulation of large conductance calcium-activated potassium channels. *Journal of General Physiology* **117**, 253–274.

THOMPSON, R. J. & NURSE, C. A. (1998). Anoxia differentially modulates multiple K+ currents and depolarizes neonatal rat adrenal chromaffin cells. *Journal of Physiology* **512**, 421–434.

TORO, L., WALLNER, M., MEERA, P. & TANAKA, Y. (1998). Maxi-K(Ca), a unique member of the voltage-gated K channel superfamily. *News in Physiological Sciences* **13**, 112–117.

TRISTANI-FIROUZI, M., REEVE, H. L., TOLAROVA, S., WEIR, E. K. & ARCHER, S. L. (1996). Oxygen-induced constriction of rabbit ductus arteriosus occurs via inhibition of a 4-aminopyridine-, voltage-sensitive potassium channel. *Journal of Clinical Investigation* **98**, 1959–1965.

TSENG-CRANK, J., FOSTER, C. D., KRAUSE, J. D., MERTZ, R., GODINOT, N., DICHIARA, T. J. & REINHART, P. H. (1994). Cloning, expression, and distribution of functionally distinct Ca2+-activated K+ channel isoforms from human brain. *Neuron* **13**, 1315–1330.

TSENG-CRANK, J., GODINOT, N., JOHANSEN, T. E., AHRING, P. K., STROBAEK, D., MERTZ, R., FOSTER, C. D., OLESEN, S. P. & REINHART, P. H. (1996). Cloning, expression, and distribution of a Ca2+-activated K+ channel beta-subunit from human brain. *Proceedings of the National Academy of Sciences of the USA* **93**, 9200–9205.

VERGARA, C., LATORRE, R., MARRION, N. V. & ADELMAN, J. P. (1998). Calcium-activated potassium channels. *Current Opinion in Neurobiology* **8**, 321–329.

WANG, D., YOUNGSON, C., WONG, V., YEGER, H., DINAUER, M. C., VEGA-SAENZ DE MIERA, E., RUDY, B. & CUTZ, E. (1996). NADPH-oxidase and hydrogen peroxide sensitive K+ channel may function as an oxygen sensor complex in airway chemoreceptors and small cell lung carcinoma cell lines. *Proceedings of the National Academy of Sciences of the USA* **93**, 13182–13187.

WEIR, E. K. & ARCHER, S. L. (1995). The mechanism of acute hypoxic pulmonary vasoconstriction: the tale of two channels. *FASEB Journal* **9**, 183–189.

WYATT, C. N. & PEERS, C. (1995). Ca2+-activated K+ channels in isolated type-I cells of the neonatal rat carotid-body. *Journal of Physiology* **483**, 559–565.

WYATT, C. N., WRIGHT, C., BEE, D. & PEERS, C. (1995). O2-sensitive K+ currents in carotid-body chemoreceptor cells from normoxic and chronically hypoxic rats and their roles in hypoxic chemotransduction. *Proceedings of the National Academy of Sciences of the USA* **92**, 295–299.