Kvβ1.2 Subunit Coexpression in HEK293 Cells Confers O_2 Sensitivity to Kv4.2 but not to *Shaker* Channels

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ABSTRACT Voltage-gated K^+ (K_V) channels are protein complexes composed of ion-conducting integral membrane α subunits and cytoplasmic modulatory β subunits. The differential expression and association of α and β subunits seems to contribute significantly to the complexity and heterogeneity of K_V channels in excitable cells, and their functional expression in heterologous systems provides a tool to study their regulation at a molecular level. Here, we have studied the effects of Kvβ1.2 coexpression on the properties of *Shaker* and Kv4.2 K_V channel α subunits, which encode rapidly inactivating A-type K^+ currents, in transfected HEK293 cells. We found that Kvβ1.2 functionally associates with these two α subunits, as well as with the endogenous K_V channels of HEK293 cells, to modulate different properties of the heteromultimers. Kvβ1.2 accelerates the rate of inactivation of the *Shaker* currents, as previously described, increases significantly the amplitude of the endogenous currents, and confers sensitivity to redox modulation and hypoxia to Kv4.2 channels. Upon association with Kvβ1.2, Kv4.2 can be modified by DTT (1,4 dithiothreitol) and DTDP (2,2′-dithiodipyridine), which also modulate the low pO_2 response of the Kv4.2+β channels. However, the physiological reducing agent GSH (reduced glutathione) did not mimic the effects of DTT. Finally, hypoxic inhibition of Kv4.2+β currents can be reverted by 70% in the presence of carbon monoxide and remains in cell-free patches, suggesting the presence of a hemoproteic O_2 sensor in HEK293 cells and a membrane-delimited mechanism at the origin of hypoxic responses. We conclude that β subunits can modulate different properties upon association with different K_V channel subfamilies; of potential relevance to understanding the molecular basis of low pO_2 sensitivity in native tissues is the here described acquisition of the ability of Kv4.2+β channels to respond to hypoxia.

KEY WORDS: potassium channels • β subunit • hypoxia

introduction

Voltage-gated K^+ (K_V)[1] channels help establish the resting membrane potential and modulate the frequency and duration of the action potentials in excitable cells. Molecular biology techniques have identified several mammalian genes encoding the pore-forming α subunits of K_V channels that can give rise to delayed rectifier or A-type currents upon expression in heterologous systems (Chandy and Gutman, 1995). The functional and structural diversity of the K_V channels' α subunits is further increased by their capacity to form functional heterotetrameric structures and to associate with modulatory β subunits (for review see Pongs, 1995; Jan and Jan, 1997). For example, association of β subunits with some members of the *Shaker* subfamily results in αβ heteromultimers with inactivation kinetics more rapid than those of the corresponding α homomultimers (Rettig et al., 1994; Chouinard et al., 1995; Heinemann et al., 1995; Majumder et al., 1995; McCormack et al., 1995; Morales et al., 1995), and, even further, some of these β subunits can convert a delayed rectifier into a rapidly inactivating channel (Rettig et al., 1994; England et al., 1995; Majumder et al., 1995; Morales et al., 1995; Heinemann et al., 1996).

In some tissues, K^+ currents exhibit specific properties, such as regulation by oxygen levels (Lopez-Barneo et al., 1988; Post et al., 1992; Youngson et al., 1993). It has been hypothesized that O_2 sensitivity of K^+ currents could be intrinsic to the channels themselves (Ruppersberg et al., 1991; Duprat et al., 1995; Weir and Archer, 1995) or, alternatively, that a membrane-bound O_2 sensor or a regulatory subunit of the K^+ channels confers the observed sensitivity (Gonzalez et al., 1992; Lopez-Barneo, 1994; Patel et al., 1997).

In the present work, we have used an heterologous expression system to study the association of the auxiliary subunit Kvβ1.2 (formerly Kvβ3) with some cloned K_V channels and its possible contribution to the hypoxic sensitivity of the heteromultimers. The K_V channels used (*Shaker* B and Kv4.2) express rapidly inactivat-

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[1]*Abbreviations used in this paper:* GFP, green fluorescent protein; GSH, reduced glutathione; K_V channel, voltage-gated K^+ channel; (M)ANOVA, fully factorial analysis of variance.

ing currents comparable to the oxygen-sensitive K^+ currents described in some preparations (Lopez-Barneo et al., 1988; Gonzalez et al., 1992). We found subfamily-specific functional interactions between Kvβ1.2 and the different K_V channels studied, so that Kvβ1.2 coexpression is able to regulate the amplitude of the endogenous HEK293 K_V currents, the rate of inactivation of the *Shaker* currents, and the redox and oxygen sensitivity of the Kv4.2 currents. The hypoxic response of the Kv4.2+Kvβ1.2 heteromultimers was unaffected by application of reduced glutathione (GSH) in the pipette solution or in the bath, but was prevented by treatment with DTT (1,4 dithiothreitol) and restored with DTDP (2,2′-dithiodipyridine), suggesting that reduction of some, but not all, of the residues susceptible to redox modulation can disrupt the mechanism underlying the low pO_2 regulation of these channels. Hypoxic inhibition was reverted by carbon monoxide (suggesting the presence of an hemoproteic O_2 sensor in HEK cells) and remains in excised membrane patches, indicating that the mechanism of low pO_2 inhibition is restricted to the plasma membrane.

materials and methods

HEK293 Cell Maintenance and Transfections

HEK293 cells were maintained in DMEM supplemented with 10% fetal calf serum (GIBCO BRL), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were grown as a monolayer and plated on squared coverslips (24 × 24 mm) placed in the bottom of 35-mm Petri dishes at a density of 2–4 × 10^5 cells/dish the day before transfection. Transient transfections were performed using the calcium-phosphate method (Wigler et al., 1978) with 1 μg of plasmid DNA encoding the drosophila *Shaker* B (H4) K^+ channel α subunit (into pRcRSV; Invitrogen Corp.), or the Kv4.2 K^+ channel α subunit (into E42c) alone or in combination with 2 μg of plasmid DNA encoding the Kvβ1.2 subunit into pREP4. In a group of experiments, the cells were only transfected with 2 μg of Kvβ1.2 subunit. In all cases, 0.2 μg of green fluorescent protein (GFP) in a CMV-promoter expression plasmid (GFPPRK5), was included to permit transfection efficiency estimates (10–40%) and to identify cells for voltage-clamp analysis (Marshall et al., 1995). Voltage-clamp recordings revealed typical inactivating currents in 100% of the cells expressing GFP. A group of control cells was obtained by analyzing the currents present in cells transfected with GFP alone or in untransfected cells. All plasmids used in this study were generously provided by Drs. E. Marban and G.F. Tomaselli (John Hopkins University, Baltimore, MD).

Electrophysiological Recordings

K^+ currents were studied using either the whole-cell or the outside-out configuration of the patch-clamp technique. The holding potential was −60 or −80 mV, respectively. Isolated HEK cells were studied 1–3 d after transfection. The coverslips with the attached cells were transferred to a small recording chamber (0.2 ml) placed in the stage of an inverted microscope and perfused by gravity with (mM): 141 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 10 glucose, 10 HEPES, pH 7.4 with NaOH. The bath solution was connected to ground via a 3 M KCl agar bridge and a Ag-AgCl electrode. Patch pipettes were double pulled (PP-83; Narishige Co.) and heat polished (MF-83; Narishige Co.) to resistances ranging from 1.5–3 MΩ for whole-cell experiments to 10–15 MΩ for cell-free recordings when filled with a internal solution containing (mM): 125 KCl, 4 $MgCl_2$, 10 HEPES, 10 EGTA, 5 MgATP, pH 7.2 with KOH. Hypoxia was achieved by bubbling the reservoir that fed the perfusion chamber with 100% N_2. The final pO_2 level in the perfusion chamber was below 10 mmHg. The time course of the fall in the pO_2 was complete within 1 min of solution exchange. In selected experiments, the control solutions were also bubbled with air to exclude potential artifactual effects due to the bubbling of the solutions. Whole-cell currents were recorded using an Axopatch 200 patch-clamp amplifier, sampled at 10 and filtered at 2 kHz (−3 dB, four-pole Bessel filter). The series resistance (ranging from 4 to 10 MΩ) was routinely compensated by 60–80%. Data were leak subtracted on line by a P/4 protocol. K^+ currents from macropatches in the outside-out configuration were registered several minutes after excision and were taken as the difference between the current recorded in a 50-ms depolarizing pulse to +40 mV from a holding potential of −80 mV and the average current obtained applying four pulses to +40 mV after inactivating the K^+ channels with 200-ms prepulses to the same potential. To facilitate the subtraction of capacitative transients, the potential was held at −80 mV during 1 ms between prepulse and pulse. Currents were sampled at 5 and filtered at 1 kHz. Records were digitized with a Digidata-1200 A/D converter (Axon Instruments), and stored on disk using PCLAMP version 6.02 software. All the experiments were done at room temperature (20–22°C).

Data Analysis

Analysis of the data was performed with the CLAMPFIT subroutines of the PCLAMP software and ORIGIN 4.0 software (Microcal Software, Inc.). Pooled data are expressed as mean ± SEM. Statistical comparisons between groups of data were carried out with the two-tailed Student's *t* test for paired or unpaired data, and values of $P < 0.05$ were considered statistically significant. The analysis of the differences between two groups of data when comparing more than one variable was done with a fully factorial analysis of variance [(M)ANOVA] using commercial software (SYSTAT; Systat Inc.).

Materials

DTT, DTDP, and GSH were obtained from Sigma Chemical Co. DTT and GSH were prepared fresh and dissolved in the bath or in the pipette solution, and DTDP was first dissolved in ethanol to a concentration of 500 mM, and then diluted in bath solution to a final concentration of 100 μM.

results

Effects of Kvβ1.2 on the Amplitude of Shaker, Kv4.2, and HEK293 Endogenous K_V Currents

Untransfected or mock-transfected (GFP alone) HEK293 cells show K_V currents of variable size, ranging from 100 to 600 pA at +60 mV. As shown in Fig. 1 A, over the length of a 100-ms pulse, this endogenous current exhibited almost no inactivation. Outward current was observed at potentials above −20 mV, and peak current typically showed a plateau at potentials more positive than +40 mV. When HEK293 cells were transfected with Kvβ1.2, there was a significant increase in the current am-

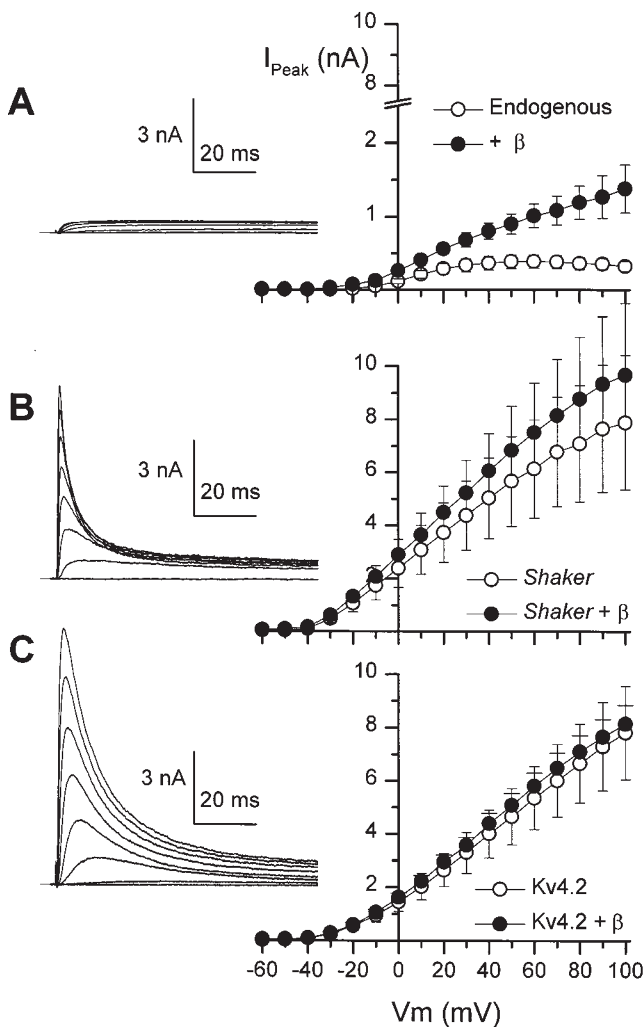

FIGURE 1. Current–voltage relationship of endogenous and heterologous K^+ currents in HEK cells. Averaged I–V relationship obtained in mock-transfected cells (A), transfected with *Shaker* channels (B), or Kv4.2 channels (C), either alone (○) or in the presence (●) of Kvβ1.2. Each curve represents the mean ± SEM of 7–20 cells. The insets show representative traces obtained from mock-transfected cells or cells transfected with *Shaker* or Kv4.2 alone in 20-mV depolarizing steps from −40 to +100 mV. The *P* values obtained with (M)ANOVA when comparing the I–V curves in the absence and presence of Kvβ1.2 were 0.006 (endogenous currents), 0.084 (*Shaker* currents), and 0.204 (Kv4.2 currents).

plitude. The peak current amplitude at +100 mV increased from 0.32 ± 0.07 nA in the mock-transfected cells ($n = 7$) to 1.38 ± 0.32 in the Kvβ1.2-transfected cells ($n = 8$). The averaged current–voltage relationships were statistically different [$P = 0.006$ with (M)ANOVA], suggesting that Kvβ1 subunit could be exerting a chaperon-like effect on the HEK293-endogenous K^+ currents.

Transfection of HEK293 with *Shaker* or Kv4.2 α subunit cDNA gave rise to large, rapid inactivating currents in all the cells studied (Fig. 1, B and C). Kvβ1.2 coexpression did not modify significantly the current amplitude, as shown in the averaged peak current–voltage relationships.

Effects of Kvβ1.2 on the Kinetics of the Cloned K^+ Channels

It has been demonstrated that Kvβ1.2 is able to modulate the rate of inactivation of some of the members of the *Shaker* family when coinjected in *Xenopus* oocytes (England et al., 1995; Majumder et al., 1995; Morales et al., 1995). However, it is not known whether this subunit is able to functionally associate with the K^+ channels of the Shal subfamily (Kv4) and modulate their electrophysiological properties. In our expression system, Kvβ1.2 also modulates the kinetics of the recombinant *Shaker* channels. The traces in Fig. 2 A show that β subunit coexpression accelerates the rate of inactivation and decreased the amplitude of the currents at the end of a 100-ms pulse. The inactivation time course for both *Shaker* and *Shaker*+β K^+ channels was best fitted to a biexponential function with time constants that ex-

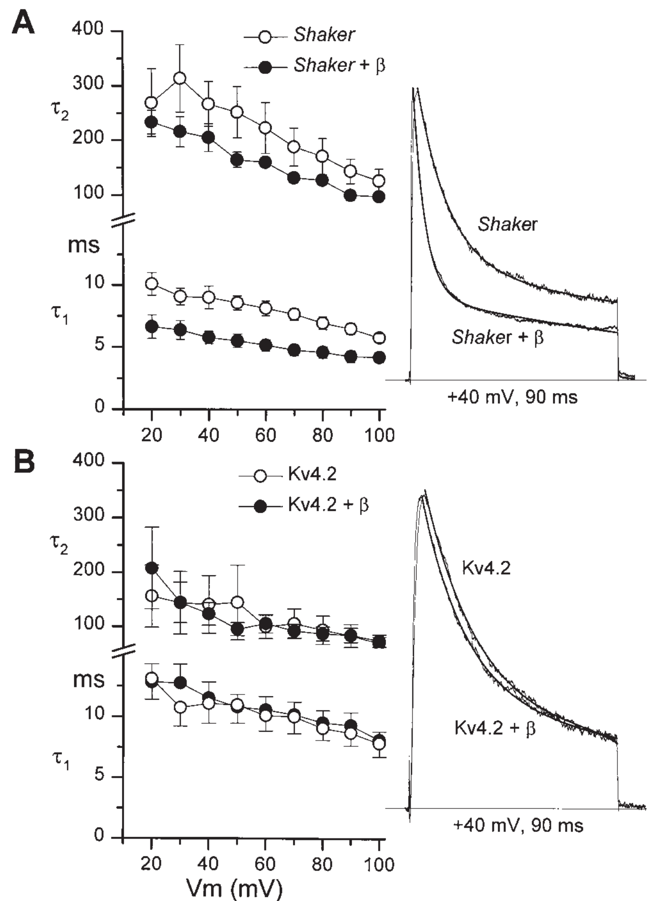

FIGURE 2. Effect of Kvβ1.2 on the inactivation kinetics of heterologously expressed K^+ currents. Inactivation time course of the K^+ currents expressed in *Shaker* (○) or *Shaker*+β (●)-transfected cells (A) and in Kv4.2 (○) or Kv4.2+β (●)-transfected cells (B). The inactivation time course was best fit to a biexponential function, and the time-constants obtained (τ_1 and τ_2) are plotted as a function of the membrane potential. Each point represents the mean ± SEM of 6–15 cells. The *P* values obtained by (M)ANOVA were 0.02 (τ_1) and 0.054 (τ_2) in A, and 0.415 (τ_1) and 0.102 (τ_2) in B. Representative traces of 90 ms normalized currents and fits obtained at +40 mV for each group are also shown.

hibited little voltage dependence. The presence of β subunit produced an acceleration of inactivation due to a significant decrease in the fast time constant at all the voltages [$P = 0.02$ with (M)ANOVA] and a less-pronounced decrease in the slow time constant that was not significant in our analysis ($P = 0.054$). However, this slow time constant is very likely distorted by the contribution of endogenous currents. Fig. 2 B shows a similar analysis of the kinetics of the Kv4.2 and Kv4.2+β recombinant channels. The time course of inactivation was also fitted to a biexponential function, but in this case it was not changed by the presence of β subunit. The same lack of effect on the activation and inactivation kinetics was observed for the endogenous channels upon association with β subunit (data not shown).

Effects of DTT and DTDP on Cloned K^+ Channels

The absence of effect of β subunit coexpression on the kinetics of the Kv4.2 channels has two possible explanations: either there is a lack of association between the two subunits, or, alternatively, β subunit associates with Kv4.2 α subunits to modulate other properties of the channel. Among these other properties, we have studied their sensitivity to sulfhydryl group reagents. Chemical redox modulation has been demonstrated for different native K^+ channels (Weir and Archer, 1995) as well as for several cloned K^+ channel α subunits (Ruppersberg et al., 1991; Duprat et al., 1995). Furthermore, a redox mechanism has been shown to modulate the effects of β subunit on the rate of inactivation of Kv1.4 K^+ channels (Rettig et al., 1994; Heinemann et al., 1995). In our work, the application of the membrane-permeable reducing agent DTT or the oxidizing agent DTDP to HEK cells expressing *Shaker* or *Shaker*+β had effects similar to those reported in the aforementioned works using other redox agents. Fig. 3 A shows one of four cells in which application of 100 μM DTDP markedly decreased the rate of inactivation of the *Shaker*+β currents in an irreversible way, whereas treatment with 2 mM DTT had the opposite effect. As an indicator of the change in the rate of inactivation, the average modification in the amplitude of the currents at the end of a 100-ms depolarizing pulse was calculated for three more cells expressing *Shaker*+β channels (Fig. 3 A, inset). DTT treatment reduced the amplitude of the current at the end of the pulse by 20.85 ± 7.1%, whereas DTDP increased this amplitude by 65.5 ± 12.1% (n = 4). The same modifications in the rate of inactivation were observed in cells transfected with *Shaker* alone (data not shown). On the contrary, treatment with DTT or DTDP failed to modify the amplitude or the kinetics of the currents expressed in cells transfected with Kv4.2 alone (Fig. 3 B, n = 3), in agreement with previous results showing the resistance of channels of the Kv4 subfamily to treatment with other redox agents (Duprat et

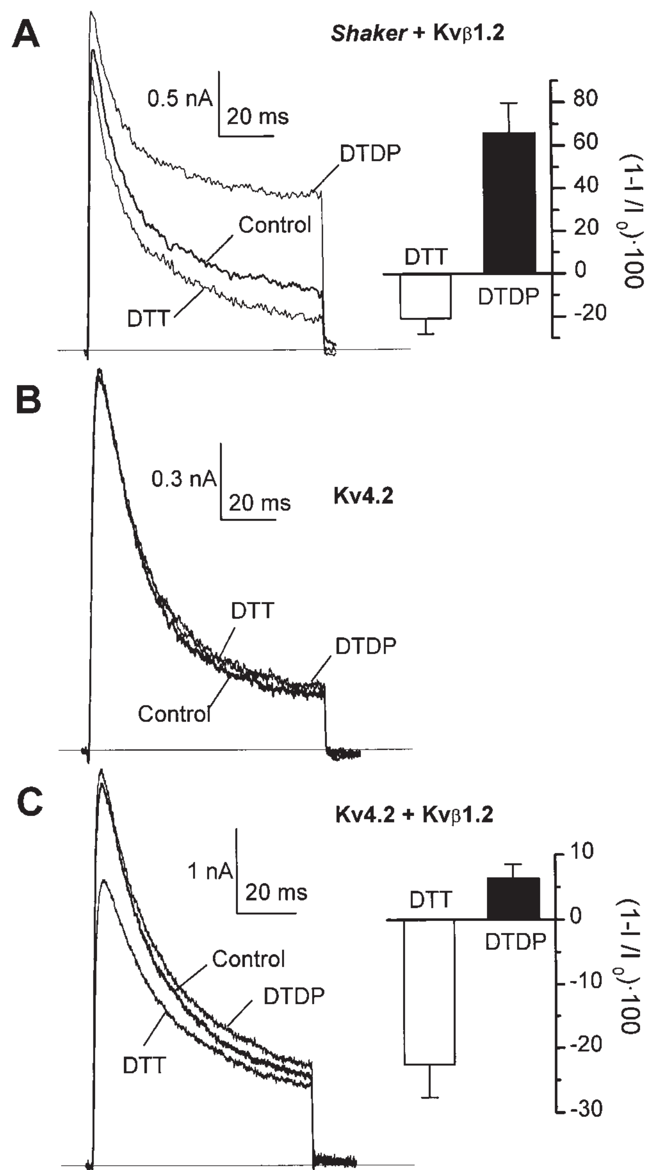

FIGURE 3. Effects of DTT and DTDP on the currents carried by cloned K^+ channels. Effect of DTT (2 mM) or DTDP (100 μM) on K^+ currents elicited in depolarizing pulses to +40 mV in cells transfected with *Shaker* + Kvβ1.2 (A), Kv4.2 (B) or Kv4.2 + Kvβ1.2 (C) channels. DTT or DTDP were applied for 3–5 min. When present, the effects did not revert upon washout with control solution. However, the effect of DTT was reverted by the application of DTDP and vice versa. A, inset, shows the average effects of these two drugs on the current amplitude in cells transfected with *Shaker* + Kvβ1.2 (n = 4). The effects are expressed as $(1 - I/I_0) \cdot 100$, I_0 being the current amplitude of the current at the end of the 100-ms depolarizing pulse in control conditions so that inhibitions appear as negative values and potentiations as positive values. C, inset, shows the results of a similar analysis in Kv4.2 + Kvβ1.2-transfected cells (n = 6), but in this case peak current amplitudes were taken.

al., 1995). When the same experiment was carried out in Kv4.2+β-transfected cells (Fig. 3 C), we could observe that application of 2 mM DTT produced an irreversible reduction of the current amplitude without

any significant change in the kinetics of the currents. Treatment with DTDP (100 μM) did not modify in a significant way the amplitude of the currents, but was able to revert the inhibition induced by DTT. Similar effects were observed in five more cells expressing Kv4.2+β channels (Fig. 3 C, inset). These results demonstrate that Kvβ1.2 is capable of associating with Kv4.2 α subunits and, upon coexpression, confers sensitivity to redox modulation to the Kv4.2+β heteromultimers.

Effects of Hypoxia on Cloned K^+ Channels

Regulation of ion channels by low pO_2 was first demonstrated in chemoreceptor cells of the rabbit carotid body, where an inactivating K^+ current was shown to be reversibly inhibited by hypoxia (Lopez-Barneo et al., 1988). It is not known whether the oxygen-sensitive K^+ channels have an O_2 sensing domain or, alternatively, the oxygen-sensitive cells have other sensor structures that can affect channel function. To address this question, we have studied the effect of lowering pO_2 on the cloned K^+ currents, as well as the possible contribution of Kvβ1.2 to the O_2 modulation of the channels. Fig. 4 A shows typical records obtained in depolarizing pulses to +40 mV from a holding potential of −60 mV for each channel (endogenous, *Shaker,* and Kv4.2) alone or in combination with Kvβ1.2. In each case, N_2-equilibrated solutions were applied for 2–5 min. Hypoxia did not modify in a significant way the amplitude or the time course of the endogenous currents in the mock-transfected (n = 5) or β-transfected (n = 6) cells. The same lack of effect was observed in all cells expressing *Shaker* channels alone (n = 8). In cells transfected with *Shaker*+β, hypoxia produced a 10% reduction in the current amplitude only in one of eight cells studied (12.5%) and, in Kv4.2-transfected cells, there was a 9% reduction in the current amplitude in 2 of 13 cells (15%). However, when the effect of hypoxia was studied in Kv4.2+β-cotransfected cells, 38 of 41 cells studied (93%) showed a reduction in the peak current amplitude that ranged from 10 to 40% and averaged 15.48 ± 0.93% at +40 mV (mean ± SEM, n = 38). Even though in some of the cells low pO_2 application seemed to slow down the time course of inactivation, this effect was not found to be statistically significant.

The time course of hypoxic inhibition of the Kv4.2+β

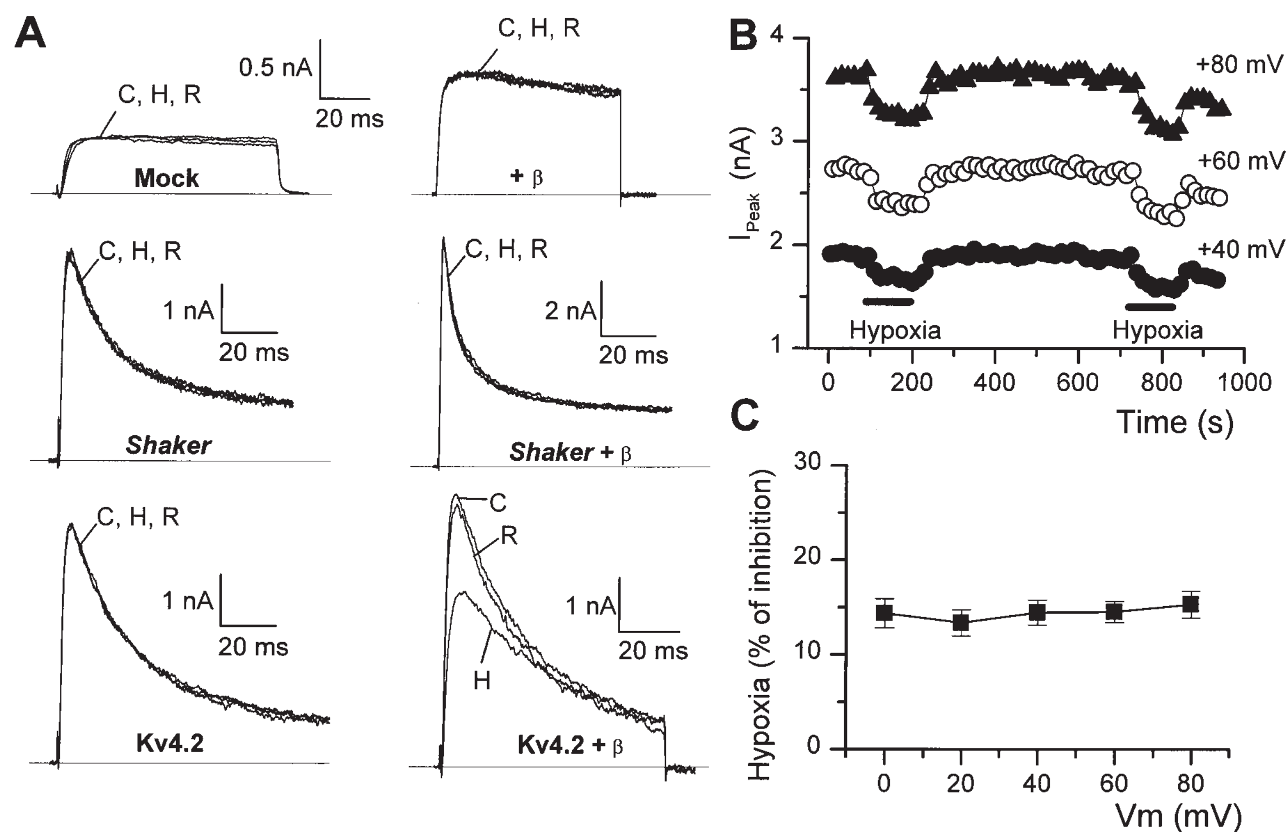

FIGURE 4. Effect of hypoxia on the endogenous and heterologous currents. (A) Current records elicited by pulses to +40 mV for each of the subunit combinations studied. The traces were obtained in control conditions (C), during the application of a N_2-equilibrated solution for 3–5 min (H), and after returning to the control solution (R). (B) Peak amplitudes of Kv4.2 + β currents obtained by depolarizing steps to three potentials applied every 5 s plotted against time. During the indicated periods, the cell was exposed to a N_2-equilibrated solution. (C) Voltage dependence of the hypoxia-induced inhibition of the current amplitude, represented as the percentage of inhibition of the K^+ current. Each point is the mean ± SEM of 8–29 cells.

cotransfected cells is shown in Fig. 4 B, where the peak current amplitude at three different voltages is represented against time. The effect of hypoxia was fully achieved within 1 min after the exchange of the solution and was readily reversible upon washout with the control solution. The hypoxia-induced inhibition of Kv4.2+β K^+ currents was voltage independent (Fig. 4 C), excluding a possible spurious effect due to a shift in the current–voltage relationship.

Effects of DTT and DTDP on the Response of Kv4.2+β to Hypoxia

The association with Kvβ1.2 invests Kv4.2 channels with two new properties, sensitivity to redox modulation and responsiveness to low pO_2 stimulation, making attractive the hypothesis that the redox status of the Kv4.2+β channels could be involved in the effect of hypoxia. This hypothesis was explored by studying the effect of hypoxic solutions on these channels after application of reducing or oxidizing agents. Fig. 5 A shows that application of 2 mM DTT produces an irreversible reduction of the current amplitude after which the response to hypoxia is lost. In these cells the effect of hypoxia was modified from a 13 ± 1.2% inhibition in control conditions to a 1.6 ± 0.6% inhibition after DTT treatment. The same protocol was used to study the effect of the oxidizing agent DTDP (100 μM) on the hypoxic inhibition of the channels (Fig. 5 B). In this case, we can see that DTDP did not modify the response to hypoxia of the Kv4.2+β currents (hypoxic inhibition averaged 16.1 ± 2.7% before and 14 ± 1.7% after DTDP treat-

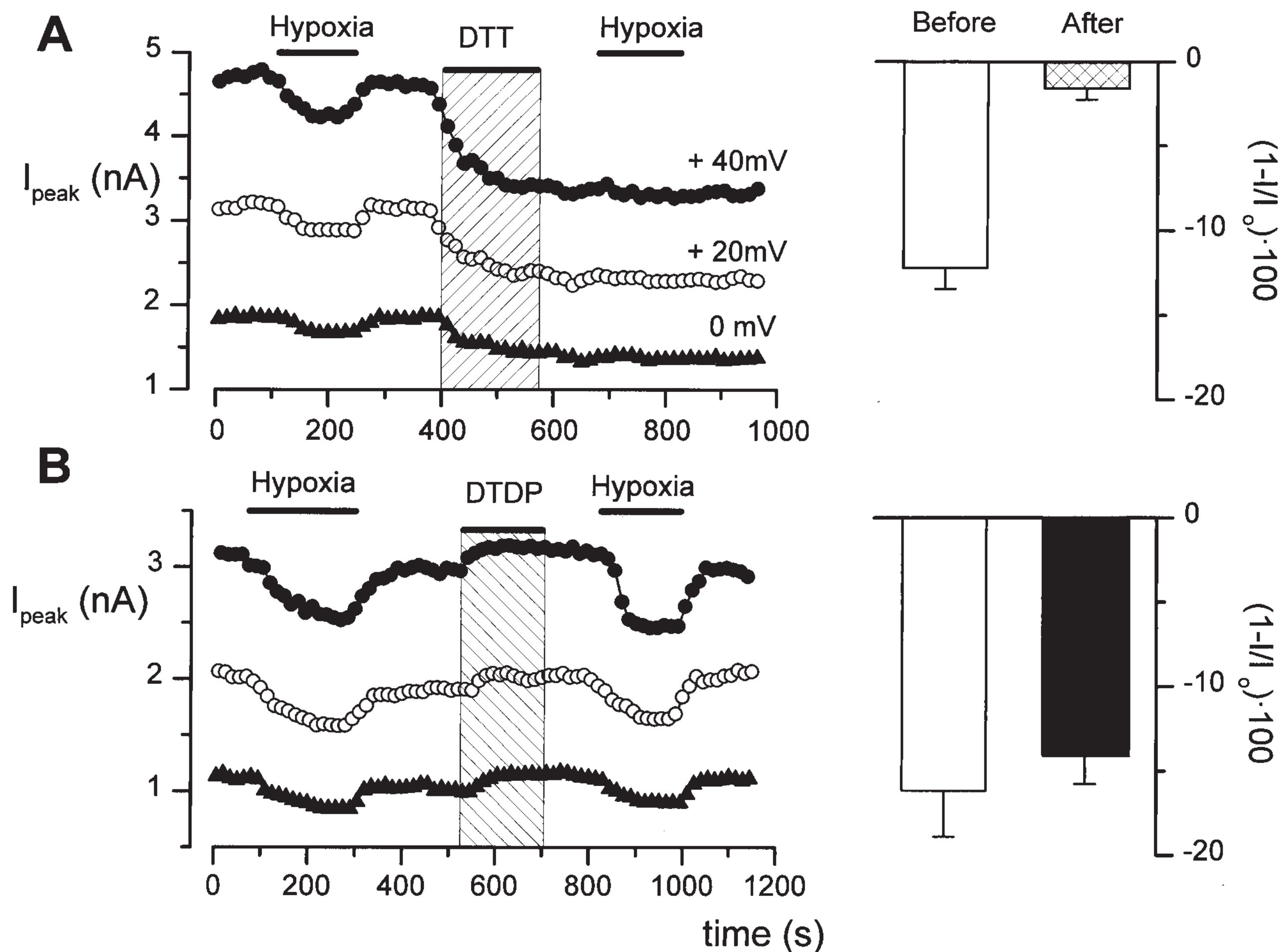

FIGURE 5. Effects of redox modulation in the hypoxic response of Kv4.2+β. (A) Effect of DTT. Peak-current amplitude to three different depolarizing voltages was measured every 5 s and plotted against time. N_2-equilibrated solutions were applied when indicated, before and after the application of 2 mM DTT for 3 min (hatched bar). In this cell, hypoxic inhibition at +40 mV was 12%, and DTT inhibition was 25%. After DTT, hypoxia produced a 1% inhibition of the current amplitude. (Right) The averaged values (mean ± SEM) of hypoxic inhibition of the currents before (open bar) and after (hatched bar) treatment with 2 mM DTT obtained in five similar experiments, and represented as in Fig. 4. (B) Effect of DTDP. A protocol similar to the one described in A was applied, but using 100 μM DTDP. In this cell, hypoxic inhibition of the peak current amplitude at +40 mV before and after treatment with DTDP amounted to 18 and 22%, respectively, and DTDP produced a 6% increase of the current amplitude. Averaged data of the effect of hypoxia before (open bar) and after (solid bar) DTDP application are represented on the right (mean ± SEM, $n = 6$).

ment, $n = 6$), although it was able to recover the hypoxic response of cells previously exposed to DTT (data not shown). These results indicate that the residues of the Kv4.2+β heteromultimers sensitive to DTT and DTDP treatment are involved in the response of the channel to acute hypoxia.

Effects of GSH on Kv4.2+β Currents

Although the previous data suggest that the redox state could be one of the mechanisms involved in the hypoxic modulation of Kv4.2+β channels, we have explored whether physiological redox modulators such as GSH have effects comparable to DTT on the currents and on their response to hypoxia. It is noteworthy that these two reducing agents modify in a similar way the time course of inactivation of cloned *Shaker* K^+ channels (Ruppersberg et al., 1991). Additionally, due to its lower membrane permeability, GSH could be helpful in indicating whether the effects are mainly due to modification of an intracellular or an extracellular site. We performed a series of experiments to explore the effect of pipette application of 5 mM GSH on the amplitude and the kinetics of Kv4.2+β currents and on their response to low pO_2 exposure. Parallel experiments in the same cultures with our normal pipette solution were used as controls. We found that inclusion of 5 mM GSH in the pipette solution did not change the amplitude of the currents (the peak current at +40 mV averaged 2.57 ± 0.46 nA in control versus 2.37 ± 0.49 nA in GSH-treated cells, $n = 9$) nor the inactivation time course (the two time constants were 11.6 ± 2.9 and 120 ± 6 ms in control cells versus 15 ± 4 and 135 ± 10.2 ms in the presence of GSH, $n = 9$). When the cells were bathed in a N_2-equilibrated solution, all cells in the two groups showed a reduction of the peak current amplitude, averaging 16.25 ± 1.6% in control and 16.86 ± 1.83% in GSH-treated cells.

The effects of extracellular application of 5 mM GSH are shown in Fig. 6, where the peak current amplitude in depolarizing pulses to +40 mV is plotted against time. In this cell, application of a N_2-equilibrated solution produced the same reduction of the current amplitude before and after bath application of GSH (23 and 22%, respectively). Treatment with 5 mM GSH reversibly decreased the amplitude and rate of inactiva-

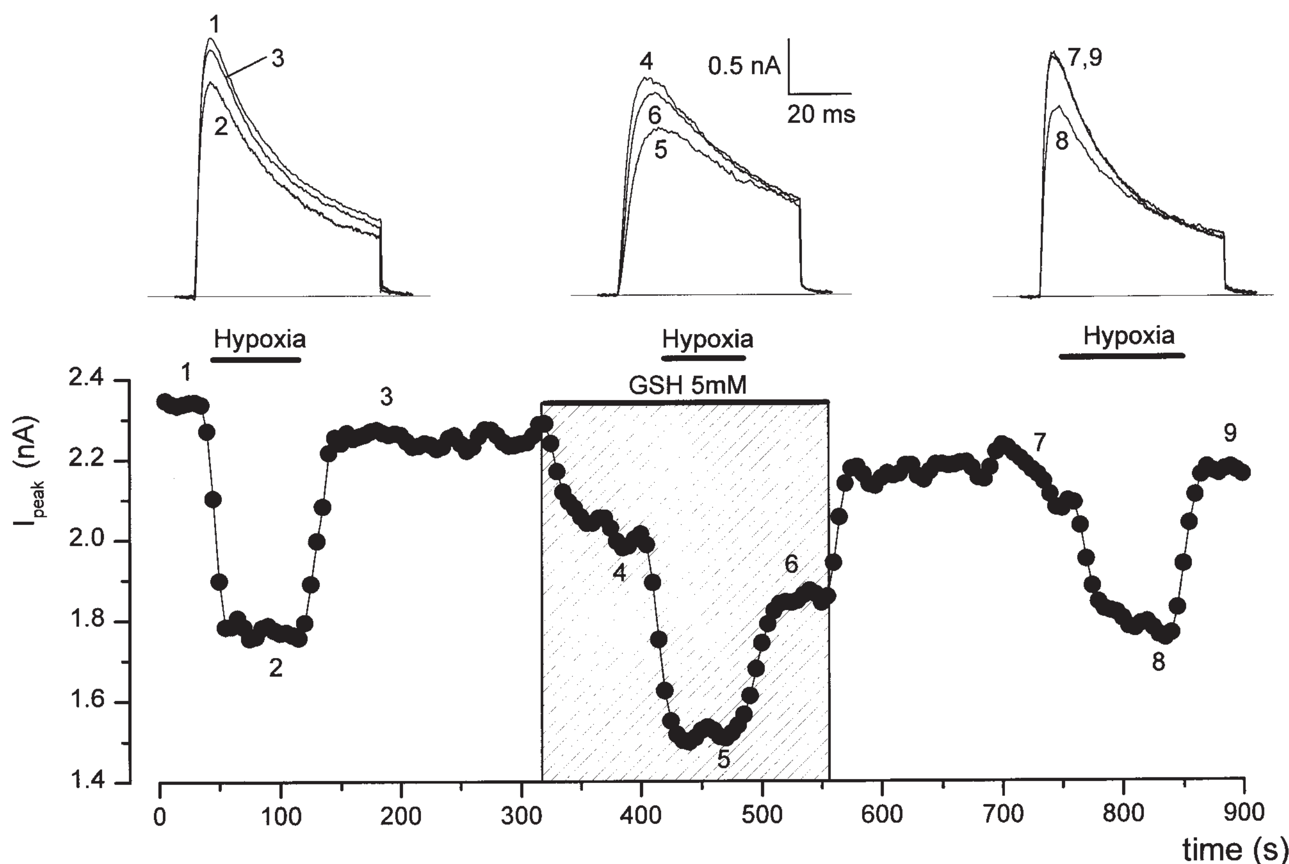

FIGURE 6. Effect of extracellular GSH on Kv4.2+β currents and on their response to hypoxia. (Bottom) The peak-current amplitude in depolarizing pulses to +40 mV was measured every 5 s and plotted against time. N_2-equilibrated solutions were applied as indicated with the solid lines, before, during, and after the application of 5 mM GSH for 4 min (hatched bar). (Top) Representative traces obtained at the times indicated by the numbers are shown.

tion of Kv4.2+β currents, but did not modify the magnitude of the effect of hypoxia. Similar results were observed in four more cells, in which the reduction produced by 5 mM GSH was somehow variable, averaging 33 ± 7%.

Molecular Mechanism of Low pO_2 Inhibition of Kv4.2+β Currents

The fact that DTT effects could not be reproduced by treatment with GSH suggests that these two agents are modifying different thiol groups. To further elucidate the underlying molecular mechanisms of hypoxic inhibition of Kv4.2+β channels, we have performed some experiments in excised membrane patches, devoid of potential intracellular mediators. Fig. 7 shows the effect of hypoxia on the peak current amplitude recorded with the protocol described in MATERIALS AND METHODS in an outside-out patch obtained from a Kv4.2+β-transfected cell. The current traces corresponding to the numbers in the plot are also shown in Fig. 7. As in the whole-cell experiments, perfusion with N_2-equilibrated solution produced a reduction in the amplitude of the current that reverted upon washout with normoxic solution. The same inhibition was observed in five more patches, with an average effect of 22.0 ± 2.5%. The average peak currents in these six patches was 106.3 ± 31.0 pA.

These data indicate that acute hypoxia is acting through a membrane-delimited pathway to produce the inhibition of Kv4.2+β currents, suggesting that either the Kv4.2+β channel proteins are intrinsically oxygen sensitive or, alternatively, there is a closely associated but distinct oxygen-sensing element that is endogenously expressed in the membrane of the host cell. The available evidence argues in favor of this latter possibility in several oxygen-sensitive tissues (Gonzalez et al., 1992), as well as in heterologous expression systems such as COS cells (Patel et al., 1997) and HEK293 cells (Fearon et al., 1999). To explore this possibility, we have studied the effect of carbon monoxide on the hypoxic response of Kv4.2+β. CO is a very inert gas that in biological systems only reacts with hemoproteins. Fig. 8 shows the effect of CO on the inhibition of Kv4.2+β currents by low pO_2. Fig. 8 shows the peak current amplitudes at two different voltages obtained in a cell while perfusing with control solution (pO_2 = 150 mmHg), with a hypoxic solution equilibrated with 100% N_2 (pO_2 < 10 mmHg), with a hypoxic solution equilibrated with a gas mixture containing 20% CO in N_2 (pO_2 < 10 mmHg, estimated pCO = 150 mmHg), and after returning to the control solution. We found

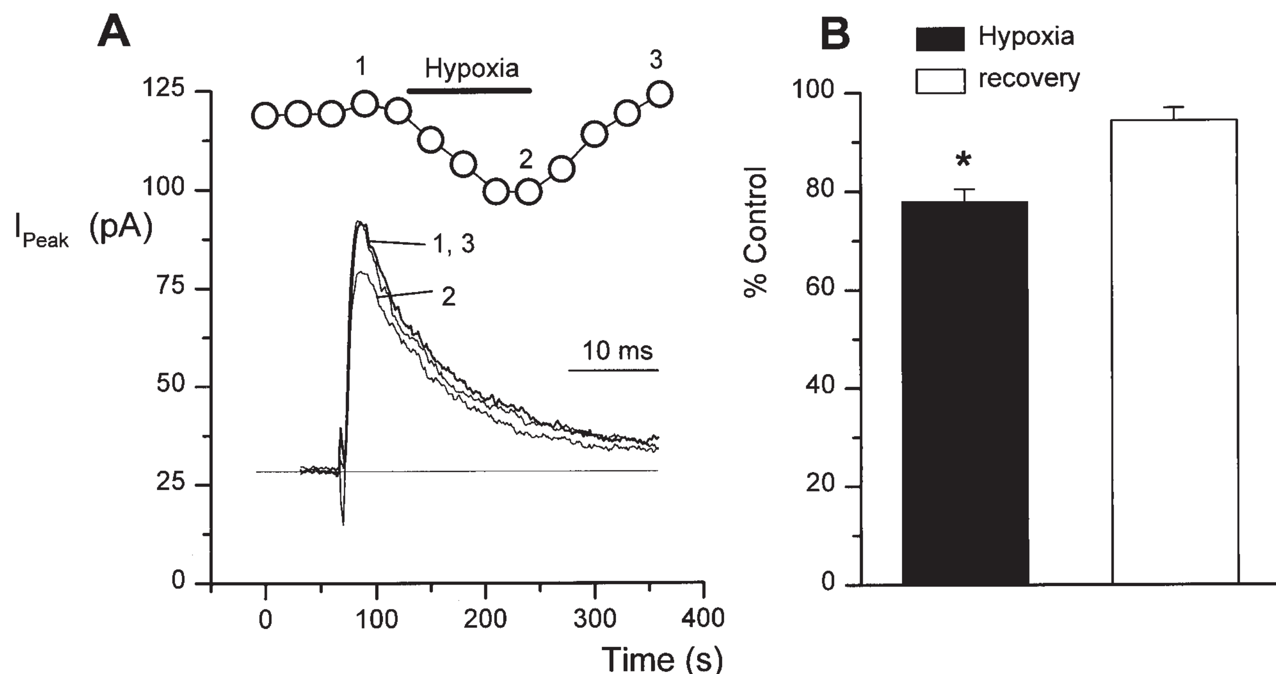

FIGURE 7. Effect of hypoxia on excised patches from Kv4.2+β-transfected cells. (A) K^+ currents from a macropatch in the outside-out configuration were obtained with depolarizing pulses with the protocol described in MATERIALS AND METHODS applied to the patch every 10 s. The figure represents a peak current versus time plot in which each point is the average of three of the depolarizing pulses. In this cell, hypoxia produced a 19% reduction of the current amplitude. The inset shows the average current traces corresponding to the indicated points, during control (1), hypoxia (2), and washout applications (3). (B) Averaged data of the current amplitude during hypoxic application and after returning to the normoxic solution in six different patches. Both current amplitudes were normalized to the peak current amplitude in control conditions. $^*P < 0.02$.

that CO is reverting in a significant extent the inhibition observed with hypoxia with a time course even faster that the onset of hypoxic application, indicating that CO is able to successfully replace O_2 at the O_2-sensing molecule, albeit with smaller affinity. The same reversion was observed in 10 more cells studied, in which CO prevented or reversed by 69.5 ± 3.2% the low pO_2-induced inhibition of Kv4.2+β currents, so that the average hypoxic inhibition decreased from 16.2 ± 1.4% to 5.04 ± 0.9% (Fig. 8 B).

discussion

The expression of recombinant channels in heterologous systems has proved a useful tool to characterize ionic channels in isolation. Transient transfection of HEK293 cells provides an efficient expression of channel proteins in a mammalian cell background, and offers an additional advantage for the present work, due to the absence of endogenous Kvβ subunits in HEK293 cells (Uebele et al., 1996). The presence of endogenous α subunits did not represent a problem due to the clear differences, both in amplitude and kinetics, between these endogenous currents and the currents through transfected K_V channels (see Fig. 1). The significant increase in the endogenous current amplitude obtained in Kvβ1.2-transfected cells suggest that this subunit interacts with the endogenous α subunits. A chaperon-like effect has been reported for several β subunits acting on specific K_V channel α subunits (Chouinard et al., 1995; Fink et al., 1996; Shi et al., 1996).

Studies on β subunit–mediated effects on K^+ channels have been primarily focused on the modifications induced in the inactivation kinetics of the heteromultimers. These studies indicate that several β subunits (Kvβ1, Kvβ3, and the *Drosophila* homologue *Hk*) are able to increase the rate of inactivation of specific members of the *Shaker* subfamily that express A-type currents or convert delayed-rectifier currents into A-type currents (see INTRODUCTION). This interaction between Kvβ1 and members of the *Shaker* subfamily has been confirmed with immunohistochemical studies that show the association and colocalization of these α-β complexes (Rhodes et al., 1995, 1997; Nakahira et

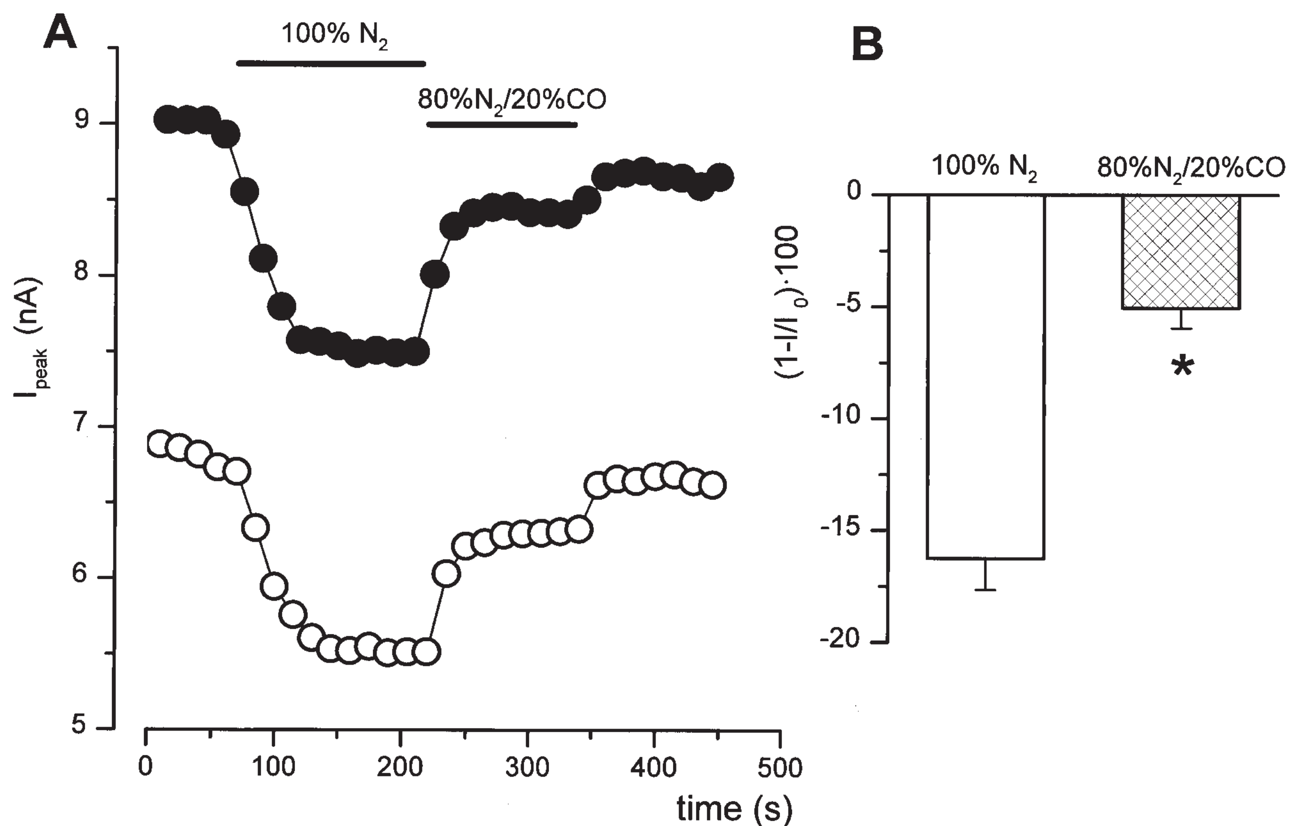

FIGURE 8. Effect of carbon monoxide on Kv4.2+β currents. (A) Peak current amplitude obtained by depolarizing steps to +60 (○) and +80 (●) mV from a holding potential of −60 mV in a cell transfected with Kv4.2+β is plotted against time. During the marked periods, the cell was superfused with solutions equilibrated with 100% N_2 or 80% N_2/20% CO as indicated in the figure. In this cell, the inhibition observed with hypoxia amounted to 15%, whereas in the presence of CO it was 4.5%; i.e., CO reversed by ~70% the effect of low pO_2. (B) Averaged values (mean ± SEM) of current inhibition by hypoxia (100% N_2, open bar) and CO (20% CO in N_2, crosshatched bar) obtained in paired data from 11 cells, and expressed as in Fig. 4. *$P < 0.001$.

al., 1996; Yu et al., 1996). It has also been reported the existence of selective interaction between both Kvβ1 and Kvβ2 and the mammalian Shal homologue Kv4.2 (Nakahira et al., 1996), but functional analysis has failed to reveal a change in the inactivation properties of the members of the Shal subfamily when coexpressed with Kvβ1 subunit (Heinemann et al., 1996; Yu et al., 1996).

In agreement with these reports, we found that coexpression with Kvβ1.2 produces a significant change in the rate of inactivation of the *Shaker* channels due to a decrease in the fast time constant. Besides, the fact that the acceleration of the channel inactivation by Kvβ1.2 does not reduce the peak current amplitude (Fig. 1 B) suggests that Kvβ1.2 is also increasing the surface expression of *Shaker* channels. Also in agreement with previous data, we found no changes in the inactivation rate of the Kv4.2 currents upon Kvβ1.2 coexpression. However, the association is functionally assessed by the acquisition by the Kv4.2+β currents of new property, namely the sensitivity to sulfhydryl group reagents (Fig. 3). Another proof of this functional association of Kv4.2 with Kvβ1.2 is the capability of Kv4.2+β currents to respond to low pO_2. This response was only consistently observed in our expression system with this particular α+β subunit combination, and consisted in a reversible reduction of the current amplitude upon exposure to hypoxic solutions (Figs. 4–8). One important aspect to consider regarding this effect is whether we are dealing with a metabolic or an allosteric-type mechanism. Given the speed of the effect of hypoxia, and the presence of 5 mM ATP in the intracellular solution, a direct action of hypoxia seems more likely than a response to altered cellular metabolism. Actually, hypoxic inhibition of native A-type K^+ channels has been slow to occur in excised membrane patches (Ganfornina and Lopez-Barneo, 1991), and the data presented in Fig. 7, showing the same effect of hypoxia in excised patches in the absence of potential intracellular mediators, strongly suggest that the response to hypoxia is a membrane-delimited mechanism. In addition, the persistence of the low pO_2 inhibition in a cell-free preparation confirms that Kv4.2 is able to coassemble with Kvβ1.2.

The modifications in the hypoxic response after application of freely membrane-permeable oxidizing and reducing agents suggest that hypoxic sensitivity can be modulated by the redox status of the channel proteins and that the same cysteine residues modified by DTT and DTDP are involved in the low pO_2 regulation of the Kv4.2+β channels. However, the absence of effect of GSH when applied intracellularly argues against a role for redox modulation under physiological conditions, and also excludes the possibility that the effect of low pO_2 on the Kv4.2+β channels could be attributable to the redox status of the cytoplasmic β subunits. On the other hand, extracellular GSH application does not interfere with the hypoxic response of the channel, supporting the idea that hypoxia and reducing agents can inhibit Kv4.2+β currents through different mechanisms. The fact that *Shaker* and *Shaker*+β channels are also modified by these agents, but insensitive to hypoxia, stresses out the fact that the effect of low pO_2 as a physiological stimulus is not simply achieved by the reduction of a sulfhydryl group. Redox modulation of *Shaker* or *Shaker*+β currents was able to change their rate of inactivation, but none of these maneuvers rendered the channels sensitive to hypoxia (data not shown). Furthermore, in contrast with DTT effect, application of hypoxic solutions did not modify the rate of inactivation of the channels (see Fig. 4 A). These observations indicate that O_2 sensing must have some specific structural requirements that seem to be achieved in our expression system by the combination of Kv4.2 α subunits with Kvβ1.2 subunits. With respect to the molecular nature of the O_2-sensing mechanism, there are two possibilities: first, the Kv4.2+β channels themselves are the O_2-sensing devices and, second, there is some other O_2-sensing molecule endogenously present in HEK293 cells (Wang and Semenza, 1993; Fearon et al., 1999) capable of interacting with Kv4.2 α subunits only when a β subunit is also present. Data on the literature showing that other structurally distinct channels are also O_2 sensitive in this cell line (Fearon et al., 1997; McKenna et al., 1998) support the second possibility, and data on the present study locate this O_2 sensor in the plasma membrane. Since the only known targets of CO in biological systems are reduced hemoproteins with accessible iron sites, our observation that CO is able to interact with this putative O_2 sensor, replacing O_2 and preventing the inhibition of K^+ currents (Fig. 8), strongly suggests that the intrinsic O_2 sensor of HEK293 cells is a hemoprotein.

The physiological relevance of the findings reported here is difficult to evaluate because neither the molecular nature of the O_2-sensitive K^+ channels nor the distribution and coexpression of Kv4.2 with Kvβ1 in native tissues are known. However, our results showing evidence that β subunits provide hypoxic sensitivity to specific K_V channel α subunits put forth the interesting possibility of the existence of tissue-specific modulatory subunit(s) that confer hypoxic sensitivity to the expressing tissues. This idea is supported by a recent report by Patel et al. (1997) in which a new K channel subunit, Kv9.3, that does not form a channel itself, is able to coassemble with Kv1.2 and increase the probability of the heteromultimeric channels to be modulated by hypoxia. Finally, our findings provide new clues in the search for the molecular mechanisms of O_2 sensing in hypoxia-sensitive tissues, raising a completely new set of questions requiring further investigation.

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references

Chandy, K.G., and G.A. Gutman. 1995. Voltage-gated potassium channel genes. *In* Ligand- and Voltage-gated Ion Channels. R.A. North, editor. CRC Press, Inc., Boca Raton, FL. 1–71.

Chouinard, S.W., G.F. Wilson, A.K. Schlimgen, and B. Ganetzky. 1995. A potassium channel beta subunit related to the aldo-keto reductase superfamily is encoded by the *Drosophila* hyperkinetic locus. *Proc. Natl. Acad. Sci. USA.* 92:6763–6767.

Duprat, F., E. Guillemare, G. Romey, M. Fink, F. Lesage, M. Lazdunski, and E. Honore. 1995. Susceptibility of cloned K^+ channels to reactive oxygen species. *Proc. Natl. Acad. Sci. USA.* 92:11796–11800.

England, S.K., V.N. Uebele, J. Kodali, P.B. Bennett, and M.M. Tamkun. 1995. A novel K^+ channel beta-subunit (hKv beta 1.3) is produced via alternative mRNA splicing. *J. Biol. Chem.* 270:28531–28534.

Fearon, I.M., A.C.V. Palmer, A.J. Balmforth, S.G. Ball, G. Mikala, A. Schwartz, and C. Peers. 1997. Hypoxia inhibits the recombinant α_{1C} subunit of the human cardiac L-type Ca^{2+} channel. *J. Physiol.* 500:551–556.

Fearon, I.M., A.C.V. Palmer, A.J. Balmforth, S.G. Ball, G. Varadi, and C. Peers. 1999. Modulation of recombinant human cardiac L-type Ca^{2+} channel α_{1C} subunits by redox agents and hypoxia. *J. Physiol.* 514:629–637.

Fink, M., F. Duprat, F. Lesage, C. Heurteaux, G. Romey, J. Barhanin, and M. Lazdunski. 1996. A new K^+ channel beta subunit to specifically enhance Kv2. 2 (CDRK) expression. *J. Biol. Chem.* 271:26341–26348.

Ganfornina, M.D., and J. Lopez-Barneo. 1991. Single K^+ channels in membrane patches of arterial chemoreceptor cells are modulated by O_2 tension. *Proc. Natl. Acad. Sci. USA.* 88:2927–2930.

Gonzalez, C., L. Almaraz, A. Obeso, and R. Rigual. 1992. Oxygen and acid chemoreception in the carotid body chemoreceptors. *Trends Neurosci.* 15:146–153.

Heinemann, S.H., J. Rettig, F. Wunder, and O. Pongs. 1995. Molecular and functional characterization of a rat brain Kvβ3 potassium channel subunit. *FEBS Lett.* 377:383–389.

Heinemann, S.H., J. Rettig, H.R. Graack, and O. Pongs. 1996. Functional characterization of Kv channel beta-subunits from rat brain. *J. Physiol.* 493:625–633.

Jan, L.Y., and Y.N. Jan. 1997. Cloned potassium channels from eukaryotes and prokaryotes. *Annu. Rev. Neurosci.* 20:91–123.

Lopez-Barneo, J. 1994. Oxygen-sensitive ion channels: how ubiquitous are they? *Trends Neurosci.* 17:133–135.

Lopez-Barneo, J., J.R. Lopez-Lopez, J. Ureña, and C. Gonzalez. 1988. Chemotransduction in the carotid body: K^+ current modulated by PO_2 in type I chemoreceptor cells. *Science.* 241:580–582.

Majumder, K., M. De Biasi, Z. Wang, and B.A. Wible. 1995. Molecular cloning and functional expression of a novel potassium channel beta-subunit from human atrium. *FEBS Lett.* 361:13–16.

Marshall, J., R. Molloy, G.W.J. Moss, J.R. Howe, and T.E. Hughes. 1995. The Jellyfish green fluorescent protein: a new tool for studying ion channel expression and function. *Neuron.* 14:211–215.

McCormack, K., T. McCormack, M. Tanouye, B. Rudy, and W. Stühmer. 1995. Alternative splicing of the human *Shaker* K^+ channel beta 1 gene and functional expression of the beta 2 gene product. *FEBS Lett.* 370:32–36.

McKenna, F., M.L.J. Ashford, and C. Peers. 1998. Hypoxia reversibly inhibits the activity of cloned hyman brain BKCa channels stably expressed in HEK 293 cells. *J. Physiol.* 509P:188P. (Abstr.)

Morales, M.J., R.C. Castellino, A.L. Crews, R.L. Rasmusson, and H.C. Strauss. 1995. A novel beta subunit increases rate of inactivation of specific voltage-gated potassium channel alpha subunits. *J. Biol. Chem.* 270:6272–6277.

Nakahira, K., G. Shi, K.J. Rhodes, and J.S. Trimmer. 1996. Selective interaction of voltage-gated K^+ channel beta-subunits with alpha-subunits. *J. Biol. Chem.* 271:7084–7089.

Patel, A.J., M. Lazdunski, and E. Honore. 1997. Kv2.1/Kv9.3, a novel ATP-dependent delayed-rectifier K^+ channel in oxygen-sensitive pulmonary artery myocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 16:6615–6625.

Pongs, O. 1995. Regulation of the activity of voltage-gated potassium channels by β subunits. *Semin. Neurosci.* 7:137–146.

Post, J.M., J.R. Hume, S.L. Archer, and E.K. Weir. 1992. Direct role for potassium channel inhibition in hypoxic pulmonary vasoconstriction. *Am. J. Physiol.* 262:C882–C890.

Rettig, J., S.H. Heinemann, F. Wunder, C. Lorra, D.N. Parcej, J.O. Dolly, and O. Pongs. 1994. Inactivation properties of voltage-gated K^+ channels altered by presence of beta-subunit. *Nature.* 369:289–294.

Rhodes, K.J., S.A. Keilbaugh, N.X. Barrezueta, K.L. Lopez, and J.S. Trimmer. 1995. Association and colocalization of K^+ channel alpha- and beta-subunit polypeptides in rat brain. *J. Neurosci.* 15: 5360–5371.

Rhodes, K.J., B.W. Strassle, M.M. Monaghan, Z. Bekele-Arcuri, M.F. Matos, and J.S. Trimmer. 1997. Association and colocalization of the Kvβ1- and Kvβ2-subunits with Kv1 α-subunits in mammalian brain K^+ channel complexes. *J. Neurosci.* 17:8246–8258.

Ruppersberg, J.P., M. Stocker, O. Pongs, S.H. Heinemann, R. Frank, and M. Koenen. 1991. Regulation of fast inactivation of cloned mammalian I_K(A) channels by cysteine oxidation. *Nature.* 352:711–714.

Shi, G., K. Nakahira, S. Hammond, K.J. Rhodes, L.E. Schechter, and J.S. Trimmer. 1996. Beta subunits promote K^+ channel surface expression through effects early in biosynthesis. *Neuron.* 16:843–852.

Uebele, V.N., S.K. England, A. Chaudhary, M.M. Tamkun, and D.J. Snyders. 1996. Functional differences in Kv1.5 currents expressed in mammalian cell lines are due to the presence of endogenous Kv beta 2.1 subunits. *J. Biol. Chem.* 271:2406–2412.

Wang, G.L., and G.L. Semenza. 1993. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc. Natl. Acad. Sci. USA.* 90:4304–4308.

Weir, E.K., and S.L. Archer. 1995. The mechanism of acute hypoxic pulmonary vasoconstriction: the tale of two channels. *FASEB J.* 9:183–189.

Wigler, M., A. Pellicer, S. Siverstein, and R. Axel. 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as a donor. *Cell.* 14:725–731.

Youngson, C., C. Nurse, H. Yeger, and E. Cutz. 1993. Oxygen sensing in airway chemoreceptors. *Nature.* 365:153–155.

Yu, W., J. Xu, and M. Li. 1996. NAB domain is essential for the subunit assembly of both α-α and α-β complexes of *Shaker*-like potassium channels. *Neuron.* 16:441–453.