

# Viral Gene Transfer of Dominant-Negative Kv4 Construct Suppresses an O<sub>2</sub>-Sensitive K<sup>+</sup> Current in Chemoreceptor Cells

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Hypoxia initiates the neurosecretory response of the carotid body (CB) by inhibiting one or more potassium channels in the chemoreceptor cells. Oxygen-sensitive K<sup>+</sup> channels were first described in rabbit CB chemoreceptor cells, in which a transient outward K<sup>+</sup> current was reported to be reversibly inhibited by hypoxia. Although progress has been made to characterize this current with electrophysiological and pharmacological tools, no attempts have been made to identify which Kv channel proteins are expressed in rabbit CB chemoreceptor cells and to determine their contribution to the native O<sub>2</sub>-sensitive K<sup>+</sup> current. To probe the molecular identity of this current, we have used dominant-negative constructs to block the expression of functional Kv channels of the *Shaker* (Kv1.xDN) or the *Shal* (Kv4.xDN) subfamilies, because members of these two subfamilies contribute to the transient outward K<sup>+</sup> currents in other preparations. Delivery of the constructs into chemoreceptor cells has been achieved

with adenoviruses that enabled ecdysone-inducible expression of the dominant-negative constructs and reporter genes in polycistronic vectors. In voltage-clamp experiments, we found that, whereas adenoviral infections of chemoreceptor cells with Kv1.xDN did not modify the O<sub>2</sub>-sensitive K<sup>+</sup> current, infections with Kv4.xDN suppressed the transient outward current in a time-dependent manner, significantly depolarized the cells, and abolished the depolarization induced by hypoxia. Our work demonstrates that genes of the *Shal* K<sup>+</sup> channels underlie the transient outward, O<sub>2</sub>-sensitive, K<sup>+</sup> current of rabbit CB chemoreceptor cells and that this current contributes to the cell depolarization in response to low pO<sub>2</sub>.

**Key words:** O<sub>2</sub>-sensitive K<sup>+</sup> current; viral gene transfer; dominant-negative constructs; carotid body chemoreceptors; hypoxia; potassium channels

The carotid body (CB) is the main peripheral arterial chemoreceptor, responsible for the increase in ventilation after exposure to hypoxia. Chemoreceptor cells are the CB elements that sense blood PO<sub>2</sub> and respond to a fall in PO<sub>2</sub> with a Ca<sup>2+</sup>-dependent release of neurotransmitters (Gonzalez et al., 1994). The presence in rabbit chemoreceptor cells of O<sub>2</sub>-sensitive K<sup>+</sup> channels (López-Barneo et al., 1988), whose open probability decreases as a function of PO<sub>2</sub> (Ganfornina and López-Barneo, 1991), led to the proposal that hypoxia could control the excitability of the cells triggering or facilitating cell depolarization, Ca<sup>2+</sup> entry, and release of neurotransmitters (Gonzalez et al., 1992, 1994).

Since the pioneer description of this low PO<sub>2</sub>-modulated channel (López-Barneo et al., 1988) in rabbit CB chemoreceptor cells, many other O<sub>2</sub>-sensitive K<sup>+</sup> channels have been found in other preparations, such as rat CB chemoreceptor cells, pulmonary artery smooth muscle cells, neuroepithelial bodies of the lung and PC12 cells (for review, see Peers, 1997). The degree of kinetic and pharmacological diversity among O<sub>2</sub>-sensitive K<sup>+</sup> channels has focused the interest toward determining the structural requirements for O<sub>2</sub>-sensing. Molecular biology techniques have identified several K<sup>+</sup> channel genes expressed in some of the hypoxia-sensitive tissues, and for some of them low pO<sub>2</sub> modulation has been studied in heterologous expression systems (Patel et al., 1997; Yuan et al., 1998; O'Kelly et al., 1999). However, there are con-

flicting reports with respect to which of these channels contributes to the native O<sub>2</sub>-sensitive K<sup>+</sup> channels, because different genes can produce channels with similar phenotypic properties (Patel et al., 1997; Hulme et al., 1999).

In the case of the rabbit CB chemoreceptors, three different voltage-dependent K<sup>+</sup> channels have been described in single-channel studies (Ganfornina and López Barneo, 1992a), and a detailed electrophysiological characterization of these currents, particularly of the O<sub>2</sub>-sensitive K<sup>+</sup> current, has been provided by several studies (Ganfornina and López-Barneo, 1991, 1992a,b; Pérez-García et al., 1992; López-López et al., 1993); however, no attempts have been made to establish the molecular identity of the different channels. Indeed, the minute size of the organ, together with its structural complexity, has delayed its characterization with conventional molecular biology techniques. In the present work, we explored the molecular nature of the O<sub>2</sub>-sensitive, transient outward K<sup>+</sup> current of rabbit CB chemoreceptor cells using selective suppression by dominant-negative constructs of Kv channels of the Kv1 and Kv4 subfamilies. Gene delivery into chemoreceptor cells was achieved with recombinant adenoviruses with ecdysone-inducible promoters (Johns et al., 1999). We found that transient K<sup>+</sup> current was not modified by infection of chemoreceptor cells with adenoviruses expressing the green fluorescent protein (GFP) reporter alone or in combination with a dominant-negative Kv1 subfamily construct (Kv1.xDN), but was almost completely blocked by a dominant-negative Kv4 subfamily construct (Kv4.xDN). The blockade of the transient K<sup>+</sup> current by Kv4.xDN increases the input resistance and depolarizes the cells; furthermore, Kv4.xDN construct suppresses hypoxia-induced depolarization, suggesting a role of this current in determining the resting membrane potential of the cells and the initiation of the low pO<sub>2</sub> chemotransduction. We conclude that genes from the Kv4 subfamily are the main contributors to the O<sub>2</sub>-sensitive K<sup>+</sup> current of rabbit CB chemoreceptor cells.

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## MATERIALS AND METHODS

**Plasmid construction and adenovirus preparation.** The plasmid pGFPK<sub>ir2.1</sub>-AAA and the adenovirus shuttle vectors pAdVgR<sub>XR</sub> and pAdEG<sub>I</sub> have been described (Johns et al., 1997, 1999; Hoppe et al., 1999). The coding sequence from rat Kv4.3 (kindly supplied by Dr. Bernardo Rudy, New York University, New York, NY) was cloned into the multiple cloning site of pAdEG<sub>I</sub> to generate pAdEG<sub>I</sub>-Kv4.3. In accordance to a previously reported dominant-negative Kv4.2 mutation (Barry et al., 1998), the point mutation W362F was introduced into Kv4.3 by site-directed mutagenesis, creating the vector pAdEG<sub>I</sub>-Kv4.3W362F (AdKv4.xDN). The Kv1.3 gene was PCR-amplified from lambdaHGK5 (ATCC 65963; American Type Culture Collection, Manassas, VA) and cloned into pAdEG<sub>I</sub>. The GYG signature sequence was mutated to AYA by site-directed mutagenesis creating pAdEG<sub>I</sub>-Kv1.3-AYA (AdKv1.xDN). The KvLQT gene (kindly provided by Dr. Mark Keating) was also subcloned into pAdEG<sub>I</sub>, and the disease causing mutation G306R was also made by site-directed mutagenesis.

Adenovirus vectors were generated by Cre-lox recombination of purified  $\psi$ 5 viral DNA and shuttle vector DNA as previously described (Hardy et al., 1997; Johns et al., 1999). The recombinant products were plaque-purified, expanded, and purified on CsCl gradients yielding concentrations on the order of 10<sup>10</sup> pfu/ml.

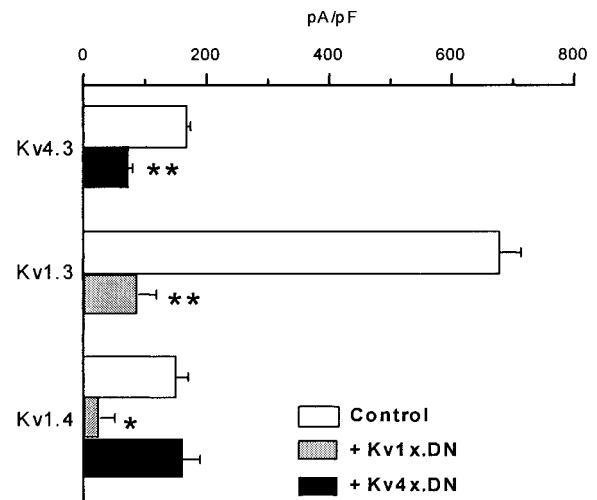
**Transient transfections.** Twenty four hours before transfection, Chinese hamster ovary (CHO)-K1 or human embryonic kidney 293 (HEK293) cells (ATCC CCL 61 and CRL 1573) were seeded at a density of  $2.0 \times 10^5$  per 35 mm. Cells were transfected with plasmid DNA (1  $\mu$ g/well total) using Lipofectamine Plus (Life Technologies, Gaithersburg, MD) as directed by the manufacturer. After 4 hr, transfection media was replaced with normal growth media. Expression was induced by addition of 10  $\mu$ M ponasterone A (Invitrogen, San Diego, CA) for 72 hr.

**CB chemoreceptor cell isolation and culture.** Adult New Zealand rabbits (1.5–2 kg) were anesthetized with pentobarbital sodium (40 mg/kg<sup>-1</sup> administered through the lateral vein of the ear). After tracheostomy, the carotid artery bifurcations were removed, and the animals were killed by an intracardiac bolus injection of pentobarbital sodium. The CBs were cleaned of surrounding connective tissue and enzymatically dispersed as described elsewhere (Pérez-García et al., 1992; López-López et al., 1997). Dispersed cells were plated onto poly-L-lysine-coated coverslips placed in 6-well dishes with 1 ml of DMEM:F-12 (1:1) with 5% FBS and maintained in culture for up to 96 hr.

**Chemoreceptor cell infections.** After 6–8 hr in culture, CB chemoreceptor cells were infected by replacing their growth media with 1 ml of new media containing 1  $\mu$ l of VgR<sub>XR</sub> and 1  $\mu$ l of the following ecdysone-inducible virus: AdEG<sub>I</sub> (control), AdKv1.xDN, or AdKv4.xDN for 12 hr (overnight). After that, expression was induced by the addition of 10  $\mu$ M ponasterone A for 24–72 hr before the experiments were performed.

**Electrophysiological recordings.** Ionic currents were recorded at room temperature (20–25°C) using the whole-cell configuration of the patch-clamp techniques (Hamill et al., 1981). Whole-cell current recordings and data acquisition from CB chemoreceptor cells were made as previously described (López-López et al., 1997). The coverslips with the attached cells were placed at the bottom of a small recording chamber (0.2 ml) on the stage of an inverted microscope and perfused by gravity with the bath solution. This solution was connected to ground via a 3 M KCl agar bridge and an Ag-AgCl electrode. Patch pipettes were made from borosilicate glass (1.5 mm o.d.; Clark Electromedical Instruments, Pangbourne, UK), double-pulled (PP-83; Narishige, Tokyo, Japan) and heat-polished (MF-83; Narishige) to resistances ranging from 1.5 to 3 M $\Omega$  when filled with the internal solution. For the recording of outward currents, the composition of the bath solution was (in mM): 141 NaCl, 4.7 KCl, 1.2 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 10 glucose, and 10 HEPES, pH 7.4 with NaOH, and the pipette was filling with a solution containing (in mM): 125 KCl, 4 MgCl<sub>2</sub>, 10 HEPES, 10 EGTA, and 5 MgATP, pH 7.2 with KOH. When studying inward currents, the CaCl<sub>2</sub> of the external solution was raised to 10 mM, and the pipette solution was (in mM): 130 CsCl, 2 MgCl<sub>2</sub>, 10 HEPES, 10 EGTA, 5 MgATP, and 2 MgGTP, pH 7.2 with CsOH. Hypoxia was achieved by bubbling the reservoir that fed the perfusion chamber with 100% N<sub>2</sub>, obtaining a final PO<sub>2</sub> level in the perfusion chamber below 10 mmHg. Oxygen levels were measured with small needle PO<sub>2</sub> electrodes (Diamond General Corporation) placed close to the cells. Whole-cell currents were recorded using an Axopatch 200 patch-clamp amplifier, filtered at 2 kHz (–3 dB, 4-pole Bessel filter) and sampled at 10 kHz. The series resistance (ranging from 4 to 10 M $\Omega$ ) was routinely compensated by 60–80%. In some experiments, data were leak-subtracted on line by a P/4 protocol. Recordings were digitized with a Digidata 1200 analog-to-digital interface, driven by Clampex 7 software (Axon Instruments, Foster City, CA) in a Pentium clone computer.

Current-clamp experiments were performed at 37°C under the whole-cell configuration of the patch-clamp technique using the Axopatch 1D amplifier (Axon Instruments). In those experiments in which the effects of hypoxia and 4-aminopyridine (4-AP) on the membrane potential were tested, the perforated patch configuration with amphotericin B was used. In these experiments the pipette solution contained (in mM): 40 KCl, 95 potassium glutamate, 10 HEPES, and 8 CaCl<sub>2</sub>, pH 7.2, with KOH and the bath solution contained (in mM): 116 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1.1 MgCl<sub>2</sub>, 10 HEPES, and 24 NaCO<sub>3</sub>H, pH 7.4, with 5% CO<sub>2</sub>. Hypoxia was achieved by bubbling the reservoir that fed the perfusion chamber with 5% CO<sub>2</sub> and



**Figure 1.** Dominant-negative constructs block the expression of K<sup>+</sup> currents in a subfamily-specific manner. The figure shows the density of the currents elicited in CHO cells after transfection with plasmids encoding for Kv4.3, Kv1.3, and Kv1.4, either alone (open bars) or together with the dominant-negative constructs Kv4.3W362F (Kv4.xDN) or Kv1.3AYA (Kv1.xDN), as indicated. Data are the mean  $\pm$  SEM of 7–15 cells. \* $p$  < 0.01; \*\* $p$  < 0.001.

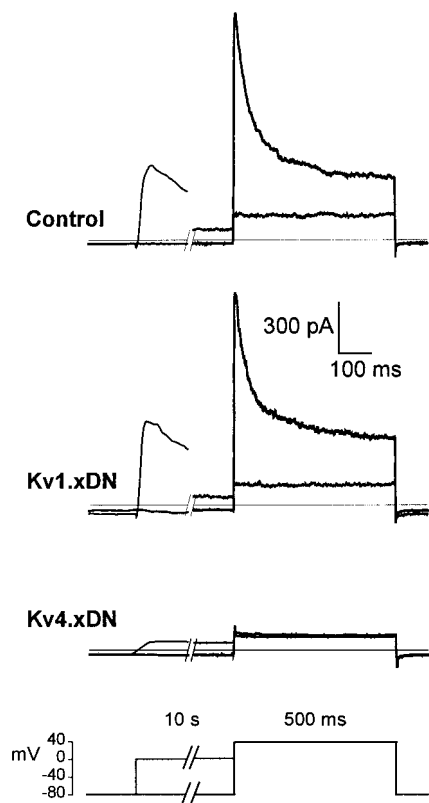
95% N<sub>2</sub>, Amphotericin B (Sigma, St. Louis, MO) was prepared fresh every 2 hr, dissolved in DMSO, and added to the perforated patch pipette solution to a final concentration of 0.23  $\mu$ g/ $\mu$ l. 4-AP was also obtained from Sigma and prepared fresh each day to a final concentration of 1 mM in bath solution. The pH of the solution was readjusted after adding the drug.

**Analysis.** Analysis of the data were performed with the Clampfit subroutine of the pClamp software and Origin 4.0 software (Microcal). Pooled data are expressed as mean  $\pm$  SEM. Statistical comparisons between groups of data were performed with the two-tailed Student's  $t$  test for paired or unpaired data, and values of  $p$  < 0.05 were considered statistically different.

## RESULTS

### Transfection of the dominant-negative constructs

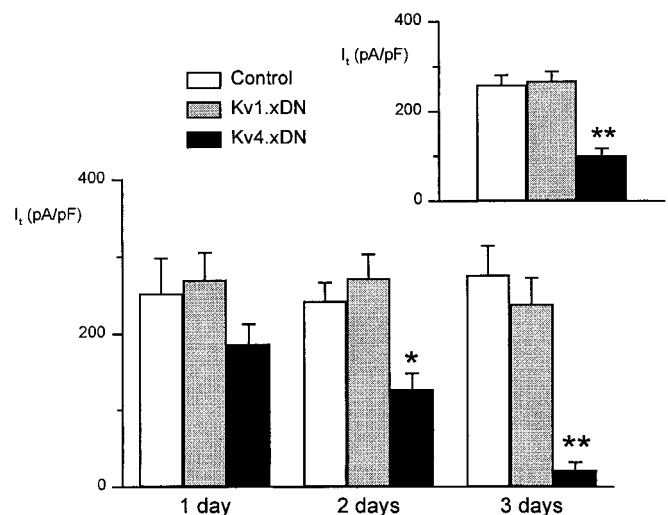
To verify that the mutants Kv1.3AYA and Kv4.3W362F act as dominant-negative suppressors of ionic current, we first investigated their ability to eliminate the corresponding wild-type channels in a heterologous expression system and the specificity of the suppression within the same subfamily. The results of these control experiments are shown in Figure 1. When CHO cells were transfected with a construct expressing Kv4.3, all the cells studied showed transient outward currents with a peak amplitude of  $167 \pm 7$  pA/pF ( $n = 13$ ). The amplitude of these currents was significantly reduced to  $75.3 \pm 4.9$  pA/pF ( $n = 17$ ) after cotransfection of Kv4.3 with Kv4.3W362F (Kv4.xDN). The effect of Kv1.3AYA (Kv1.xDN) suppressing its parent channel was even clearer, and the current density decreased from  $677.7 \pm 35.4$  pA/pF in the cells transfected with Kv1.3 ( $n = 5$ ) to  $90.2 \pm 28.8$  pA/pF in the Kv1.3 + Kv1.xDN cotransfected cells ( $n = 5$ ). We also investigate whether Kv1.xDN cross-reacts with other members of the Kv1 subfamily; in particular, for the purpose of our study, it was crucial to verify that Kv1.xDN would be able to eliminate transient outward currents carried by Kv1 channels (i.e., Kv1.4). In the bottom part of Figure 1 we show that the mean peak current amplitude obtained in six CHO cells expressing Kv1.4 channels ( $149.4 \pm 20.1$  pA/pF) is decreased by cotransfection with Kv1.xDN ( $27.5 \pm 23.4$  pA/pF;  $n = 5$ ), but is unchanged by coexpression with Kv4.xDN ( $165.6 \pm 24.1$  pA/pF;  $n = 5$ ). In all these experiments, we include as a control a dominant-negative construct for another channel family, to exclude nonspecific effects of expressing a membrane protein. The dominant-negative controls used in the experiments shown were either Kir2.1AAA or KvLQTG306R, and we did not observe any difference when comparing the currents in the absence or in the presence of these controls (data not shown).



**Figure 2.** Infection of rabbit CB chemoreceptor cells with AdKv4.xDN abolishes the transient component of K<sup>+</sup> current. The figure shows currents recorded from three individual CB chemoreceptor cells after coinfection of the receptor virus (AdVgRXR) with virus expressing either GFP alone (AdEGI, control), AdKv1.xDN, or AdKv4.xDN. In all cases, the currents were elicited with the voltage protocol shown at the bottom, in which 500 msec depolarizing steps to +40 mV follow 10 sec prepulses to two different potentials, -80 mV (to fully activate the current, *thicker trace*), and 0 mV (to inactivate the transient component). The difference between the current amplitude at +40 mV in these two pulses is defined throughout the paper as the transient outward current.

### Effects of adenoviral infection on transient outward currents of CB chemoreceptor cells

CB chemoreceptor cells infected with AdEGI, AdKv1.XDN, or AdKv4.xDN were studied 1–3 d after induction with ponasterone A. To confirm efficient infection and inductibility, all experiments included several control wells in which the cells were either not infected, infected with AdVgRXR + AdEGI and not induced with ponasterone A, or infected with adenovirus constitutively expressing GFP (AdCGI). In the two first groups we did not observe any GFP fluorescence, and in the latter group the number of GFP-fluorescent cells increased with time in culture, reaching up to 70% of the cells at day 4 after infection. When characterized electrophysiologically, there were no differences among these three groups of control cells or between them and the control cells infected with AdVgRXR + AdEGI and induced with ponasterone A (data not shown); this excludes nonspecific effects of the infections on the electrical properties of the chemoreceptor cells. Transient outward currents were studied with a two-pulse protocol (Fig. 2), in which the currents were elicited after depolarization to +40 mV after 10 sec prepulses to two different potentials, -80 mV (to obtain the fully primed current), and 0 mV (to inactivate the transient component). Thus, transient or inactivating current was defined as the difference between the current elicited by the two pulses. Figure 2 shows representative traces obtained in cells infected with AdEGI, AdKv1.xDN, and AdKv4.xDN. Apparently, there is no difference between the transient currents from chemoreceptor cells infected with AdEGI (control) or with AdKv1.xDN; in both cases, the amplitude and the time course of the inactivation of the transient



**Figure 3.** Time course of the effects of adenoviral infection on the amplitude of the transient current of CB chemoreceptor cells. Summary data for the peak current density of the transient outward current ( $I_t$ ) was obtained applying the voltage protocol described in Figure 2 and subtracting the prepulse inactivated current (0 mV prepulse) from the fully primed current (-80 mV prepulse). In all cases, CB chemoreceptor cells cultures were infected overnight with AdVgRXR together with AdEGI (control; *open bars*), AdKv1.xDN (gray bars), or AdKv4.xDN (black bars), after which expression was induced by adding 10  $\mu$ M Ponasterone A for the indicated amount of time. The *inset* shows the averaged data for all the cells studied regardless of the length of the induction period. \* $p < 0.005$ ; \*\* $p < 0.0001$ .

component of the currents was similar. On the contrary, infection with AdKv4.xDN suppresses the inactivating current in CB chemoreceptor cells. The adenoviral-induced dominant-negative construct in chemoreceptor cells acts to suppress the transient current selectively, because the sustained component of the outward current is not affected. This effect of the infection with AdKv4.xDN was evident in all cells studied, and the magnitude of the suppression was clearly related with the time of induction (see below). However, in some cells there was not a complete elimination of the transient current after 72 hr exposure to ponasterone A. This small residual current showed a time course of inactivation identical to the control currents and reflects in all likelihood the competition between the turnover rate of the functional channel subunits and the time and amount of expression of the Kv4.3W362F gene product.

### Time course of induction of dominant-negative expression

Dominant-negative suppression of K<sup>+</sup> currents involves expression of a nonfunctional subunit that is capable of assembling with wild-type subunits to disable their function. With the assumption that channels containing one or more mutant subunits are sufficient to “knock-out” function, the relative reduction of the current should increase with increasing concentrations of the nonfunctional subunit, either because the mutant subunits coassemble with endogenous channels and give rise to nonfunctional channels on the cell membrane or because the multimeric complex containing the mutant protein is recognized and degraded (Babila et al., 1994; Tinker et al., 1996; Lalli et al., 1998). With the ecdysone-inducible system, this titration of the effect of the mutant subunit can be obtained by increasing the time of induction of the adenoviral constructs (Johns et al., 1999), to confirm the behavior expected for heterotetrameric channels. Figure 3 shows the averaged current density for the transient current obtained in the three experimental groups (Control Kv1.XDN and Kv4.xDN) at different times after induction of the corresponding adenoviral constructs. The transient current density of control cells was not modified by the time in culture and remains essentially unchanged in the cells. However, chemoreceptor cells infected with Kv4.xDN exhibit a clear time-

dependent reduction in the density of the transient outward current. This current is reduced by 26% after 1 d of induction with ponasterone A, and the reduction reaches 47% after 2 d and 92% after 3 d.

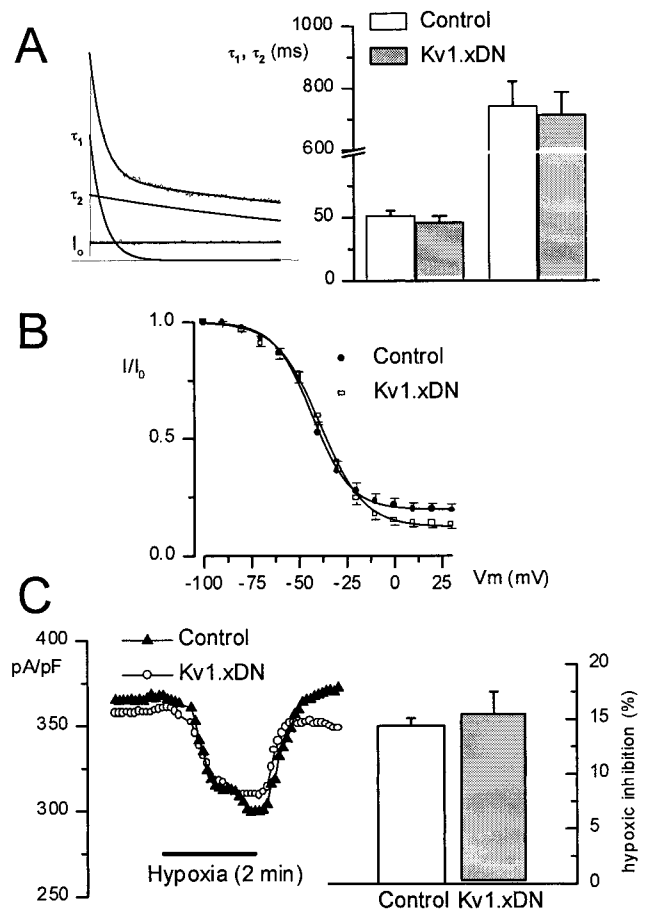
### Effect of AdKv1.xDN in the kinetics and the functional responses of transient outward currents from chemoreceptor cells

Although the data shown in Figure 3 suggest that the transient component of K<sup>+</sup> current in these cells is almost entirely carried by K<sup>+</sup> channels of the Kv4 subfamily, it is also possible that other non-Kv4 subunits contribute to a minor fraction of the transient outward current in some of the cells. To test this latter possibility, we have studied in more detail the effects of AdKv1.xDN infection on the kinetics and the functional response of A-type currents of CB chemoreceptor cells. Figure 4*A* shows the analysis of the time course of the inactivation of the transient component of K<sup>+</sup> currents in control and AdKv1.xDN infected chemoreceptor cells. In both cases, the inactivation pattern was best fitted to a biexponential function with time constants that were not significantly different between the two groups. We have also look for more subtle changes after AdKv1.xDN infection, investigating the inactivation in the steady-state for the two groups, control and Kv1.xDN cells (Fig. 4*B*). The steady-state inactivation curves were obtained by normalizing the peak current obtained by depolarizing pulses to +40 mV preceded by a 10 sec prepulse to potentials between -100 and +30 mV to the peak current obtained after the -100 mV prepulse ( $I_0$ ). The figure shows the fit of the pooled data of each group to Boltzmann functions. However, when the data obtained for each individual cell was fitted to a Boltzmann function, we found that whereas the slope of the functions ( $8.98 \pm 0.53$  in control cells vs  $9.9 \pm 0.49$  in Kv1.xDN cells) and the midpoint of inactivation ( $V_{1/2}$ ,  $-41.6 \pm 2.0$  mV in control cells vs  $-39.2 \pm 1.7$  mV in Kv1.xDN cells) were not statistically different between the two groups, the fraction of noninactivating current was significantly decreased in the cells infected with Kv1.xDN, averaging  $0.18 \pm 0.025$  in the control and  $0.12 \pm 0.014$  in the Kv1.xDN cells ( $p < 0.05$ ).

Nevertheless, low pO<sub>2</sub> inhibition represents only a small fraction of the amplitude of the transient outward K<sup>+</sup> current of chemoreceptor cells (López-López et al., 1993), making conceivable the hypothesis that Kv1.4 could contribute to the component of the transient outward K<sup>+</sup> current that is inhibited by hypoxia, so that its blockade by the Kv1.xDN construct could be unperceived when looking at the transient current amplitude caused by the cell to cell variability (Pérez-García et al., 1992). To explore this possibility, we have studied if hypoxic inhibition of the transient K<sup>+</sup> current is still present in Kv1.xDN cells. In Figure 4*C* we show the peak current amplitude elicited by depolarizing steps to +40 mV applied every 5 sec in a control (AdEGI-infected) CB chemoreceptor cell (top graph) or in a cell infected with the Kv1 dominant-negative construct (bottom graph). At the indicated times, the bath solution was replaced with an N<sub>2</sub>-equilibrated solution, and a reduction in the amplitude of the current can be observed in both cases (17% in the control cell and 14.5% in the Kv1.xDN cell). This effect was consistently obtained in another eight cells in each group, in which hypoxic inhibition of the transient K<sup>+</sup> current averaged  $14.5 \pm 0.7\%$  in control cells and  $15.5 \pm 2\%$  in the Kv1.xDN cells.

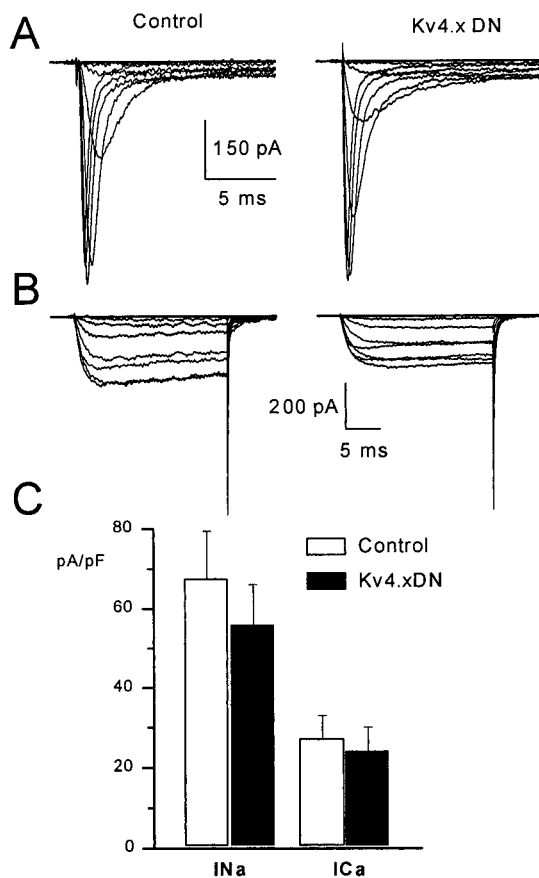
### AdKv4.xDN infections in CB chemoreceptor cells do not affect inward currents

The results presented so far indicate that the genes of the Kv4 subfamily could represent the molecular constituents of the transient outward current of CB chemoreceptor cells. However, a toxic, nonspecific effect of AdKv4.xDN infection in our preparation needs to be excluded. The specificity of the effect was insinuated by the fact that we did not see any modification in the sustained component of the outward current in Kv4.xDN cells (Fig. 2); furthermore, inward currents kinetically similar to those described through voltage-dependent Na<sup>+</sup> channels were recorded when studying the outward currents in several cells with a complete removal of the



**Figure 4.** AdKv1.xDN does not modify transient outward K<sup>+</sup> currents in chemoreceptor cells. The effects of infection with Kv1.xDN were studied with the same two-pulse voltage protocol described in Figure 2. *A*, The inactivation time course of the transient currents elicited at +40 mV in control (AdEGI infected) and AdKv1.xDN-infected cells was fitted by the following function:  $I = I_0 + A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2}$ , where  $I_0$  was selected as a fix parameter during the fitting procedure, being in each cell the sustained current obtained in the pulse after the 0 mV prepulse. One example of  $I$ ,  $I_0$ ,  $A_1 e^{-t/\tau_1}$ , and  $A_2 e^{-t/\tau_2}$  is shown overlaid to the current traces obtained in a cell. The average of the two time constants ( $\tau_1$  and  $\tau_2$ ) are represented in the right graph. Each point is the mean  $\pm$  SEM of 36 and 69 data for control and Kv1.xDN, respectively. *B*, The steady-state inactivation curves were constructed by normalizing the peak current elicited with a depolarizing pulse to +40 mV preceded by a 10 sec prepulse to potentials between -100 and +30 mV in chemoreceptor cells infected with AdEGI (Control) or with AdKv1.xDN. Each point is the mean  $\pm$  SEM of 22–25 determinations. Lines represent the best fit of the data to Boltzmann functions. *C*, Peak current densities of currents recorded in AdEGI (control, filled triangles) or AdKv1.xDN (open circles)-infected cells obtained by applying depolarizing steps to +40 mV every 5 sec are plotted against time. During the period indicated with the bar, the cell was exposed to an N<sub>2</sub>-equilibrated solution. The column plot shows the average inhibition (mean  $\pm$  SEM) obtained in eight similar experiments in each group.

transient component (data not shown). To strengthen this point, we have studied in more detail the effects of AdKv4.xDN infections on the voltage-dependent inward currents present in chemoreceptor cells. Representative traces of families of Na<sup>+</sup> and Ca<sup>2+</sup> currents obtained in control (AdEGI) and AdKv4.xDN-infected cells 3–4 d after induction with ponasterone A are shown in Figure 5, *A* and *B*. Na<sup>+</sup> currents (Fig. 5*A*) were obtained in the presence of 100  $\mu$ M Cd<sup>2+</sup> in the bath solution to block the Ca<sup>2+</sup> component of the inward currents, and Ca<sup>2+</sup> currents (Fig. 5*B*) were isolated with the application of 100 nM TTX. The average Na<sup>+</sup> and Ca<sup>2+</sup> current density obtained in 10–18 cells in each group (control and Kv4.xDN) is shown in Figure 5*C*. For Na<sup>+</sup> currents, the mean current density was  $75.4 \pm 18.8$  pA/pF in control cells and  $56.1 \pm 10.0$  pA/pF in the Kv4.xDN cells, and for Ca<sup>2+</sup> currents  $27.3 \pm 5.7$

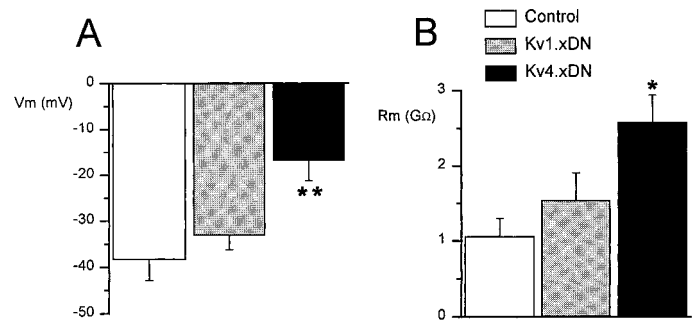


**Figure 5.** AdKv4.x infection does not modify voltage-dependent inward currents in CB chemoreceptor cells. *A*, Sodium currents recorded in a control cell (AdEGI-infected) and in a AdKv4.xDN-infected cell 3 d after induction with ponasterone A. The currents were elicited by 20 msec depolarizing pulses to potentials from  $-60$  to  $+60$  mV in  $10$  mV intervals from a holding potential of  $-80$  mV. The bath solution contained  $100$   $\mu$ M Cd<sup>2+</sup>. *B*, Calcium currents were elicited with the same voltage protocol in the presence of  $100$  nM TTX. The figure shows records obtained in each of the two experimental conditions (AdEGI and AdKv4.xDN infection). *C*, Summary of the peak Na<sup>+</sup> and Ca<sup>2+</sup> current densities obtained in control and Kv4.xDN cells. The voltage pulse at which the peak current amplitude was measured was  $+10$  mV for the Ca<sup>2+</sup> current and  $+20$  mV for the Na<sup>+</sup> current. Each bar represents the mean  $\pm$  SEM of 10–18 determinations.

in the control and  $24.4 \pm 7.7$  in the Kv4.xDN group. These results confirm the specificity of AdKv4.xDN infections suppressing the transient component of the outward current of CB chemoreceptor cells.

#### Modifications of resting membrane potential by overexpression of the dominant-negative constructs

The most accepted model of chemotransduction process in the rabbit CB chemoreceptors proposes that low pO<sub>2</sub> inhibition of K<sup>+</sup> current is linked to depolarization of the cells, leading to Ca<sup>2+</sup> entry and neurotransmitter release (Gonzalez et al., 1994). For this model to be sustained, transient outward K<sup>+</sup> currents must contribute to the resting membrane potential of chemoreceptor cells. We have explored this possibility looking at the effects of Kv1.xDN and Kv4.xDN on the resting membrane potential of the cells under current-clamp conditions (Fig. 6*A*). The resting membrane potential measured in nine control cells in the whole-cell configuration was  $-38.3 \pm 4.6$  mV, not differing from the membrane potential found in Kv1.xDN cells ( $-33.6 \pm 3.1$  mV;  $n = 18$ ). However, the cells infected with the Kv4.xDN showed a significant change in their membrane potential toward more depolarized values, which averaged  $-16.9 \pm 4.3$  in 16 cells studied. These data suggest that in fact Kv4 channels contribute to fix the resting membrane potential



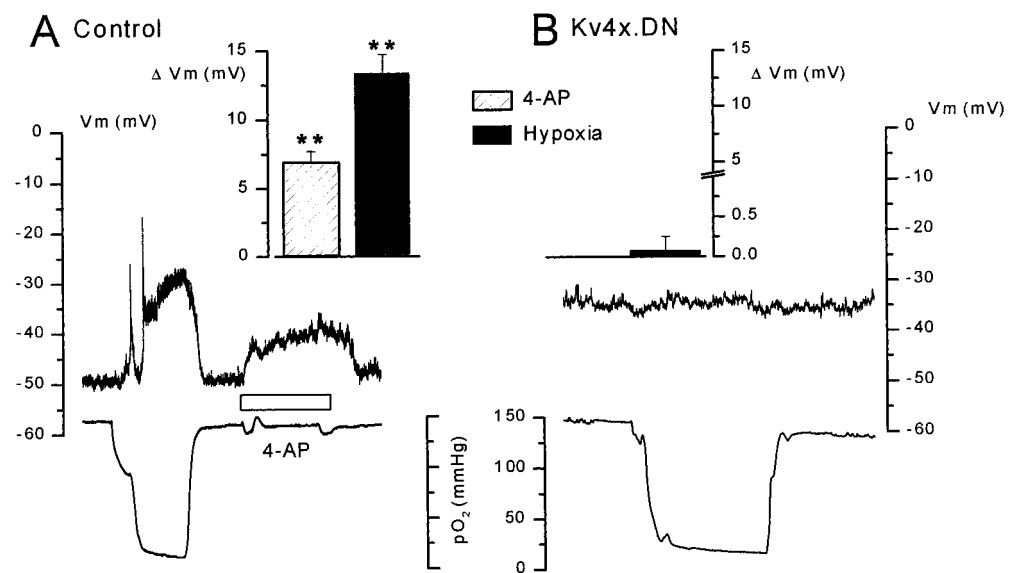
**Figure 6.** Effect of adenoviral infection with dominant-negative constructs on resting membrane potential and input resistance. *A*, Values of resting membrane potential obtained in current-clamp experiments in control (open column), Kv1.xDN (gray column), and Kv4.xDN (black column) cells in the whole-cell configuration 3 d after induction with ponasterone A. The measured values of resting membrane potential averaged  $-38.3 \pm 4.6$  in control cells ( $n = 9$ ),  $-33.2 \pm 3.1$  in Kv1.xDN-infected cells ( $n = 18$ ), and  $-16.9 \pm 4.3$  in Kv4.xDN-infected cells ( $n = 16$ ).  $**p < 0.005$  as compared to control. *B*, Average values for input membrane resistance obtained in the same conditions for the three experimental groups. Each bar is the mean  $\pm$  SEM of 5–10 cells.  $*p < 0.05$  as compared to control.

of CB chemoreceptor cells and are also in concordance with the observation that the input membrane resistance of AdKv4.xDN-infected cells ( $2.58 \pm 0.35$  GΩ) is significantly increased when compared with AdEGI ( $1.05 \pm 0.24$  GΩ) or AdKv1.xDN-infected cells ( $1.6 \pm 0.36$  GΩ; Fig. 6*B*). To confirm that this effect of Kv4.xDN can be explained by the blockade of the transient outward current, we have investigated if other maneuvers that inhibit this current also lead to chemoreceptor cell depolarization. In Figure 7*A*, the effect of hypoxia and 4-AP on the resting membrane potential of a control cell recorded in the perforated-patch configuration is shown. Decrease in pO<sub>2</sub> by perfusion with an N<sub>2</sub>-equilibrated solution leads to a significant depolarization of the cell ( $24$  mV in the example shown) that is readily reversible after returning to normoxic bath solution. In this same cell, perfusion with a solution containing  $1$  mM 4-AP, which has been reported to selectively block the transient component of the outward K<sup>+</sup> current in CB chemoreceptor cells (López-López et al., 1993), also produced a marked depolarization. The bottom part of the graph shows simultaneous recording of the pO<sub>2</sub> in the bath obtained with an oxygen microelectrode placed close to the cell. Similar results were obtained in 15 more cells, in which the average depolarization obtained was  $13.3 \pm 1.4$  mV with low pO<sub>2</sub> and  $7.0 \pm 0.7$  with  $1$  mM 4-AP (see inset). In Figure 7*B* records from an experiment in which effect of hypoxia was studied in a cell infected with AdKv4.xDN under the same conditions are shown. In the Kv4.xDN cells, the resting membrane potential was always more depolarized than in control cells from parallel cultures, averaging (with the perforated-patch configuration)  $-23.02 \pm 3.3$  for Kv4.xDN ( $n = 22$ ) versus  $-52.0 \pm 3.3$  for control cells ( $n = 15$ ). In addition, hypoxia was without effect in all the cells studied (see inset), suggesting that the blockade of the transient component of the current abolishes the low pO<sub>2</sub>-induced depolarization of CB chemoreceptor cells.

#### DISCUSSION

In the present study we have used dominant-negative constructs to eliminate the main contributors to the transient outward K<sup>+</sup> currents described in several tissues, namely Kv1.4, Kv4.2 and Kv4.3 channels (Sheng et al., 1992, 1993; Barry and Nerbonne, 1996; Dixon and McKinnon, 1996; Serodio and Rudy, 1998), and we demonstrate that *Shal* K<sup>+</sup> channels represent the major constituent of the O<sub>2</sub>-sensitive, transient K<sup>+</sup> current of rabbit CB chemoreceptor cells. These Kv4 channels have been shown to be the principal contributors to the transient outward current in other preparations such as rat atrial and ventricular myocytes (Fiset et al., 1997; Johns et al., 1997; Bou-Abboud and Nerbonne, 1999; Xu

**Figure 7.** Hypoxia induced depolarization in control but not in AdKv4.xDN-infected CB chemoreceptor cells. The experiments shown in this figure were obtained in current-clamp experiments using the perforated-patch technique. The bath solutions (which were kept at 37°C) contained bicarbonate buffer, so that pH was equilibrated by bubbling with a 5% CO<sub>2</sub>–20% O<sub>2</sub>–75% N<sub>2</sub> gas mixture. **A**, The graph shows the value of the resting membrane potential in one control cell during the application of a hypoxic solution (equilibrated with 5% CO<sub>2</sub> and 95% N<sub>2</sub>) and a solution containing 1 mM 4-AP during the time indicated with the bar. Simultaneous register of the pO<sub>2</sub> value in the bath solution is shown in the bottom part of the graph. The average depolarization (mean ± SEM) obtained with these two maneuvers is represented in the inset as  $\Delta V_m$  ( $n = 16$  in each group). The values of resting membrane potential before and during application of the stimuli were statistically significant when compared with the two-tailed Student's *t* test for paired data (\*\* $p < 0.001$ ). **B**, The effect of hypoxia was studied in a AdKv4.xDN-infected cell using the same experimental protocol. Hypoxia did not produce any significant change on the resting membrane potential in all the Kv4.xDN studied ( $n = 22$ ), as shown in the inset.



et al., 1999) as well as to the A-type currents in several neuronal tissues (Baro et al., 1997; Serodio and Rudy, 1998).

The O<sub>2</sub>-sensitive, transient K<sup>+</sup> current of rabbit CB chemoreceptor cells has been characterized with electrophysiological and pharmacological approaches (see introductory remarks), but nothing is known about its molecular nature, because the small size of the rabbit CB (~300  $\mu$ g), together with the cellular heterogeneity of this chemoreceptor, has precluded the use of conventional molecular biology techniques to identify the K<sup>+</sup> channels present in the organ. However, determination of the molecular constituents of the O<sub>2</sub>-sensitive K<sup>+</sup> currents in native tissues is a relevant issue, not only to understand the molecular mechanisms of O<sub>2</sub> detection in hypoxia-sensitive tissues, but also to provide a physiological meaning to the reported O<sub>2</sub> modulation of cloned channels expressed in heterologous systems (Patel et al., 1997; Fearon et al., 1999; Hulme et al., 1999; Pérez-García et al., 1999).

Selective gene suppression by using antisense methodologies or dominant-negative constructs has become a useful method for elucidating protein function experimentally. The use of dominant-negative constructs is particularly helpful in manipulating K<sup>+</sup> channels because they form multimers in the plasma membrane, and one dysfunctional K<sup>+</sup> channel subunit can be enough to disable an otherwise normal tetrameric complex (Tinker et al., 1996; Johns et al., 1997). Delivery of dominant-negative constructs into native cells can be achieved by transgenic approaches, but developmental adaptations or possible lethal effects may complicate the interpretation of such experiments (Barry et al., 1998). These problems can be partially circumvented by the use of recombinant adenoviruses to introduce the dominant-negative constructs into native cells. In the present work, we have benefit of a recent improvement of this strategy by using ecdysone-inducible adenoviral constructs (No et al., 1996; Johns et al., 1999). The ecdysone regulatory system exhibits lower basal activity and higher inducibility, and the use of polycistronic vectors allows sensitive detection of the infected cells without toxicity caused by overexpression of the channel constructs (Johns et al., 1999).

Our control experiments in CHO cells (Fig. 1) show that Kv channel  $\alpha$  subunits with critical mutations in the pore-forming region (Kv1.3AYA and Kv4.3W362F) behave as dominant-negatives, coassembling with wild-type channels in a subfamily-specific way to functionally suppress the current through the heteromultimeric channels. When introduced in chemoreceptor cells of the rabbit CB, only Kv4.3W362F (Kv4.xDN) infections produce a time-dependent suppression of the transient outward current (Figs. 2, 3), whereas this current was unaffected by adenoviral

transfer of Kv1.3AAYA (Kv1.xDN; Figs. 2, 4). Taking into account that Kv1.3AAYA was able to reduce in a significant way the amplitude of the Kv1.4 currents when expressed in an heterologous system (Fig. 1), the lack of effect of Kv1.xDN on the O<sub>2</sub>-sensitive K<sup>+</sup> current of rabbit CB chemoreceptor cells rules out a possible contribution of Kv1.4 channels to this current; moreover, we can also exclude the presence in the cells of heterotetrameric complexes of other members of the Kv1 subfamily that, in association with Kv $\beta$  subunits, could give rise to rapidly inactivating currents, as described in other preparations (Pongs et al., 1999). However, the observed reduction in the magnitude of the noninactivating current in the Kv1.xDN-infected chemoreceptor cells (Fig. 4) indicates that there are some Kv1 channels expressed in the cells, which functionally contribute to the sustained outward current.

Regarding the O<sub>2</sub>-sensitive transient outward K<sup>+</sup> current of rabbit CB chemoreceptor cells, with the findings reported here it is reasonable to conclude that it is carried mainly, if not exclusively, by channels of the Kv4 subfamily. We have not studied the contribution to this current of channels from other subfamilies, such as Kv3.4, which also expresses transient outward currents in neuronal tissues (Rudy et al., 1999); however, the fact that we often see a complete disappearance of the transient current after 3 d of infection with AdKv4.xDN makes unlikely a significant contribution of non-Kv4 channels to this current. In the light of this observation, our recent report showing that Kv4.2 but not *Shaker* channels are sensitive to hypoxia when coexpressed with Kv $\beta$ 1.2 in an heterologous expression system (Pérez-García et al., 1999) acquires physiological relevance; provided that low pO<sub>2</sub> modulation of Kv4 channels does actually happen in native tissues, it will be interesting to study the presence, distribution, and function of Kv $\beta$  subunits in the CB chemoreceptor cells in order to examine if they constitute a structural requirement for the O<sub>2</sub>-sensitive K<sup>+</sup> currents.

Viral gene transfer of the dominant-negative constructs into chemoreceptor cells not only provides a tool to determine the molecular nature of their macroscopic ionic currents, but could also help to understand the contribution of these channels to excitability of the cells. As already mentioned in the introductory remarks, the most accepted hypothesis for the chemotransduction of hypoxic stimuli in the CB sustains that hypoxic inhibition of the O<sub>2</sub>-sensitive K<sup>+</sup> channel would lead to the Ca<sup>2+</sup>-dependent release of neurotransmitters via cell depolarization. In open contradiction with this hypothesis, it has been recently reported that 4-aminopyridine effects are not related to changes in [Ca<sup>2+</sup>]<sub>i</sub>, dopamine release, and chemosensory discharge from rat and cat

CBs (Roy et al., 1998); however, it is important to note that there is not a transient component in the outward currents described in these two species (Peers and O'Donnell, 1990; Chou and Shirahata, 1996), and, furthermore, that it has not been shown that 4-AP, at the concentrations used by Roy et al. (1998), is able to inhibit the O<sub>2</sub>-sensitive component of the K<sup>+</sup> currents in the two preparations. Our results in Figure 7A show that 4-AP and hypoxia are in fact able to depolarize rabbit CB chemoreceptor cells. Furthermore, we demonstrate that the selective removal of the transient outward current is able to abolish the hypoxia-induced depolarization of the cells (Fig. 7B). Although we cannot rule out the presence of other targets for the effect of hypoxia on resting membrane potential, the fact that all the maneuvers tested (AdKv4.xDN infection, low pO<sub>2</sub>, and 1 mM 4-AP application) are able to inhibit transient outward currents on the one hand and also to depolarize CB chemoreceptor cells on the other, suggests a causal relationship between these two effects, and thereby a contribution of the transient outward current to set the normal resting membrane potential of rabbit CB chemoreceptor cells. Additionally, the data presented here provide support to the hypothesis that inhibition of the O<sub>2</sub>-sensitive K<sup>+</sup> current by hypoxia could represent the trigger of the low pO<sub>2</sub> chemotransduction process in rabbit CB chemoreceptors.

In conclusion, our findings show that the inducible expression of dominant-negative Kv constructs in the CB chemoreceptor provides a powerful tool to identify the molecular components of the K<sup>+</sup> currents present in the cells and to dissect the contribution of these channels to the global response of the organ by allowing one to manipulate the excitability of the cells.

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