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Regulation of the resting potential of rabbit pulmonary artery myocytes by a low threshold, O_2-sensing potassium current*

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1 The contributions of specific K^+ currents to the resting membrane potential of rabbit isolated, pulmonary artery myocytes, and their modulation by hypoxia, were investigated by use of the whole-cell, patch-clamp technique.

2 In the presence of 10 μM glibenclamide the resting potential (-50 ± 4 mV, $n=18$) was unaffected by 10 μM tetraethylammonium ions, 200 nM charybdotoxin, 200 nM iberiotoxin, 100 μM ouabain or 100 μM digitoxin. The negative potential was therefore maintained without ATP-sensitive (K_{ATP}) or large conductance Ca^{2+}-sensitive (BK_{Ca}) K channels, and without the Na^+-K^+ATPase.

3 The resting potential, the delayed rectifier current ($I_{K(V)}$) and the A-like K^+ current ($I_{K(A)}$) were all reduced in a concentration-dependent manner by 4-aminopyridine (4-AP) and by quinine.

4 4-AP was equally potent at reducing the resting potential and $I_{K(V)}$, 10 mM causing depolarization from -44 mV to -22 mV with accompanying inhibition of $I_{K(V)}$ by 56% and $I_{K(A)}$ by 79%. In marked contrast, the effects of quinine on resting potential were poorly correlated with its effects on both $I_{K(A)}$ and $I_{K(V)}$. At 10 mM, quinine reduced $I_{K(V)}$ and $I_{K(A)}$ by 47% and 38%, respectively, with no change in the resting potential. At 100 μM, both currents were almost abolished while the resting potential was reduced $<50\%$. Raising the concentration to 1 mM had little further effect on $I_{K(A)}$ or $I_{K(V)}$, but essentially abolished the resting potential.

5 Reduction of the resting potential by quinine was correlated with inhibition of a voltage-gated, low threshold, non-inactivating K^+ current, $I_{K(N)}$. Thus, 100 μM quinine reduced both $I_{K(N)}$ and the resting potential by around 50%.

6 The resting membrane potential was the same whether measured after clamping the cell at -80 mV, or immediately after a prolonged period of depolarization at 0 mV, which inactivated $I_{K(A)}$ and $I_{K(V)}$, but not $I_{K(N)}$.

7 When exposed to a hypoxic solution, the O_2 tension near the cell fell from 125 ± 6 to 14 ± 2 mmHg ($n=20$), resulting in a slow depolarization of the myocyte membrane to -35 ± 3 mV ($n=16$). The depolarization occurred without a change in the amplitude of $I_{K(V)}$ or $I_{K(A)}$, but it was accompanied by 60% inhibition of $I_{K(N)}$ at 0 mV.

8 Our findings suggest that the resting potential of rabbit pulmonary artery myocytes depends on $I_{K(N)}$, and that inhibition of $I_{K(N)}$ may mediate the depolarization induced by hypoxia.

Keywords: Pulmonary artery; pulmonary artery myocytes; K current; potassium current; K channel; oxygen-sensitive K channel; hypoxia; 4-aminopyridine; quinine

Introduction

It is well known that alveolar hypoxia causes pulmonary vasoconstriction, although the primary mechanisms underlying the reponse are unclear. The ability to contract to hypoxia is thought to be intrinsic to the pulmonary arteries, because it is retained in pulmonary arteries that have been removed from the lung (Wadsworth, 1994; Weir & Archer, 1995). The endothelium appears to be involved in the response (Wadsworth, 1994), but it is unclear whether the endothelium initiates the response or modulates the responsiveness of the smooth muscle to hypoxia. Contractions in response to hypoxia have been observed after removal of the endothelium (Yuan *et al*., 1990; Jin *et al*., 1992; Leach *et al*., 1994), and hypoxia-induced contraction of pulmonary artery myoctyes has been observed in the absence of other cell types (Murray *et al*., 1990; Madden *et al*., 1992). Furthermore, pulmonary artery smooth muscle cells have also been shown to depolarize in response to acute hypoxia, both in the intact vessel (Madden *et al*., 1985) and after cell isolation (Post *et al*., 1992; Yuan *et al*., 1993; Cornfield *et al*., 1994). The depolarization is associated with inhibition of K^+ current (Post *et al*., 1992; Yuan *et al*., 1993; Weir & Archer, 1995), although the nature of the K^+ channel responsible for the depolarization is not yet clear. It is most likely that hypoxia inhibits a 4-aminopyridine (4-AP) sensitive, voltage-gated K^+ channel (Yuan *et al*., 1993; Post *et al*., 1995), rather than a Ca^{2+} activated K^+ channel (Post *et al*., 1992), since the former appear to be more important in regulating the membrane potential of pulmonary artery smooth muscle cells at rest (Yuan, 1995).

We recently described a novel 4-AP-sensitive K^+ current in rabbit pulmonary artery myocytes, denoted $I_{K(N)}$, which was active at the resting membrane potential (Evans *et al*., 1996). $I_{K(N)}$ is voltage-gated with a threshold for activation between -80 and -65 mV. It activates slowly (1.6 s time constant) without a delay, and does not inactivate during prolonged depolarization. Moreover, $I_{K(N)}$ could be distinguished from the delayed rectifier ($I_{K(V)}$) and transient, A-like ($I_{K(A)}$) currents by its different voltage dependence, kinetics and pharmacology. The aim of this study was to elucidate the contributions of different K^+ currents, including $I_{K(N)}$, to the resting potential of rabbit pulmonary artery myocytes, and to determine their roles in mediating membrane depolarization induced by hypoxia. Preliminary accounts of part of this work have been published in abstract form (Evans *et al*., 1995; Osipenko *et al*., 1995), where $I_{K(N)}$ was referred to as I_{KRP}.

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Methods

Cell isolation

Male New Zealand white rabbits (2–3 kg) were killed by intravenous injection of sodium pentobarbitone (80 mg kg^{-1}) and exsanguinated. The main pulmonary artery was dissected out and placed in physiological salt solution (PSS) of the following composition (mM): NaCl 124, KCl 5, $NaHCO_3$ 15, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.5, KH_2PO_4 0.5, glucose 10, 5-N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) 15, phenol red 0.04, adjusted to pH 7.3 by gassing with 95% O_2/5% CO_2. Connective tissue was carefully removed, the vessels opened along the longitudinal axis and cut into 1 mm wide strips. Single smooth muscle cells were isolated by a previously described method (Clapp & Gurney, 1991). Briefly, the muscle strips were washed in a low [Ca^{2+}] dissociation medium of composition (mM): NaCl 110, KCl 5, $NaHCO_3$ 15, $CaCl_2$ 0.16, $MgCl_2$1, NaH_2PO_4 0.5, KH_2PO_4 0.5, glucose 10, HEPES 15, phenol red 0.04, ethylenediaminetetraacetic acid (EDTA) 0.49, taurine 10, adjusted to pH 7.0 with 95% air/5% CO_2. They were then placed in 5 ml dissociation medium containing 0.25 mg ml^{-1} papain (Fluka Chemicals Ltd, Dorset, UK; cat. no. 76218) and 0.02% bovine serum albumin (Sigma Ltd, Poole, UK: fraction V, fatty acid and globulin free) and stored overnight in a refrigerator. The following day 0.2 mM dithiothreitol (Sigma) was added to the enzyme solution containing the tissue and the solution warmed to 37°C for 10 min. The tissue was then transferred to enzyme-free dissociation medium, and the smooth muscle cells isolated by trituration. Cells were stored in dissociation medium in a refrigerator until required and were studied within 2–5 h after isolation.

Electrophysiology

A 10 μl aliquot of cell suspension was placed on the glass bottom of a 400 μl experimental chamber, mounted on the stage of an inverted microscope. After a few minutes had been allowed for the cells to adhere to the bottom, they were superfused at 0.5–1 ml min^{-1} with PSS containing 10 μM glibenclamide, at room temperature (22–25°C). Except where indicated, the superfusing solution also contained 10 mM tetraethylammonium chloride (TEA). In some experiments, the K^+ concentration was raised to 130 mM by replacing the NaCl in the PSS with equimolar KCl. Solutions were continuously bubbled with a 95% O_2/5% CO_2 gas mixture to give pH 7.3.

Resting potential was measured as the zero-current potential under current-clamp conditions, whereas K^+ currents were recorded under voltage clamp. In each case, the whole-cell, patch-clamp technique was used with an Axopatch 1A or 200A patch-clamp amplifier (Axon Instruments Inc., Foster City, CA, U.S.A.). Patch pipettes (1–2 MΩ) were pulled from filamented borosilicate glass capillaries (Clark Electromedical Instruments, Pangbourne, U.K.) and filled with recording solution of the following composition (mM): KCl 130, $MgCl_2$ 1, ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) 1, HEPES 20, Na_2GTP 0.5, pH adjusted to 7.2 with KOH. The junction potential between the pipette and bath solution (2–4 mV) was cancelled before pipette-cell contact. Reported voltages were not corrected for junction potential errors, arising when the whole-cell configuration had been established. Series resistance was 4.5±0.3 MΩ (n=30) and was further minimized by electronic compensation. The input resistance and cell capacitance were measured from the current response to a voltage step from −80 to −90 mV. Voltage commands were generated with pCLAMP data acquisition software (versions 5.5 and 5.7; Axon Instruments), with a Labmaster TM-40 (Scientific Solutions Inc., Ohio, U.S.A.) or Digidata 1200 (Axon Instruments) interface. Currents were filtered at 0.5–5 kHz and stored on DAT or video tape, or digitized at 1–16 kHz and stored on disc with pCLAMP. Data analysis employed pCLAMP and ORIGIN (Microcal Software, Northampton, MA, U.S.A.) software. Current records were not leak subtracted unless stated. Data are quoted as mean±s.e.mean, unless otherwise noted. Statistical comparison employed one way analysis of variance (ANOVA), with significance assumed if $P<0.05$.

Solutions for studies on hypoxia

Experiments designed to study the influence of hypoxia on I_K and membrane potential employed a HEPES-buffered PSS of the following composition (mM): NaCl 112, $CaCl_2$ 1.8, KCl 5, $MgCl_2$ 1, glucose 10, TEA 10, glibenclamide 0.01, HEPES 21 (pH 7.3 with NaOH), phenol red 0.04. Normoxic solutions were prepared by equilibration with room air and hypoxic solutions by equilibration with N_2 gas. The oxygen tension (P_{O_2}) of the bathing solution during the experiments was monitored with an oxygen electrode (W.P.I. Inc., Sarasota, Florida) placed in the experimental chamber, 1–2 mm from the cell under study. The electrode was calibrated in the experimental salt solution.

Drugs

Stock solutions of TEA (1M; Fluka), charybdotoxin (10 μM; Peninsula Laboratories Europe Ltd, Merseyside, U.K.) and iberiotoxin (10 μM; Sigma) were prepared in 18 MΩ H_2O. Ouabain (1 mM; RBI, through Semat Technical (U.K.) Ltd, St. Albans), digitoxin (1 mM; Sigma), quinine sulphate (1 mM; Merck) and 4-aminopyridine (4-AP, 10 mM; Sigma) were prepared daily in PSS, the pH of the 4-AP solution being adjusted to 7.3 before dilution. Glibenclamide (10 mM; Sigma)

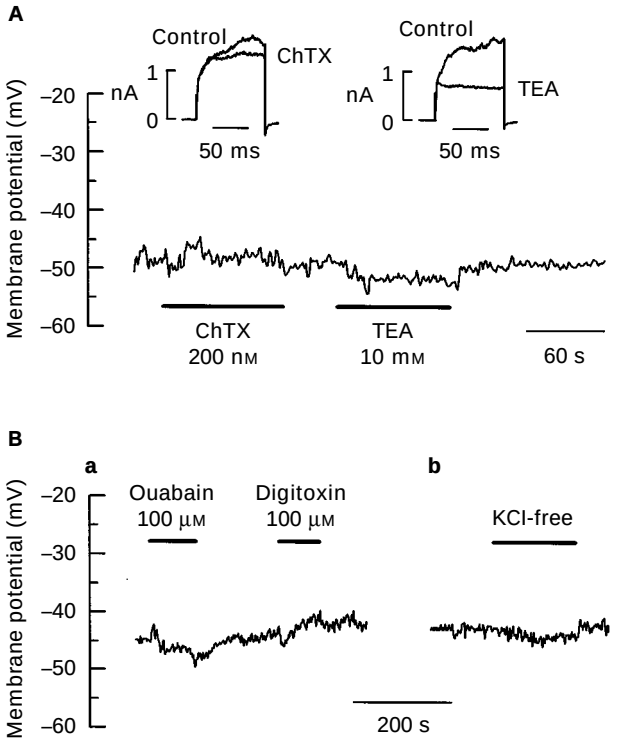

Figure 1 Effects of K^+ channel suppression and inhibitors of Na^+-K^+-ATPase on the resting potential of rabbit pulmonary artery myocytes. Resting potential recorded as the zero current potential under current clamp. (A) Records of resting potential with charybdotoxin (ChTX; 200 nM) and TEA (10 mM) applied as indicated by the bars. Inset records show I_K from the same cell, under control conditions and in the presence of ChTX or TEA. I_K activated by a step to 40 mV from a holding potential of −80 mV, is composed of $I_{K(A)}$, $I_{K(V)}$ and I_{KCa} (B) Effects on the resting potential of (a) ouabain (100 μM) and digitoxin (100 μM) and (b) extracellular KCl replacement with CsCl, applied as indicated by the bars. Glibenclamide (10 μM) was present throughout all experiments.

was dissolved in dimethylsulphoxide (DMSO), which was present at 0.1% after dilution. At this concentration, DMSO by itself had no effect on the K^+ currents recorded. Fresh experimental solutions were prepared daily by dilution in PSS. Drugs were applied close to the cell through plastic microcapillary tubes, with a home-made, gravity-driven, multichannel rapid perfusion system, which exchanged the solution around the cell within 0.5 s. The same system was used to change the O_2 tension around the cells, by switching between one channel containing the normoxic solution and another containing pre-equilibrated hypoxic solution.

Results

Resting membrane potential regulation in pulmonary artery myocytes

Previous studies showed that the resting potential of isolated pulmonary artery myocytes remains within the range measured in the intact blood vessel, following complete block of $I_{K(ATP)}$ with glibenclamide (Clapp & Gurney, 1992; Yuan, 1995). Our experiments were all carried out with 10 μM glibenclamide added to the PSS to block K_{ATP} channels. Under these conditions, the resting potential of rabbit pulmonary artery myocytes, measured as the zero current potential under current clamp, was -50 ± 4 mV ($n=18$). As shown in Figure 1A, this was unaffected by the additional application of 200 nM charybdotoxin (ChTX), 100 nM iberiotoxin (IbTX) or 10 mM tetraethylammonium chloride (TEA), in the presence of which the mean resting potentials were -52 ± 4 mV ($n=4$), -50 ± 3 mV ($n=3$) and -50 ± 1 mV ($n=78$), respectively. At the concentrations used, these drugs would all be expected to block large-conductance K_{Ca} channels maximally (Miller *et al*., 1985; Benham *et al*., 1986; Candia *et al*., 1992). Indeed, as illustrated for ChTX and TEA in Figure 1A (inset), they all reduced the K current (I_K) activated by steps to 40 mV from a holding potential of -80 mV. The amplitude of I_K at the end of a 100 ms step was reduced by $14\pm11\%$ ($n=4$) in the presence of 200 nM ChTX, $14\pm8\%$ ($n=5$) in the presence of 200 nM IbTX and by $33\pm5\%$ ($n=7$) in the presence of 10 mM TEA. Since TEA had a larger effect than ChTX or IbTX, it may have inhibited an additional current, probably $I_{K(V)}$. Nevertheless, the results imply that $I_{BK(Ca)}$ was not necessary to maintain the resting potential, so it was routinely blocked in all subsequent experiments by including 10 mM TEA in the perfusing solution. Thus all solutions contained 10 μM glibenclamide and 10 mM TEA.

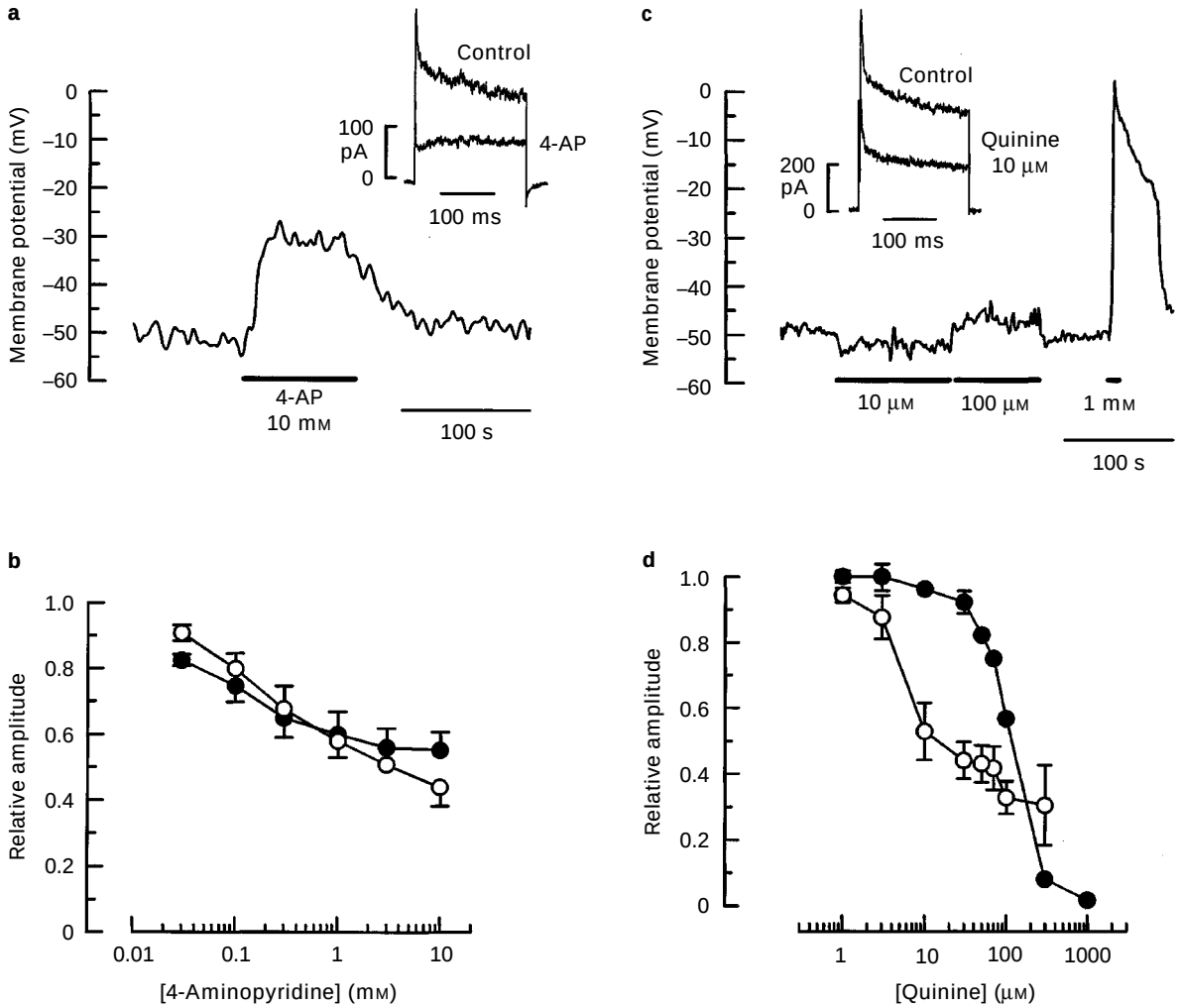

Figure 2 Reduction of resting potential and $I_{K(V)}$ by K channel blockers. (a) Effect of 10 mM 4-aminopyridine (AP) on resting potential, applied as indicated by horizontal bar. Inset records show I_K from the same cell, in the absence (control) and presence of 10 mM 4-AP. (b) Amplitudes of the resting potential (E_m; ●) and $I_{K(V)}$ (○) in the presence of 4-AP, measured relative to the values recorded under control conditions, plotted as a function of concentration. (c) Effect of quinine on resting potential, applied at the concentrations indicated under the bars. Inset records show the effect of 10 μM quinine on I_K recorded from the same cell. (d) Amplitudes of the resting potential (E_m; ●) and $I_{K(V)}$ (○) in the presence of quinine, measured relative to the values recorded under control conditions, plotted as a function of concentration. $I_{K(V)}$ amplitude was measured as the current at the end of a 100 ms step to 40 mV from -80 mV. Symbols and vertical lines represent mean$\pm$s.e.mean of $n=3-8$ (b) or $n=3-10$ (d). In this and all subsequent figures glibenclamide (10 μM) and TEA (10 mM) were present throughout.

Maintenance of a negative resting potential in a variety of vascular smooth muscles has been proposed to involve the membrane Na^+-K^+-ATPase (Kuriyama *et al*., 1982). If this were the case in rabbit pulmonary artery myocytes, then inhibition of Na^+-K^+-ATPase activity should lead to membrane depolarization. On the contrary, as illustrated in Figure 1Ba, the resting potential was unaffected by the Na^+-K^+-ATPase inhibitors ouabain (100 μM, $n=5$) and digitoxin (100 μM, $n=5$). Furthermore, perfusing the cells with PSS in which the KCl was replaced with equimolar CsCl, caused a slight hyperpolarization (-5 ± 2 mV, $n=4$); Figure 1Bb). In contrast, the resting potential collapsed to -0.4 ± 1 mV ($n=6$) when the K^+ in the pipette solution was replaced with equimolar Cs^+; this procedure essentially abolished I_K. Along with the hyperpolarization induced by K^+-free PSS, this supports the view that a background K^+ conductance was responsible for the negative resting potential.

Rabbit pulmonary artery myocytes are known to express a number of K^+ channels that are sensitive to block by 4-AP (Okabe *et al*., 1987; Clapp & Gurney, 1991; Evans *et al*., 1996). These include the transient current, $I_{K(A)}$, and delayed rectifier current, $I_{K(V)}$ (Clapp & Gurney, 1991; Evans *et al*., 1996), which are the main K^+ currents recorded in response to brief depolarizing voltage steps to 40 mV after $I_{BK(Ca)}$ and $I_{K(ATP)}$ have been inhibited (Evans *et al*., 1996). When exposed to 10 mM 4-AP, cells were found to depolarize reversibly from -44 ± 1 mV to -22 ± 2 mV ($n=12$, $P<0.001$) and, as illustrated in Figure 2a (inset), this was associated with $56\pm6\%$ and $79\pm1\%$ inhibition of $I_{K(V)}$ and $I_{K(A)}$, respectively ($n=4$). The transient current, $I_{K(A)}$, was estimated as the peak current activated within the first 20 ms of a step to 40 mV (Clapp & Gurney, 1991), while the sustained current at the end of the 200 ms step was taken as a measure of the more slowly activating $I_{K(V)}$ (Evans *et al*., 1994; 1996). The concentration-dependence of the inhibitory effects of 4-AP on the resting potential and on $I_{K(V)}$ were well correlated, as shown in Figure 2b, with 10 mM 4-AP required to reduce both parameters by 50%. Resting membrane potential suppression was less well correlated with block of $I_{K(A)}$ which, as previously found (Clapp & Gurney, 1991), was more sensitive than $I_{K(V)}$ to 4-AP. Another K channel-blocking drug, quinine, was also found to depolarize the membrane and to suppress $I_{K(V)}$ and $I_{K(A)}$ reversibly (Figure 2c). However, in marked contrast to 4-AP, quinine was more potent at reducing $I_{K(V)}$ than the resting membrane potential. It was also more effective at reducing $I_{K(A)}$ than the resting potential. Thus at 10 μM, quinine reduced $I_{K(V)}$ and $I_{K(A)}$ by $47\pm9\%$ and $38\pm4\%$, respectively ($n=5$), while having little, or no effect, on the resting potential (Figure 2c). The concentration-dependence of quinine inhibition of $I_{K(V)}$ is compared with its suppression of resting potential in Figure 2d. It is clear that inhibition of $I_{K(V)}$ (and $I_{K(A)}$; not shown) was near maximal (around 70%) at 100 μM, when the resting potential was re-

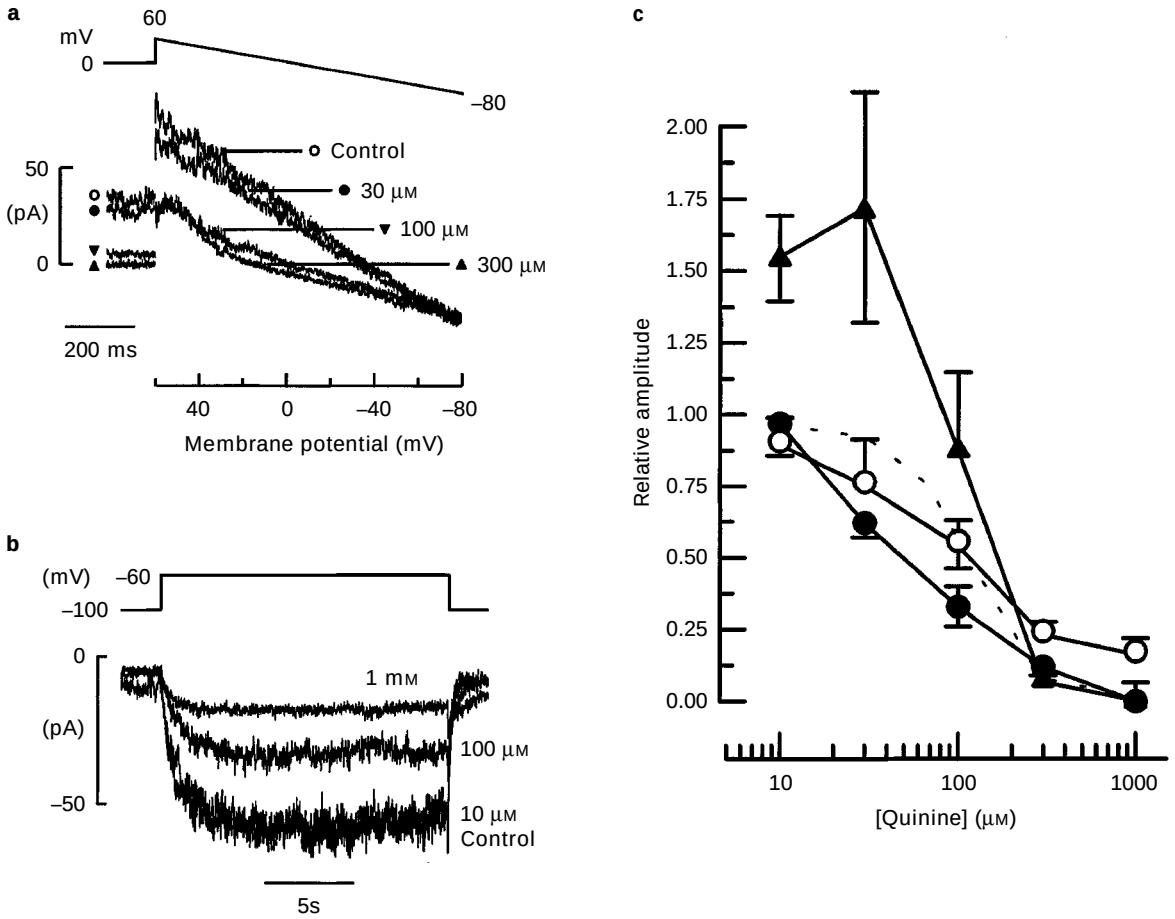

Figure 3 Comparison of the effects of quinine on $I_{K(N)}$ and resting potential. (a) Cells were voltage clamped at 0 mV for 5 min to inactivate $I_{K(A)}$ and $I_{K(V)}$, then stepped to 60 mV, followed immediately by a ramp to -80 mV at 0.2 mVms^{-1} to record $I_{K(N)}$ over a range of potentials. Current at the end of the 5 min period at 0 mV is shown along with the current during the ramp, under control conditions and during exposure to increasing concentrations of quinine, as indicated. Symbols indicate corresponding traces before and during ramp. (b) $I_{K(N)}$ activated in the presence of 130 mM extracellular K^+ by steps to -60 mV, from a holding potential of -100 mV. Records shown under control conditions and in the presence of increasing concentrations of quinine, as indicated. (c) Amplitudes of $I_{K(N)}$ at 0 mV (●) and -60 mV (○) in the presence of 5 mM K^+ (recorded with the ramp protocol), and at -60 mV in the presence of 130 mM K^+ (▲) (recorded with the step protocol), measured relative to the control current recorded in the same condition before drug addition and plotted as a function of quinine concentration. Symbols and vertical lines represent mean$\pm$s.e.mean of 3–7 (5 mM K^+) and 10 (130 mm K^+) observations. The broken line indicates the normalised resting potential measured in 5 mM K^+ as a function of quinine concentration (same data as in Figure 2d).

duced by less than 50%. Moreover, raising the quinine concentration further, to 1 mM, collapsed the resting potential almost completely without further inhibition of $I_{K(V)}$ or $I_{K(A)}$.

Evidence that the non-inactivating current, $I_{K(N)}$, controls the resting membrane potential

The results above suggest that the resting potential is set by a mechanism which is senstitive to block by 4-AP, but is relatively insensitive (compared to $I_{K(A)}$ and $I_{K(V)}$) to block by quinine. The low threshold, non-inactivating K^+ current, $I_{K(N)}$, recently identified in rabbit pulmonary artery smooth muscle cells and suggested to play a role in membrane potential regulation, was found to have these properties (Evans *et al.*, 1996). To investigate the role of $I_{K(N)}$ in setting the negative resting potential, we examined its sensitivity to quinine in more detail and compared it directly with the quinine sensitivity of the resting membrane potential.

To isolate $I_{K(N)}$ from $I_{K(A)}$ and $I_{K(V)}$, cells were clamped at 0 mV for at least 5 min, during which the latter two currents

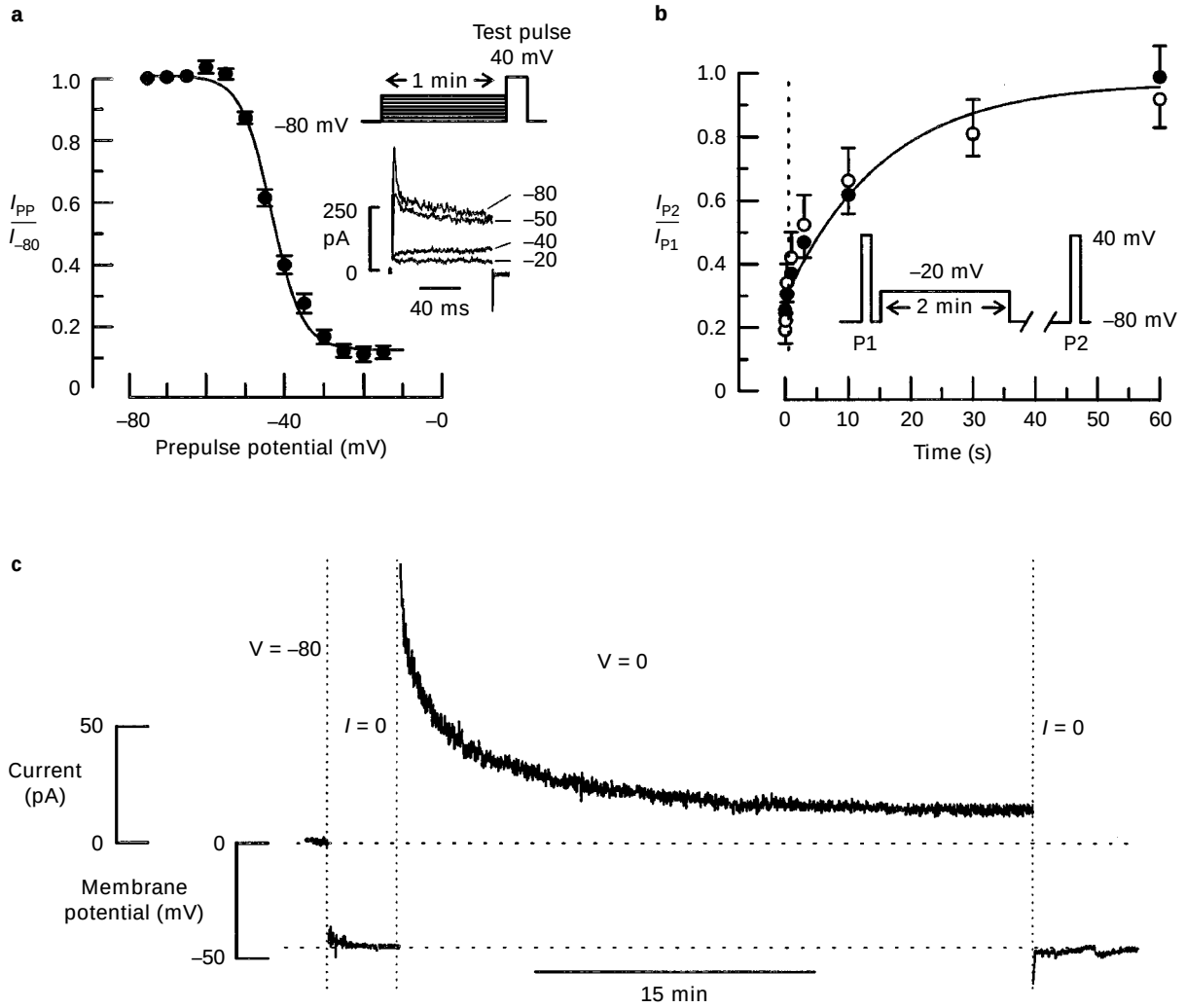

Figure 4 The resting potential is maintained following inactivation of $I_{K(A)}$ and $I_{K(V)}$. (a) Steady-state inactivation of $I_{K(V)}$, determined with the voltage protocol illustrated. Records of I_K activated by a test pulse to 40 mV from a holding potential of -80 mV, with or without a 1 min prepulse to various potentials (as indicated), are inset. The plot shows the amplitude of $I_{K(V)}$ activated after a prepulse (I_{PP}), relative to that evoked from -80 mV (I_{-80}), as a function of the prepulse potential. $I_{K(V)}$ was measured at the end of the 100 ms test pulse. The continuous line shows the best fit to equation 1, with $V_{0.5} = -43$ mV. (b) Recovery from inactivation at -80 mV, measured with the voltage protocol illustrated in the inset for $I_{K(A)}$ (○) and $I_{K(V)}$ (●). $I_{K(A)}$ was estimated as the peak current activated within the first 20 ms of the test pulse. Inactivation was induced by 2 min at -20 mV, followed by varying periods at -80 mV to allow recovery. The amplitudes of the currents activated by test pulse P2 (I_{P2}), relative to those activated by P1 (I_{P1}), are plotted as a function of the time the cell was held at -80 mV. The continuous line represents the best fit to $I_{K(V)}$ recovery of the exponential function:

$$\frac{I_{P1}}{I_{P2}} = c + A(1 - e^{-t/\tau}) \qquad (2)$$

where $c = 0.29$ represents the degree of inactivation induced at -20 mV, $A = 0.68$ and $\tau = 16$ s are the amplitude and time constant of the exponential and t is the time after return to -80 mV. (c) Continuous record of current under voltage clamp and resting potential under current clamp, with the periods of voltage clamp at -80 mV and 0 mV indicated, respectively, by $V = -80$ and $V = 0$ above the record; periods of current clamp indicated by $I = 0$. The cell was initially voltage clamped at -80 mV for ~ 5 min, then switched to current clamp to record the resting potential. After a steady level had been established, the cell was voltage-clamped at 0 mV for 35 min, before returning again to current clamp. In this cell, the steady-state resting potential was -44 mV after equilibration at -80 mV and -46 mV following the period at 0 mV. The latter level was reached within 130 ms of the switch from voltage to current clamp. Symbols and vertical lines represent mean ± s.e.mean of $n = 8$(a) or $n = 4$(b).

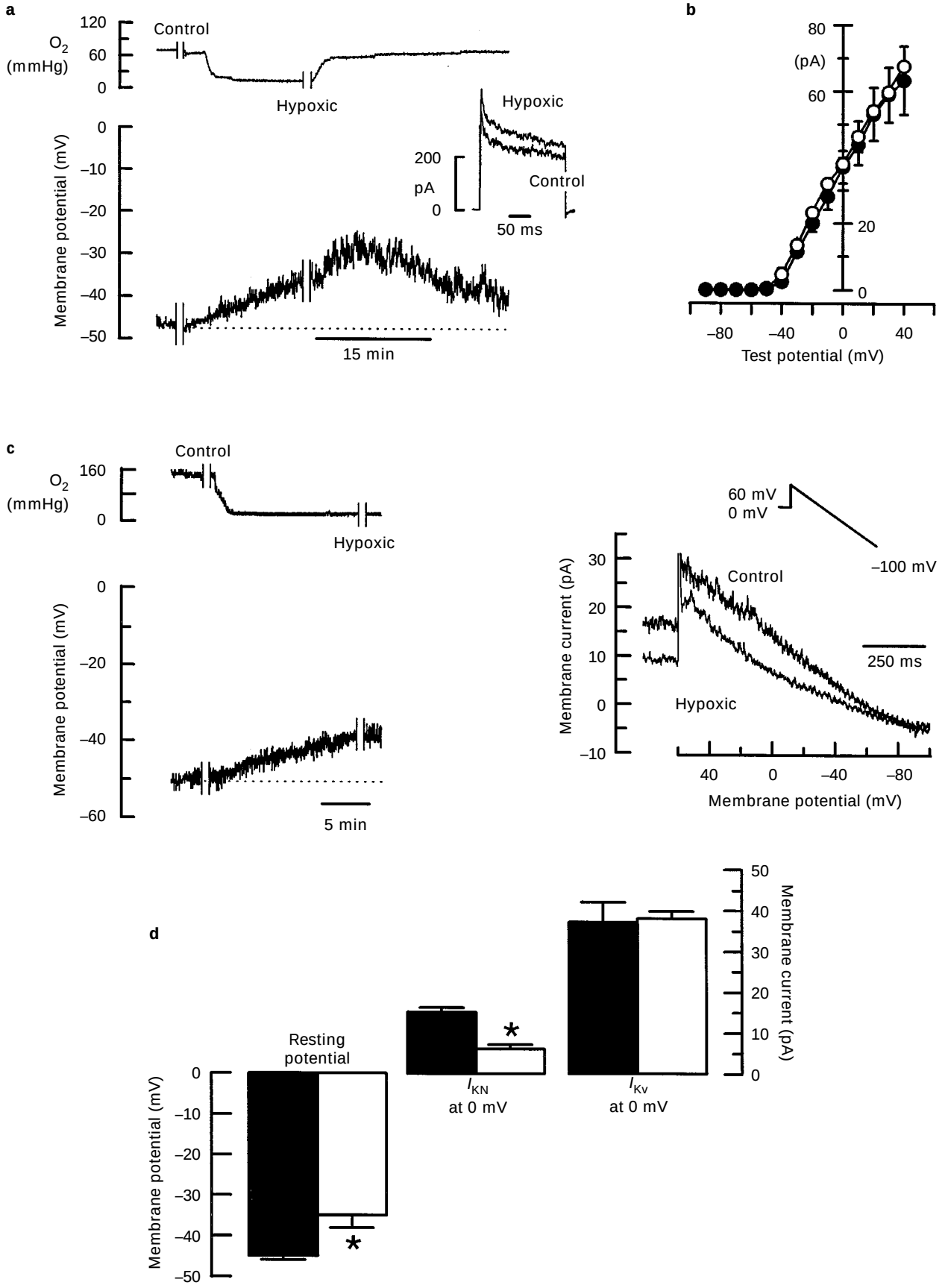

Figure 5 The influence of hypoxia on membrane potential, $I_{K(V)}$, $I_{K(A)}$ and $I_{K(N)}$. (a) Continuous records of resting membrane potential and O_2 tension near the cell while changing the perfusion solution from one equilibrated with air to one bubbled with N_2. Inset records of I_K were activated by steps to 40 mV from −80 mV, under control and hypoxic conditions, and were obtained during the gaps in the record of membrane potential. (b) Current versus voltage relationship for $I_{K(V)}$ measured in control (●) and hypoxic (○) conditions. (c) (Left hand) Continuous records of resting membrane potential and O_2 tension near the cell while changing the perfusion solution from one equilibrated with air to one bubbled with N_2. (Right hand) Records of $I_{K(N)}$ obtained during the gaps in the record of membrane potential. For each record the cell was clamped at 0 mV for 5 min to inactivate $I_{K(V)}$ and $I_{K(A)}$, followed by a voltage ramp from 60 to −100 mV. (d) Histogram comparing the values of resting potential with the

inactivated. The voltage was then stepped to 60 mV, followed by a voltage ramp at 0.2 mV ms^{-1} to −80 mV in order to obtain the current-voltage relationship for $I_{K(N)}$. As shown in Figure 3a, this gave rise to a current that displayed a sigmoidal dependence on membrane potential, the reduction of current at negative potentials being proposed to reflect the closure/deactivation of the ion channels that underlie $I_{K(N)}$ (Evans *et al*., 1996). At 10 μM, quinine had little effect on $I_{K(N)}$ recorded in this way (not shown), but as the quinine concentration was raised, there was an increasing reduction of the steady-state current recorded at 0 mV, and of the current observed during the subsequent voltage ramp (Figure 3a). Half maximal inhibition of the current was observed at around 100 μM quinine, with complete block at 1 mM. At 1 mM only, there appeared to be some relief of quinine block following the step to 60 mV, suggesting some voltage sensitivity to its blocking action.

The activation of $I_{K(N)}$ can also be recorded at negative potentials, below the activation threshold for $I_{K(V)}$ and $I_{K(A)}$, provided it is studied with an elevated extracellular potassium concentration ($[K^+]_o$; Evans *et al*., 1996). This amplifies K^+ currents at negative potentials, which become inward in direction. In the presence of 130 mM $[K^+]_o$, a 15 s step to −60 mV from a holding potential of −100 mV induced a slowly developing inward current, characteristic of $I_{K(N)}$, as illustrated in Figure 3b. In 5 out of 10 cells, $I_{K(N)}$ recorded under these conditions was unaffected by 10 μM quinine, but above this concentration $I_{K(N)}$ was inhibited in a concentration-dependent manner (Figure 3b). Thus, in these cells, the effect of quinine on $I_{K(N)}$ in the presence of 130 mM $[K^+]_o$ exhibited a similar dependence on concentration to that observed when $I_{K(N)}$ was recorded during a voltage ramp in the presence of 5 mM $[K^+]_o$ (Figure 3a). However, in 5 other cells, $I_{K(N)}$ was potentiated by quinine at concentrations below 100 μM (see below), as shown previously (Evans *et al*., 1996). Figure 3c shows the concentration-dependence for the inhibition of $I_{K(N)}$ by quinine, for all the cells studied and under all conditions. Below 100 μM, quinine clearly had a different effect on $I_{K(N)}$ at −60 mV when studied in 5 or 130 mM $[K^+]_o$. Thus the observed facilitation of $I_{K(N)}$ appears to be a consequence of the high $[K^+]_o$ rather than the voltage sensitivity of quinine binding. Allowing for this, then in the presence of 5 and 130 mM $[K^+]_o$, quinine inhibited $I_{K(N)}$ at −60 mV with a similar concentration-dependence, which was also comparable with that observed at 0 mV in the presence of 5 mM $[K^+]_o$. In Figure 3c, inhibition of $I_{K(N)}$ under all conditions is compared with the inhibition of the resting membrane potential, indicated by the broken line. The reduction of the resting potential and inhibition of $I_{K(N)}$ exhibited a similar dependence on quinine concentration, with 100 μM causing approximately 50% inhibition in each case. Furthermore, raising the quinine concentration above 300 μM essentially abolished $I_{K(N)}$ and the measured resting potential.

The potential role of $I_{K(N)}$ in maintaining the resting potential was further investigated by use of voltage instead of drugs to inhibit $I_{K(A)}$ and $I_{K(V)}$, both of which inactivate during maintained depolarization. This is illustrated in the inset to Figure 4a, which shows records of I_K activated by a 100 ms test pulse to 40 mV, applied either directly from a holding potential of −80 mV, or after a 1 min prepulse to increasingly more positive potentials. As previously shown, $I_{K(A)}$ inactivated at more negative potentials and more rapidly than $I_{K(V)}$ (Clapp & Gurney, 1991), and the inactivation of $I_{K(V)}$ took around 1 min to reach steady-state (Evans *et al*., 1994). Steady-state inactivation of $I_{K(V)}$ was quantified by measuring the amplitude of the current sustained at the end of the 100 ms test pulse following a 1 min prepulse (I_{pp}), relative to the sustained current activated without a prepulse (I_{-80}), and is plotted as a function of the prepulse potential in Figure 4a. The superimposed curve shows the best fit to a Boltzman function of the form:

$$\frac{I_{pp}}{I_{-80}} = \frac{1}{1 + \exp[(V - V_{0.5})/k]} \quad (1)$$

where V is the prepulse potential, $V_{0.5}$ (= −43 mV) is the voltage of half-maximal inactivation and k (= 3.9 mV) is a slope factor. Steady-state inactivation was clearly maximal by −20 mV, leaving the residual, non-inactivating $I_{K(N)}$. Figure 4b shows the rate of recovery from inactivation at −80 mV, following a 2 min conditioning pulse at −20 mV. Results are shown for both the sustained ($I_{K(V)}$) and transient ($I_{K(A)}$) currents activated by a 100 ms test pulse. The recovery of $I_{K(V)}$ was best described by a single exponential with time constant of 16 s, as shown by the curve superimposed upon the data in Figure 4b. Although the transient current appeared to recover earlier than $I_{K(V)}$, the difference was small. From these data it is clear that neither $I_{K(A)}$ nor $I_{K(V)}$ would be expected to recover substantially until the membrane was clamped at −80 mV for several seconds.

Figure 4c shows the effect that inactivating $I_{K(V)}$ and $I_{K(A)}$ had on the resting membrane potential. The cell illustrated was initially voltage clamped at −80 mV for around 5 min to allow for equilibration. The clamp was then switched to the zero-current mode, and the resting membrane potential recorded. After a stable resting potential had been established, the cell was voltage clamped at 0 mV, thereby inducing a large, transient outward current due mainly to activation and subsequent inactivation of $I_{K(A)}$ and $I_{K(V)}$. The current declined in amplitude to a steady level within 15 min in the example shown, but even after 40 min (the longest time tested), a non-inactivating component of outward current, $I_{K(N)}$, remained. In 7 cells, the outward current at 0 mV took 8±4 min to decline to a steady-state minimum, at which point it had an amplitude of 10±2 pA. After maximal current inactivation had been observed, the clamp was again switched to zero-current mode. As soon as we could resolve it after the switch to current clamp, the resting potential in these 7 cells measured −41±6 mV, which was not significantly different from the steady-state resting potential of −45±1 mV observed following equilibration at −80 mV. Switching between clamp modes was carried out manually, so the time taken to complete the switch was somewhat variable. The shortest time taken for the clamp to settle at zero current was 25 ms, and in that cell the resting potential achieved at 25 ms was −41 mV, compared with −42 mV in the steady-state. The longest time taken for the resting potential to become established at the steady-state level was 500 ms following the switch to current clamp from voltage clamp at 0 mV, although in most cells it was established well within this time. Little recovery of $I_{K(V)}$ or $I_{K(A)}$ would be expected to occur within 500 ms (~8% at −80 mV; broken line, Figure 4b), so it seems unlikely that these currents were responsible for the negative resting potential. Taken together with the similar potency of quinine for blocking $I_{K(N)}$ and the measured resting potential, these findings strongly suggest that $I_{K(N)}$, and not $I_{K(V)}$ or $I_{K(A)}$, maintains the resting potential in rabbit pulmonary artery myocytes.

Effect of hypoxia on membrane potential and potassium currents

The influence of hypoxia on resting potential and K^+ currents was investigated with a HEPES-buffered PSS, in order to prevent pH changes during exposure to hypoxia. Similar results were observed when the usual bicarbonate-based-PSS was

amplitudes of $I_{K(N)}$ and $I_{K(V)}$, both measured at 0 mV, under control (solid columns) and hypoxic (open columns) conditions. Means±s.e.mean of 16 (resting potential) or 8 ($I_{K(V)}$ and $I_{K(N)}$) cells. The resting potential and $I_{K(N)}$, but not $I_{K(V)}$, were significantly (*$P<0.01$) reduced by hypoxia.

used (not shown). Figure 5a illustrates the effect of hypoxia on membrane potential, $I_{K(V)}$ and $I_{K(A)}$. When the solution around the cell was changed to one bubbled with N_2, the O_2 tension close to the cell declined from 125 ± 6 to 14 ± 2 mmHg ($n = 20$). This was accompanied by a slowly developing depolarization from -45 ± 1 to -35 ± 3 mV ($n = 16$), which was partially reversed on return to normoxic conditions. This effect of hypoxia was not due to a decrease in the amplitude of $I_{K(V)}$ or $I_{K(A)}$, since no decrease was observed in any of 8 cells studied, regardless of the O_2 level present before the hypoxic challenge (range 60–170 mmHg). In fact, in the cell illustrated in Figure 5a, depolarization was associated with an increase in the amplitude of $I_{K(V)}$ and $I_{K(A)}$. Overall, hypoxia had no significant effect on the amplitude of $I_{K(V)}$ over a wide voltage range (Figure 5b). However, the depolarization induced by hypoxia was associated with 60% inhibition of $I_{K(N)}$. Figure 5c shows the changes in resting potential and $I_{K(N)}$ induced by hypoxia in a single cell. Reducing the O_2 tension around the cell caused a slowly developing depolarization with concomitant inhibition of $I_{K(N)}$ throughout its voltage range of activation. Unfortunately, due to the slow time course of the depolarization and subsequent recovery, combined with the time required to record $I_{K(N)}$ (minimum of 5 min at 0 mV to inactivate $I_{K(A)}$ and $I_{K(V)}$), we were unable to hold cells long enough to confirm that recovery of $I_{K(N)}$ accompanies repolarization upon returning to normoxic conditions. The mean results from all these experiments are summarized in Figure 5d. Membrane depolarization was clearly associated with inhibition of $I_{K(N)}$, measured at 0 mV after inactivating $I_{K(A)}$ and $I_{K(V)}$, with no effect on $I_{K(V)}$ recorded at the same potential. The effect of hypoxia was unlikely to be due to changes in the intracellular ATP concentration, because similar results were obtained whether the recording pipette contained 2 mM ATP (results not shown) or ATP was omitted from the pipette (Figure 5). Similar experiments were also carried out with the nystatin, perforated-patch technique to minimize the loss of second messengers from the cell, but with similar results (not shown) to those obtained with the standard whole-cell configuration.

Discussion

The resting potential depends on $I_{K(N)}$, not $I_{K(V)}$

The resting membrane potential of pulmonary artery smooth muscle cells, measured in the intact tissue with microelectrodes, lies between -50 and -60 mV (Casteels *et al*., 1977; Hara *et al*., 1980; Haeusler & Thorens, 1980; Suzuki & Twarog, 1982; Madden *et al*., 1985). Values of resting potential measured in the rabbit isolated myocytes were around -50 mV, even in the presence of glibenclamide to block K_{ATP} channels (see also Clapp & Gurney, 1992), or when BK_{Ca} channels were also blocked with TEA, charybdotoxin or iberiotoxin. This concurs with a recent study in rat pulmonary artery myocytes (Yuan, 1995). On the other hand, TEA causes contraction of isolated pulmonary arteries (Casteels *et al*., 1977; Haeusler & Thorens, 1980; Suzuki & Twarog, 1982) and vasoconstriction in the intact lung (Hasunuma *et al*., 1991; Post *et al*., 1992). It is possible that in the present experiments we artificially reduced the contribution of $I_{K(Ca)}$ by buffering intracellular Ca^{2+} with millimolar EGTA. Alternatively, in the intact blood vessel, TEA could cause contraction by blocking endothelial K_{Ca} channels (Demirel *et al*., 1994). Our results show that K_{Ca} channels were not necessary to maintain the negative resting potential, so although they may be important for the resting potential and tone in pressurised systemic arteries (Brayden & Nelsen, 1992), as argued previously (Evans *et al*., 1996), they are unlikely to be major contributors in the low pressure pulmonary circulation. The resting membrane potential was also found to be independent of Na^+-K^+-ATPase activity, although the ATPase will play a role *in vivo* by maintaining the transmembrane K^+ gradient.

That 4-AP caused depolarization is consistent with recent studies in other species (Smirnov *et al*., 1994; Post *et al*., 1995; Yuan 1995; Archer *et al*., 1996). Such findings have been interpreted as indicating an important contribution of delayed rectifier channels to the resting membrane potential (Smirnov *et al*., 1994; Post *et al*., 1995; Archer *et al*., 1996). However, several of our findings argue against this. Although 4-AP reduced the resting potential and $I_{K(V)}$ with similar potency, such a correlation was absent with quinine. In fact, quinine had little effect on the resting potential at concentrations causing pronounced inhibition of $I_{K(V)}$ and $I_{K(A)}$. Thus the drug sensitivity of the resting potential was poorly correlated with inhibition of $I_{K(V)}$ and $I_{K(A)}$. The sensitivity of the resting potential to quinine was more closely paralleled by the inhibition of $I_{K(N)}$, a recently-identified current shown to be active at the resting potential in these cells (Evans *et al*., 1996). Although $I_{K(N)}$ could be detected at potentials as low as -60 mV, its amplitude was small in the presence of physiological K^+ concentrations. It could be resolved more accurately when the $[K^+]_o$ was raised. Unfortunately, this appeared to modify the effects of quinine, with facilitation of the current seen at low concentrations in half of the cells. Previous studies also found the actions of quinine to vary with the $[K^+]_o$ (Reichstein & Rothstein, 1981). Thus, although raising the $[K^+]_o$ amplifies K^+ currents around the resting potential, it is not an ideal manipulation for studying drug interactions with $I_{K(N)}$. Even so, a similar relationship between quinine concentration and the inhibition of $I_{K(N)}$ was recorded in 5 and 130 mM $[K^+]_o$. Furthermore, the sensitivity of $I_{K(N)}$ to block by quinine was similar at -60 and 0 mV, and this was additionally comparable to suppression of the resting membrne potential by quinine.

Perhaps more convincing was the finding that prolonged depolarization at 0 mV, which inactivated $I_{K(A)}$ and $I_{K(V)}$, but not $I_{K(N)}$, had no effect on the resting membrane potential measured immediately upon switching from voltage to current clamp ($I = 0$ pA). It is unlikely that sufficient $I_{K(A)}$ or $I_{K(V)}$ could have recovered from inactivation during the switch to current clamp to explain this result. Even when the resting potential took 500 ms to reach its steady-state level, recovery of $I_{K(A)}$ and $I_{K(V)}$ would have been minimal. The 16 s recovery time constant for $I_{K(V)}$ predicts that in 500 ms less than 5% of the channels would have recovered at -80 mV, with only slightly more recovery of $I_{K(A)}$ (see Figure 4b); recovery would be even slower at the more positive potentials experienced during the switch to current clamp. Even when the maximum number of channels is available for opening, there is no resolvable $I_{K(V)}$ or $I_{K(A)}$ at -50 mV in physiologcal conditions (Evans *et al*., 1996), due to the normally low open probability between -50 and -40 mV. The few channels that could recover from inactivation while switching from voltage to current clamp would be subject to the same low probability of opening, so overall, channel availability should be grossly depleted. If $I_{K(V)}$ or $I_{K(A)}$ were major contributors to the resting potential, depleting channel availability in this way would be predicted to suppress substantially the resting potential attained immediately after a prolonged period at 0 mV. In contrast, after inactivating $I_{K(V)}$ and $I_{K(A)}$ by prolonged clamping at 0 mV, the resting potential was re-established as soon as it could be measured, which was as early as 25 ms. This finding rules out an important contribution from $I_{K(V)}$ or $I_{K(A)}$. Thus we conclude that $I_{K(N)}$ is the predominant K^+ current that regulates the membrane potential of rabbit pulmonary artery myocytes under resting conditions.

Hypoxia causes depolarization by suppressing $I_{K(N)}$

The membranes of pulmonary artery myocytes from several species have been shown to be depolarized when exposed to hypoxic solutions (Post *et al*., 1992; Yuan *et al*., 1993; Cornfield *et al*., 1994), an effect associated with reduction of a K^+ current, which could be activated by brief voltage steps to positive potentials (Post *et al*., 1992; Yuan *et al*., 1993; Yuan, 1995). The depolarization was proposed to be mediated by

inhibition of a 4-AP sensitive, voltage-gated K^+ channel (Post *et al*., 1995; Yuan, 1995), possibly a delayed rectifier (Post *et al*., 1995). In contrast, a recent study on rabbit pulmonary artery smooth muscle cells found that hypoxia enhanced the K^+ current activated by a similar voltage step (Bonnet *et al*., 1994). Since the enhancement could be suppressed by raising the intracellular EGTA concentration, it probably reflected a Ca^{2+}-dependent increase in $I_{K(Ca)}$. Our results are consistent with this, in that hypoxia had no significant effect on I_K activated by brief voltage steps in the presence of TEA to block $I_{BK(Ca)}$ when the current mainly reflected $I_{K(A)}$ and $I_{K(V)}$. Despite this, hypoxia did induce depolarization. This result indicates that neither $I_{K(A)}$, $I_{K(V)}$ nor $I_{BK(Ca)}$) was responsible for the hypoxia-induced depolarization in rabbit pulmonary artery smooth muscle cells. The hypoxia-induced depolarization is most likely mediated by the inhibition of $I_{K(N)}$ observed here, because $I_{K(N)}$ is active within the range of resting potentials measured in isolated cells and intact blood vessels, and it activates slowly in response to depolarization, therefore contributing little to I_K activated during 100 ms voltage steps (Evans *et al*., 1996).

Along with the finding that $I_{K(N)}$ is important in the control of the resting potential, its sensitivity to hypoxia suggests that it may be important in the physiological control of the pulmonary circulation. The small pulmonary vessels contribute most to the rise in pressure in the pulmonary circulation induced by hypoxia (Barnes & Liu, 1995), and the large pulmonary arteries of the rabbit have even been shown to relax in response to hypoxia (Bonnet *et al*., 1994). This corresponds with differential effects of hypoxia on cytosolic $[Ca^{2+}]$ in myocytes from conduit and resistance branches of the rabbit pulmonary artery (Ureña *et al*., 1996). However, $I_{K(N)}$ is present in smooth muscle cells at all levels of the rabbit pulmonary arterial tree (Evans *et al*., 1996), although we do not yet know if it is evenly distributed. Other studies suggest that the density of K_{Ca} channels varies in pulmonary artery smooth muscle cells according to the size of the vessel, with the lowest density found in the small resistance-sized vessels (Archer *et al*., 1996). It is therefore possible that in large arteries, inhibition of $I_{K(N)}$ by hypoxia is counteracted by an increased activity of K_{Ca} channels, as suggested by the results of Bonnet *et al*., (1994). In small vessels, depolarization resulting from the inhibition of $I_{K(N)}$ would be less effectively opposed by a concomitant increase in $I_{K(Ca)}$, and could therefore lead to contraction.

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