O₂-sensitive K⁺ currents in carotid body chemoreceptor cells from normoxic and chronically hypoxic rats and their roles in hypoxic chemotransduction

(neurosecretory type I cell/hypoxia)

C. N. WYATT*, C. WRIGHT†, D. BEE†, AND C. PEERS*‡

*Department of Pharmacology, Leeds University, Leeds, LS2 9JT, United Kingdom; and †Department of Medicine, Sheffield University Medical School, Beech Hill Road, Sheffield, S10 2RX, United Kingdom

ABSTRACT   Carotid body-mediated ventilatory increases in response to acute hypoxia are attenuated in animals reared in an hypoxic environment. Normally, O₂-sensitive K⁺ channels in neurosecretory type I carotid body cells are intimately involved in excitation of the intact organ by hypoxia. We have therefore studied K⁺ channels and their sensitivity to acute hypoxia (P_{O_2} 12–20 mmHg) in type I cells isolated from neonatal rats born and reared in normoxic and hypoxic environments. When compared with cells from normoxic rats, K⁺ current density in cells from hypoxic rats was significantly reduced, whereas Ca²⁺ current density was unaffected. Charybdotoxin (20 nM) inhibited K⁺ currents in cells from normoxic rats by ≈25% but was without significant effect in cells from hypoxic rats. However, hypoxia caused similar, reversible inhibitions of K⁺ currents in cells from the two groups. Resting membrane potentials (measured at 37°C using the perforated-patch technique) were similar in normoxic and hypoxic rats. However, although acute hypoxia depolarized type I cells of normoxic rats, it was without effect on membrane potential in type I cells from hypoxic animals. Charybdotoxin (20 nM) also depolarized cells from normoxic rats. Our results suggest that type I cells from chronically hypoxic rats, like normoxic rats, possess O₂-sensing mechanisms. However, they lack charybdotoxin-sensitive K⁺ channels that contribute to resting membrane potential in normoxically reared rats, and this appears to prevent them from depolarizing (and hence triggering Ca²⁺ influx and neurosecretion) during acute hypoxia.

The ventilatory responses to acute hypoxia of animals and humans change dramatically from fetal to adult life: in fetal animals, exposure to hypoxia is inhibitory to breathing movements (1), whereas in the adult, hypoxia causes a sustained increase in ventilation (2). Neonatal animals produce an intermediate biphasic response, with ventilation increasing and then falling again during sustained hypoxia (3). This transient increase, along with the sustained increase seen in adults, is a result of stimulation of peripheral chemoreceptors, primarily the carotid body (4, 5). Ventilatory responses to acute hypoxia of neonatal animals born and raised in hypoxic environments are blunted or absent (6), and a similar lack of ventilatory response to hypoxia has also been noted in adult animals exposed to hypoxia chronically (7) and in high-altitude residents (8). It is conceivable that common mechanisms underlie the lack of ventilatory response to hypoxia of chronically hypoxic neonatal animals and high-altitude residents.

The carotid bodies of chronically hypoxic animals or humans show dramatic morphological changes following prolonged hypoxia: most notably, type I carotid body cells undergo hyperplasia and hypertrophy (9, 10). Type I cells are widely accepted as the chemosensory element of the carotid body, and various stimuli including hypoxia stimulate Ca²⁺-dependent release of neurotransmitters from these cells in a manner that correlates with increased discharge of afferent chemosensory fibers (5, 11). In recent years, several groups have used patch-clamp techniques to investigate ion channels in type I cells, and there are several reports describing O₂-sensitive K⁺ channels in these cells (12–16). These findings have given rise to a proposed mechanism for hypoxic chemotransduction in which inhibition of K⁺ channels by hypoxia leads to depolarization and increased excitability of type I cells sufficient to activate voltage-gated Ca²⁺ channels. This leads to Ca²⁺ influx and triggering of neurosecretion, an essential step in the chemotransductive pathway (5). Here we have compared ionic channels and their modulation by acute hypoxia in type I cells isolated from neonatal rats born and raised in normoxia and hypoxia in order to investigate whether the lack of chemoreceptor-mediated increases in ventilation seen in animals reared under chronically hypoxic conditions can be attributed to altered electrophysiological properties of type I cells.

MATERIALS AND METHODS

These studies used type I cells isolated from Wistar rats (9–14 days old) born and raised either in normoxia or in a normobaric hypoxic chamber (10% O₂) and whose ventilatory responses to acute hypoxia were measured by whole-body plethysmography as follows. Awake rats were placed inside a plethysmograph with their heads protruding through a soft rubber collar. Changes in pressure were monitored using a 0–25 mmH₂O manometer (Furniss Controls, Bexhill on Sea, U.K.) and the plethysmograph was calibrated for volume by repeated 0.1-ml injections. Tidal volume and frequency were measured from a chart recording to give minute ventilation. Inspired gases were delivered by a hood for 1 min each at 2–3 liters/min. The change in minute ventilation caused by dropping the inspired O₂ levels from 21% to 10% was taken as an index of the hypoxic ventilatory response.

To isolate type I cells, carotid bodies were dissected from halothane-anesthetized rats and placed in phosphate-buffered saline (containing 0.05% collagenase, 0.025% trypsin, and 50 μM Ca²⁺) for 20 min at 37°C. The tissues were then teased apart, incubated for a further 7 min, triturated, and centrifuged at 200 × g for 5 min. The supernatant was removed and cells were resuspended in Ham's F-12 culture medium containing insulin (84 units/liter), penicillin (100 international units/ml), streptomycin (100 μg/ml), and 10% fetal calf serum. The dispersed cells were then placed on poly(lysine)-coated coverslips

Abbreviations: I–V, current–voltage; ChTx, charybdotoxin.
‡To whom reprint requests should be addressed at: Institute for Cardiovascular Research, University of Leeds, Leeds LS2 9JT, U.K.

and kept in culture for up to 48 hr (5% CO_2/95% air; type I cells from hypoxic rats were kept in an atmosphere of 10% O_2/5% CO_2/85% N_2). For patch-clamp studies (17), fragments of coverslip containing attached cells were placed in a perfused chamber (volume $\approx$80 μl; flow rate = 0.5–1.0 ml/min) at room temperature (21–24°C). To record K^+ currents from type I cells, patch pipettes (resistance, 6–10 MΩ) were filled with a solution of (in mM) KCl, 117; $CaCl_2$, 1; $MgSO_4$, 2; NaCl, 10; EGTA, 11; Hepes, 11; and ATP, 2 (pH 7.2). In some experiments, the amphotericin B perforated patch technique (18) was used. For these studies, amphotericin B (240 μg/ml) was added to the intracellular solution (from a stock solution of 60 mg/ml in dimethyl sulfoxide) and ATP was omitted. The perfusate contained (in mM) NaCl, 135; KCl, 5; $MgSO_4$, 1.2; $CaCl_2$, 2.5; Hepes, 5; and glucose, 10 (pH 7.4). Where indicated in *Results*, solutions were made hypoxic by bubbling the reservoir with N_2 and passing a stream of N_2 over the surface of the recording chamber. The final P_{O_2} achieved ranged from 12 to 20 torr (1 torr = 133 Pa), as determined on each experimental day using a commercial O_2 electrode (Strathkelvin Instruments, Glasgow, U.K.). Type I cell membrane potentials were also measured, using the perforated-patch technique. For these studies, cells were bathed in the above-mentioned solutions, but at the physiological temperature of 37°C, and potentials were recorded with the patch amplifier (Axopatch 1D, Axon Instruments, Burlingame, CA) in current-clamp ($I = 0$) mode. To study Ca^{2+} currents, pipettes contained (in mM) CsCl, 130; EGTA, 1.1; $MgCl_2$, 2; $CaCl_2$, 0.1; Hepes, 10; and NaCl, 10 (pH 7.2), and the perfusate was composed of (in mM) NaCl, 110; CsCl, 5; $MgCl_2$, 0.6; $BaCl_2$, 10; Hepes, 5; glucose, 10; and tetraethylammonium chloride, 20 (pH 7.4). Whole-cell currents were evoked using 50-ms depolarizing steps applied at 0.2 Hz to cells clamped at -70 mV. Currents were filtered at 1–2 kHz, digitized at 5 kHz, and measured for amplitude using VCAN software (J. Dempster, Strathclyde University, Strathclyde, Scotland). Current–voltage (I–V) relationships were plotted following leak subtraction (19). All data are presented as means $\pm$ SEM, and statistical comparisons were made using the paired Student's t test, unless otherwise indicated.

RESULTS

Ventilatory measurements demonstrated that the chronically hypoxic rats used in these studies had a depressed ventilatory response. Using whole-body plethysmography, we found in control, normoxically reared rats, that minute ventilation increased by 27.1% $\pm$ 5.9% (mean $\pm$ SEM, n = 6 rats) when inspired O_2 was reduced from atmospheric levels (21%) to 10%. Responses to an identical fall of inspired O_2 levels in chronically hypoxic rats produced a mean change in minute ventilation of $-3.7\% \pm 8.4\%$ (n = 20), significantly lower ($P < 0.01$, unpaired t test) than seen in normoxic animals. These findings are in agreement with previous studies (6).

Under phase-contrast microscopy used for visualizing cells for patch clamp recordings, the most immediately striking observation was that type I cells isolated from chronically hypoxic animals were larger than those from normoxic animals. To quantify this more accurately, we measured cell membrane capacitance, an indicator of cell surface area. For normoxic rats, cell capacitance was 3.52 $\pm$ 0.15 pF (n = 35). If type I cells are assumed to be spherical and membrane capacitance is a near-constant value of 1 μF/cm^2, our results correspond to a mean cell diameter in normoxic animals of $\approx$10.4 μm. The capacitance of cells taken from hypoxic animals was significantly greater ($P < 0.0001$, unpaired t test) at 6.33 $\pm$ 0.33 pF (n = 38), corresponding to a cell diameter of $\approx$14.2 μm. Thus, our isolated type I cells corresponded in terms of cell size to the known morphology of cells taken from chronically hypoxic animals (9, 10).

Fig. 1 shows examples of whole-cell K^+ currents and corresponding I–V relationships taken from example normoxic

and hypoxic type I cells. For normoxic cells, currents began to activate noticeably at between -30 mV and -20 mV and showed a pronounced shoulder in the I–V plot at positive test potentials (e.g., Fig. 1A). This shoulder is known to arise as a result of voltage-gated Ca^{2+} influx activating a charybdotoxin (ChTx)-sensitive, Ca^{2+}-dependent K^+ current (IK_{Ca}) (19). By contrast, I–V relationships taken from hypoxic animals activated at between -20 mV and -10 mV and increased thereafter in a more linear fashion with increasing test potentials, without an obvious shoulder at low, positive test potentials. We also commonly noticed a more prominent, transient inward current that preceded the outward current in cells from hypoxic animals (e.g., Figs. 1C and 2C).

It was noteworthy that although type I cells from hypoxic animals were larger (see above), their K^+ currents were not, indicating that K^+ channel density in hypoxic cells was decreased. This is quantified in Fig. 2A, which plots mean current density (calculated by dividing currents obtained in each cell by that cell's membrane capacitance) for hypoxic and normoxic type I cells. For normoxic cells, the outward shoulder at low positive test potentials is less apparent, since in individual cells the potential at which the peak of the shoulder occurs varies between $+10$ mV and $+50$ mV (15, 19, 20), leading to a smoothing of the mean data. K^+ current density was significantly ($P < 0.05$ to $P < 0.0002$, unpaired t test) reduced at all activating test potentials studied. A similar analysis was made of inward currents, recorded using solutions designed to block outward currents and using 10 mM Ba^{2+} as charge carrier through Ca^{2+} channels (20). As suggested earlier, those seen in type I cells from hypoxic animals were larger in amplitude but, when corrected for cell capacitance, the inward current density was not significantly different at any activating test potential studied. For example, step depolarizations from -70 mV to 0 mV evoked current densities of -9.1 ± 1.0 pA/pF for normoxic rats (n = 6) and -11.5 ± 2.3 pA/pF for hypoxic rats (n = 11).

The findings described above suggest that K^+ current density in type I cells from hypoxic animals is reduced as compared with cells from normoxic animals, whereas the inward Ca^{2+} current density is unaffected. We also noted that K^+ I–V relationships in hypoxic cells lacked the shoulder in the I–V plots seen in normoxic cells (Fig. 1). This shoulder arises due to activation of IK_{Ca}, and so to test whether the hypoxic type I cells were specifically lacking these currents, we examined their responses to ChTx, which selectively blocks IK_{Ca} in normoxic type I cells (16, 19). Fig. 2B shows that although 20 nM ChTx inhibited K^+ currents in normoxic type I cells (mean inhibition of 25.0% $\pm$ 2.7%; n = 11; test potential, $+30$ mV), it was a significantly ($P < 0.001$) less effective inhibitor of K^+ currents in type I cells from hypoxic animals (e.g., Fig. 2C; mean inhibition, 7.1% $\pm$ 3.0%; n = 8; test potential, $+30$ mV). This finding suggests that type I cells from hypoxic animals are specifically lacking IK_{Ca} channels. Such a finding was of particular interest, since IK_{Ca} is selectively inhibited in this species by chemostimuli such as hypoxia and acidity (15, 19, 20).

The effects of acute hypoxia (P_{O_2}=12–20 mmHg) were investigated on K^+ currents in cells from normoxic and hypoxic rats (Fig. 3). As previously reported for type I cells from normoxic rats (14, 15), even at room temperature K^+ currents were reversibly inhibited by hypoxia (e.g., Fig. 3A). In 12 cells studied, hypoxia caused significant current inhibitions over the test potential range -10 mV to $+60$ mV. The degree of inhibition ranged from 18.2% $\pm$ 3.5% to 32.9% $\pm$ 5.3% ($P < 0.005$ to $P < 0.0001$). Given the previous observation that K^+ current inhibition by hypoxia is selective for ChTx-sensitive IK_{Ca} (15, 16), it might be anticipated that hypoxia would not inhibit K^+ currents in type I cells from hypoxic rats. However, we found that hypoxia also inhibited K^+ currents in these cells, over the same test potential range of -10 mV to $+60$ mV (e.g., Fig. 3B). In 8 cells studied, hypoxia caused significant current

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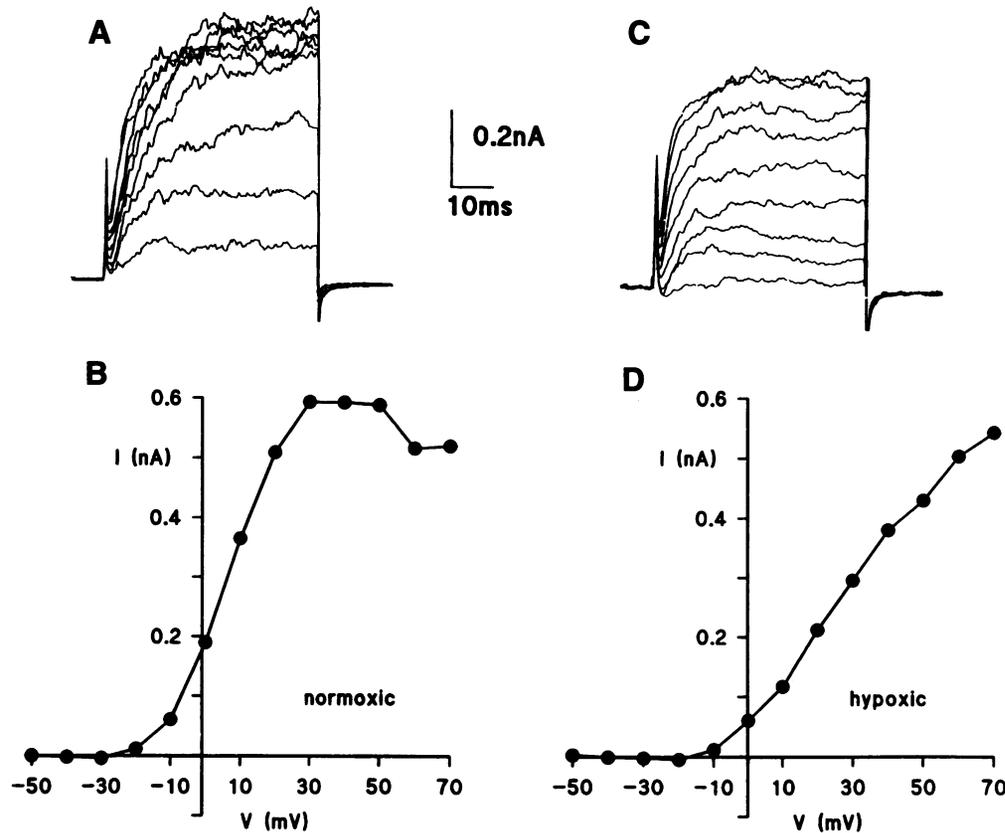*Proc. Natl. Acad. Sci. USA 92 (1995)*   297

FIG. 1.   Whole-cell K^+ currents in type I carotid body cells isolated from normoxic and hypoxically reared neonatal rats. (*A*) Currents evoked in a representative normoxic type I cell by 50-ms step depolarizations to between -10 mV and $+70$ mV (in 10-mV increments) from a holding potential of -70 mV. (*B*) Complete *I-V* relationship for the same cell as in *A*, following leak subtraction. (*C*) Currents evoked in a representative chronically hypoxic type I cell by 50-ms step depolarizations to between -10 mV and $+70$ mV (in 10-mV increments) from a holding potential of -70 mV. (*D*) Complete *I-V* relationship for the same cell as in *C*, following leak subtraction.

inhibitions ranging from 32.2% ± 4.1% to 51.2% ± 5.2% (*P* < 0.01 to *P* < 0.0001). Furthermore, at any given test potential the degree of inhibition caused by acute hypoxia was not significantly different from inhibitions seen in normoxic rats.
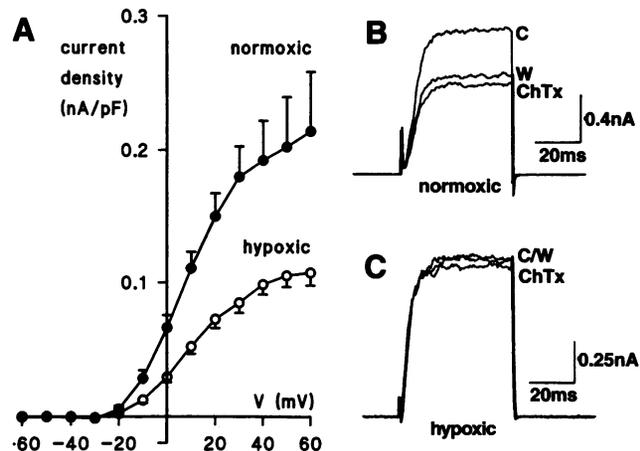

FIG. 2.   Whole-cell K^+ current-density and sensitivity to ChTx in type I cells isolated from normoxic and chronically hypoxic neonatal rats. (*A*) Plot of mean K^+ current density (with vertical SEM bars) versus membrane potential for type I cells isolated from normoxic (●; *n* = 17 cells) and chronically hypoxic (○; *n* = 20) neonatal rats. (*B*) K^+ currents evoked by step depolarizations from -70 mV to $+30$ mV in a normoxic type I cell before, during, and after bath application of 20 nM ChTx, as indicated. (*C*) K^+ currents evoked as in *B*, but from a type I cell of a chronically hypoxic neonatal rat. Note the smaller effect of ChTx as compared with the effect in cells from the normoxic animal (*B*). C, control; W, wash.

As described earlier, there is an emerging consensus that K^+ channel inhibition by hypoxia leads to cell depolarization and, hence, Ca^{2+} influx and consequent neurosecretion. However, the link between K^+ channel inhibition and membrane depolarization remains to be demonstrated in rat type I cells. We investigated this by recording membrane potential in type I cells using the perforated-patch technique at 37°C. Mean resting potential in type I cells from normoxic rats was -42.5 ± 0.9 mV (range, -60 to -34 mV; *n* = 29) and, as exemplified in Fig. 4*A*, hypoxia (12–20 mmHg) reversibly and significantly (*P* < 0.0001) depolarized these cells, on average by 8.6 ± 0.9 mV (range, 5 to 13 mV; *n* = 8). Resting potentials measured in cells from hypoxic rats were not significantly different from those of normoxically reared rats (mean, -45.1 ± 1.0 mV; range, -58 to -38 mV; *n* = 21), but, by contrast, membrane potential was not discernibly altered by hypoxia in these cells (*n* = 8; e.g., Fig. 4*B*). It was possible that acute hypoxia only depolarized cells from normoxic rats because the O_2-sensitive K^+ channels in these cells ($I_{K_{Ca}}$) contribute to the resting membrane potential. To test this, we examined the effects of 20 nM ChTx on membrane potential and found that it depolarized normoxic type I cells by 6.8 ± 0.5 mV (*n* = 10; *P* < 0.0001; e.g., Fig. 4*C*). The depolarizing effects were poorly reversible as were the effects of ChTx on K^+ currents in voltage-clamp experiments (Fig. 2*B*).

DISCUSSION

The present study describes ionic channels in type I carotid body cells isolated from chronically hypoxic animals. One major finding was that K^+ current density was significantly reduced in such cells (Fig. 2*A*), and this can be attributed, at
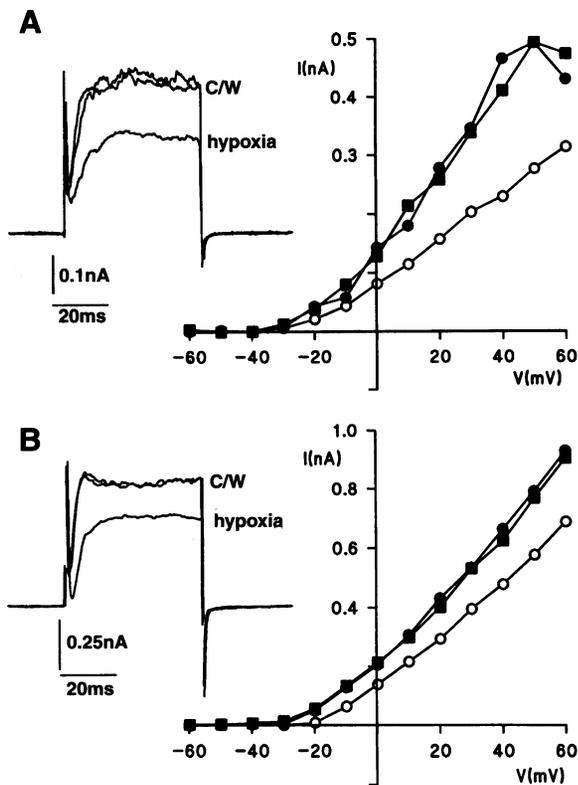
A

B

FIG. 3. Effect of acute hypoxia on whole-cell K$^+$ currents in type I cells isolated from normoxically reared and chronically hypoxically reared neonatal rats, as measured by using the perforated-patch technique. (*A*) *I–V* relationships obtained from a type I cell isolated from a normoxically reared neonatal rat before (●), during (○), and after (■) exchange of normoxic perfusate to a hypoxic perfusate (P$_{O_2}$ between 12 and 20 mmHg). (*Inset*) Example traces obtained from the same cell under the same three conditions (C, control; W, wash; test potential, +20 mV). (*B*) Experiment identical to that in *A*, but in this case the K$^+$ currents were obtained from a hypoxically reared neonatal rat. In *A* and *B* the holding potential was −70 mV.

least in part, to a down-regulation or lack of expression of IK$_{Ca}$ since ChTx was without significant effect on the whole-cell K$^+$ current (Fig. 2*C*). It is impossible to say whether any particular single factor acting *in vivo* causes the lack of expression of IK$_{Ca}$; for example, in addition to being hypoxic, animals reared under such conditions are also acidotic (3, 6). Nevertheless, this observation was of particular interest, since IK$_{Ca}$ in rat type I cells has previously been shown to be selectively inhibited by chemostimuli including hypoxia and acidity (15, 16, 19, 20). Perhaps surprisingly, therefore, we found that acute hypoxia was still able to inhibit K$^+$ currents in cells from chronically hypoxic rats. This observation raises questions about O$_2$-sensing mechanisms in type I cells. Ganfornina and Lopez-Barneo (21) have shown that rabbit type I cells [which have strikingly different electrophysiological properties from those of rats (12, 22)] possess specific O$_2$-sensitive K$^+$ channels that are inhibited by hypoxia even in excised patches. However, the P$_{O_2}$-K$^+$ channel relationship in such studies is displaced so that channel inhibition is seen at P$_{O_2}$ levels that do not excite the intact carotid body (21), which suggests that other factors influence the effects of hypoxia on K$^+$ channels. Indeed, intracellular factors such as cyclic AMP or NAD(P)H oxidase have been implicated in O$_2$ chemoreception (23, 24). The present study suggests that the O$_2$-sensing mechanism in type I cells, whatever it may be, can result in the regulation of different K$^+$ channels in cells from normoxic as compared with chronically hypoxic animals. This might in turn suggest that type I cells are specialized in terms of O$_2$ sensing, not because
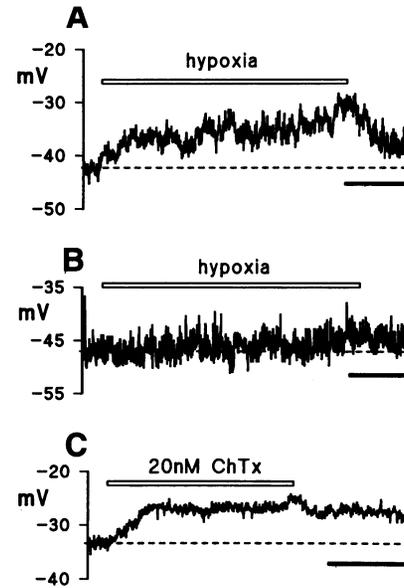
A

B

C

FIG. 4. Recordings of membrane potential obtained at 35–37°C using the perforated-patch technique in current-clamp (*I* = 0) mode. (*A*) Depolarizing effect of hypoxia (P$_{O_2}$ 12–20 mmHg) in a type I cell isolated from a normoxically reared neonatal rat. The period of exposure to hypoxic solution is indicated by the horizontal bar. (*B*) Experiment identical to that in *A*, but the recording was made from a type I cell isolated from a hypoxically reared neonatal rat. (*C*) Membrane potential recording from a normoxically reared neonatal rat type I cell under normoxic conditions throughout, showing the effects of bath application of 20 nM ChTx applied for the period indicated by the horizontal bar. In *A–C* the solid bars indicate 30 s.

they possess specific O$_2$-sensitive K$^+$ channels, but because they possess (as yet unidentified) specialized intracellular O$_2$-sensing mechanisms, which can couple to different K$^+$ channel types.

Our finding that resting membrane potential in rat type I cells is approximately −43 mV is in good agreement with other recent studies (25, 26), but the mechanisms that contribute to maintenance of this potential have not been examined. Here we have shown, using the perforated-patch technique (to prevent loss of cell constituents), that at the physiological temperature of 37°C ChTx can depolarize type I cells, which indicates that ChTx-sensitive K$^+$ channels contribute to the maintenance of membrane potential. This lends further support to the widely accepted mechanism for hypoxic chemotransduction (i.e., K$^+$ channel inhibition leading to depolarization, Ca^{2+} influx, and, so, neurosecretion), since hypoxia selectively inhibits these channels in type I cells from normoxic rats (15, 16). It should be noted that depolarizations caused by hypoxia or ChTx were modest (usually <10 mV), but such small changes can cause substantial rises of intracellular [Ca^{2+}] in these cells; although Fieber and McCleskey (27) reported that type I cells needed to depolarize to −20 mV or more positive in order to activate Ca^{2+} channels, their studies did not account for possible membrane surface charge screening effects of high (110 mM) Ba^{2+} concentrations used to record Ca^{2+} channel currents. Instead, under physiological conditions, Buckler and Vaughan-Jones (25) have demonstrated that small depolarizations to between −40 mV and −20 mV can cause substantial elevations of intracellular Ca^{2+} concentration. Acute hypoxia failed to depolarize type I cells of chronically hypoxic rats, despite inhibition of K$^+$ currents, a finding that suggests that the O$_2$-sensitive K$^+$ channels present in these cells do not appreciably influence resting membrane potential.

The carotid body mediates ventilatory responses to acute hypoxia in normoxic animals, and these ventilatory responses

are attenuated in animals born and reared in hypoxic environments (see above). The present study suggests that this lack of ventilatory response might arise because O_2 chemoreception by the type I cells of chronically hypoxic carotid bodies involves modulation of a K^+ channel that (unlike in normoxic cells) does not appear to influence resting membrane potential, even at 37°C in undialyzed type I cells. Such effects at the level of ion channels in type I cells may also be involved in ventilatory adaptation to high altitude: ventilation initially increases in adults when exposed to altitude or hypoxia (28), and type I cells isolated from normoxic animals and cultured under chronically hypoxic conditions become more excitable (29) as well as being enlarged and showing a reduced K^+ current density (29), as we have shown here for chronically hypoxic animals. However, prolonged exposure to hypoxia leads to the development of a blunted hypoxic response (8, 30), as seen in animals born and reared in hypoxia (5). It will be of interest to compare the electrophysiological properties of type I cells from long-term high-altitude or chronically hypoxic animals with the findings of the present study.

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