Anoxia differentially modulates multiple K^+ currents and depolarizes neonatal rat adrenal chromaffin cells

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1. Using perforated-patch, whole cell recording, we investigated the membrane mechanisms underlying O_2 chemosensitivity in neonatal rat adrenomedullary chromaffin cells (AMC) bathed in extracellular solution containing tetrodotoxin (TTX; 0·5–1 μM), with or without blockers of calcium entry.

2. Under voltage clamp, low P_{O_2} (0–15 mmHg) caused a graded and reversible suppression in macroscopic outward K^+ current. The suppression during anoxia ($P_{O_2} = 0$ mmHg) was ~35% (voltage step from −60 to +30 mV) and was due to a combination of several factors: (i) suppression of a cadmium-sensitive, Ca^{2+}-dependent K^+ current, $I_{K(CaO_2)}$; (ii) suppression of a Ca^{2+}-insensitive, delayed rectifier type K^+ current, $I_{K(VO_2)}$; (iii) *activation* of a glibenclamide- (and Ca^{2+})-sensitive current, $I_{K(ATP)}$.

3. During normoxia ($P_{O_2} = 150$ mmHg), application of pinacidil (100 μM), an ATP-sensitive potassium channel (K_{ATP}) activator, increased outward current density by $45{\cdot}0 \pm 7{\cdot}0$ pA pF^{-1} (step from −60 to + 30 mV), whereas the K_{ATP} blocker glibenclamide (50 μM) caused only a small suppression by $6{\cdot}3 \pm 4{\cdot}0$ pA pF^{-1}. In contrast, during anoxia the presence of glibenclamide resulted in a substantial reduction in outward current density by $24{\cdot}9 \pm 7{\cdot}9$ pA pF^{-1}, which far exceeded that seen in its absence. Thus, activation of $I_{K(ATP)}$ by anoxia appears to reduce the overall K^+ current suppression attributable to the combined effects of $I_{K(CaO_2)}$ and $I_{K(VO_2)}$.

4. Pharmacological tests revealed that $I_{K(CaO_2)}$ was carried predominantly by maxi-K^+ or BK potassium channels, sensitive to 50–100 nM iberiotoxin; this current also accounted for the major portion (~60%) of the anoxic suppression of outward current. Tetraethylammonium (TEA; 10–20 mM) blocked all of the anoxia-sensitive K^+ currents recorded under voltage clamp, i.e. $I_{K(CaO_2)}$, $I_{K(VO_2)}$ and $I_{K(ATP)}$.

5. Under current clamp, anoxia depolarized neonatal AMC by 10–15 mV from a resting potential of ~−55 mV. At least part of this depolarization persisted in the presence of either TEA, Cd^{2+}, 4-aminopyridine or charybdotoxin, suggesting the presence of anoxia-sensitive mechanisms additional to those revealed under voltage clamp. In Na^+/Ca^{2+}-free solutions, the membrane hyperpolarized, though at least a portion of the anoxia-induced depolarization persisted.

6. In the presence of glibenclamide, the anoxia-induced depolarization increased significantly to ~25 mV, suggesting that activation of K_{ATP} channels may function to attenuate the anoxia-induced depolarization or receptor potential.

Adrenomedullary chromaffin cells (AMC) mediate the elevation in plasma catecholamine (CA) that occurs when animals are exposed to stressors, e.g. acute hypoxia. In the neonatal rat, this CA surge is vital for the animal's ability to survive hypoxic stress associated with the transition to extrauterine life, but occurs through a 'non-neurogenic' mechanism that is present prior to the onset of mature sympathetic innervation (Seidler & Slotkin, 1985). We recently reported that rat AMC possess a developmentally regulated oxygen sensing mechanism, since in the majority of cells derived from neonatal (postnatal (P) day 1–P3), but not juvenile (P13–P21) animals, acute hypoxia caused suppression of the outward K^+ current, membrane depolarization and CA secretion (Thompson *et al.* 1997). These responses appear qualitatively similar to those of prototypic O_2 chemoreceptors, i.e. type 1 cells of the carotid body (Buckler & Vaughan-Jones, 1994; Gonzalez *et al.* 1994; Peers & Buckler, 1995; Lopez-Barneo, 1996; Jackson &

Nurse, 1997) and interestingly, both cell types derive from a similar lineage, the sympathoadrenal branch of the neural crest. However, hypoxia is known to modulate differentially several types of K^+ channels and cause either membrane depolarization or hyperpolarization in a number of other cell types (Haddad & Jiang, 1997), including arterial myocytes (Post *et al.* 1992), pulmonary neuroepithelial bodies (Youngson *et al.* 1993), PC12 cells (Zhu *et al.* 1996) and central neurons (Jiang *et al.* 1994).

The K^+ channel subtypes that are inhibited by hypoxia in carotid body type 1 cells include large conductance Ca^{2+}-dependent K^+, or maxi-K^+ channels (Wyatt & Peers, 1995) and voltage-independent, small conductance K^+ 'leak' channels (Buckler, 1997) in the rat, and Ca^{2+}-independent, slow-inactivating, delayed rectifier type K^+ channels in the rabbit (Lopez-Lopez *et al.* 1989). In PC12 cells, a cell line derived from adrenal chromaffin cells, hypoxia inhibits a slow-inactivating, delayed rectifier type K^+ channel which mediates membrane depolarization (Zhu *et al.* 1996). However, in these cells hypoxia also appears to activate a large conductance Ca^{2+}-dependent K^+ channel (Conforti & Millhorn, 1997). In central neurons, hypoxia activates a glibenclamide-sensitive ATP-sensitive K^+ current, $I_{K(ATP)}$, which is thought to play a protective role in low oxygen conditions by inducing hyperpolarization and preventing action potential generation (Jiang *et al.* 1994). These results suggest that hypoxia can modulate multiple K^+ channels in different tissues, and that the effect on a particular channel (i.e. closure *vs.* opening) may be both species and cell-type dependent.

In our initial study (Thompson *et al.* 1997), it was unclear which K^+ channel types mediate hypoxic chemosensitivity in *neonatal* rat AMC. This is of additional interest, since, as discussed above, hypoxia inhibits K^+ channels with different calcium sensitivities in two cell types, carotid body type 1 and PC12 cells, that are related developmentally to AMC. Furthermore, in a recent study K_{ATP} channels were presumed to play a crucial role in hypoxia-induced responses in *adult* rat chromaffin cells (Mochizuki-Oda *et al.* 1997). In the present study we used perforated-patch, whole cell recording and pharmacological tools to characterize the types of O_2-sensitive K^+ currents in neonatal rat AMC and to investigate whether these currents can account for the hypoxia-induced membrane depolarization or receptor potential (Thompson *et al.* 1997). Preliminary results of some of these findings were reported in a recent abstract (Thompson & Nurse, 1997).

METHODS

Cell culture

Pregnant or lactating Wistar rats and pups (Charles River, Quebec, Canada) were housed in our animal facility under a 12 h light–12 h dark cycle. All animal handling and tissue removal conformed to guidelines established by the Canadian Council on Animal Care. Primary cultures enriched in dissociated adrenomedullary chromaffin cells (AMC) were prepared as previously described (Thompson *et al.* 1997). Adrenal glands were dissected from neonatal rats (i.e. postnatal (P) day P1–P2) that were rendered unconscious by a blow to the head and killed by decapitation. Most of the surrounding cortex was trimmed and discarded. The remaining (medullary) tissue was dissociated by incubation (at 37 °C) in an enzymatic solution containing v/v 0·1 % trypsin, 0·1 % collagenase (Gibco) and 0·01 % deoxyribonuclease (Millipore). After 1 h incubation, most of the enzyme was removed and the remainder inactivated by addition of growth medium consisting of F-12 nutrient medium (Gibco) supplemented with 10 % v/v fetal calf serum (Gibco), 80 U l^{-1} insulin (Sigma), 0·6 % v/v glucose, 2 mM glutamine, 1 % v/v penicillin–streptomycin (Gibco) and 0·01 % v/v dexamethasone (Sigma). Tissue was then triturated using a Pasteur pipette, and the final cell suspension was pre-plated for 1–24 h on a collagen-coated culture dish to remove most of the cortical cells. The non-adherent AMC were then plated onto the central region of Nunclon culture dishes that had been previously coated with a thin layer of Matrigel (Collaborative Research, Bedford, MA, USA). The cells were grown at 37 °C in a humidified atmosphere of 95 % air, 5 % CO_2 for 3–72 h before they were used in patch clamp experiments.

Electrophysiology

All voltage and current clamp data were obtained using the perforated-patch configuration of the whole cell technique as previously described (Thompson *et al.* 1997). The seal resistance was typically 2–10 GΩ, and most (~75 %) of the series resistance (range, 12–50 MΩ) was compensated in voltage clamp experiments; voltage errors due to series resistance were minimal due to the high input resistance (~2 GΩ) of the cells. Junction potentials were typically 2–5 mV in the standard bathing solution, and were cancelled prior to seal formation. The pipette solution for perforated-patch recording contained (mM): potassium gluconate, 105; KCl, 30; NaCl, 5; $CaCl_2$, 0·1; Hepes, 10; at pH 7·2, plus nystatin (450 μg ml^{-1}). The standard bathing solution for both voltage clamp and current clamp experiments consisted of (mM): NaCl, 135; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 2; glucose, 10; Hepes, 10; tetrodotoxin (TTX), 0·0005–0·001; pH adjusted to 7·4 with NaOH. In experiments where a Ca^{2+}-free bathing solution was required, $CaCl_2$ (2 mM) was replaced with equimolar $MgCl_2$ and 1 mM EGTA. In some experiments, 200 μM $CdCl_2$ was added to block Ca^{2+} currents and, indirectly, Ca^{2+}-dependent K^+ currents. In experiments requiring a Na^+-free solution, Na^+ was replaced with equimolar *N*-methyl-D-glucamine (NMDG) and the pH was adjusted with HCl.

All recordings were obtained at room temperature (20–23 °C) with an Axopatch-1D amplifier equipped with a 500 MΩ head stage feedback resistor (Axon Instruments). Records were digitized with a Digidata 1200 computer interface (Axon) and stored on hard disk in an IBM-compatible computer using pCLAMP software version 6.0.3 (Axon). In the majority of experiments anoxia was used as the low P_{O_2} stimulus. Anoxia was generated by bubbling 100 % N_2 into the perfusion reservoir in the presence of the O_2 scavenger sodium dithionite; the pH was adjusted to 7·4 with NaOH. For hypoxic solutions, 100 % N_2 was bubbled in the absence of dithionite. Measurements of P_{O_2} were obtained with an O_2 microelectrode (Diamond Electro-Tech Inc., Ann Arbor, MI, USA) placed near the recording site. During calibration, 0 mmHg (anoxia) was the designated P_{O_2} of a solution equilibrated with 100 % N_2 in the presence of 1 mM sodium dithionite. The solution in the recording chamber (volume, 750 μl–1 ml) was exchanged by perfusion under gravity and simultaneous removal by suction at a rate of 5–6 ml min^{-1}.

The effects of anoxia and/or hypoxia on voltage-activated currents were examined by comparing peak currents from the average of four records at each step potential, over the range of −50 to +50 mV (10 mV increments) from a holding potential of −60 mV. Records were taken before (control), during, and after (recovery) application of the stimulus, and the averages were obtained either during data collection (on-line), or during subsequent analysis (off-line). All measurements of membrane potential were obtained under current clamp in zero current ($I = 0$) mode. Membrane capacitance (pF) was obtained by first integrating the capacitative transient elicited by a hyperpolarizing voltage step from −60 to −100 mV, then dividing by the magnitude of the step. Currents (pA) or current densities (pA pF^{-1}) were compared using either paired or independent Student's t tests, with the level of significance set at $P < 0{\cdot}05$. Voltage clamp current traces are shown in the text with 'leak currents' unsubtracted; current or current density *vs.* voltage (I–V) plots are leak subtracted. All data are presented as means ± S.E.M.

Drugs

In order to block various types of K^+ currents (as indicated in the text) the following drugs were added directly to the perfusion fluid: tetraethylammonium (TEA), glibenclamide and 4-aminopyridine (4-AP), obtained from Sigma; and iberiotoxin (IbTx) and charybdotoxin (ChTx) obtained from Alomone Laboratories (Jerusalem, Israel). In some experiments the K_{ATP} channel activator pinacidil, a gift of Dr Jan Huizinga, was used. Additionally, in some experiments cadmium at 200 μM ($CdCl_2$) was used to block Ca^{2+} entry and, indirectly, Ca^{2+}-dependent K^+ currents. To block voltage-dependent Na^+ currents, tetrodotoxin (TTX; Sigma) was added to the bathing solution.

RESULTS

Neonatal rat adrenomedullary chromaffin cells (AMC) were first tested for hypoxic sensitivity between 3 h and 3 days in culture, using the perforated-patch technique for whole cell recording. As in our previous report (Thompson *et al.* 1997), low P_{O_2} caused a reversible suppression of outward current (Figs 1 and 2) and/or membrane depolarization (e.g. Fig. 7) in >80% of cells tested ($n = 68/78$). As shown in Fig. 1, the magnitude of the effect was graded, with the maximum current suppression occurring in anoxia ($P_{O_2} = 0$ mmHg). During a voltage step from −60 to +30 mV, the mean (± S.E.M.) percentage suppression was 12·2 ± 0·02% during hypoxia (P_{O_2}, ~15 mmHg) compared with 43·6 ± 0·08% in anoxia, for the same group of five cells examined at both P_{O_2} levels. Thus, in the experiments reported below, an anoxic stimulus was routinely used in order to optimize the cell response. O_2-sensitive AMC were observed after acute cell isolation (i.e. 3 h *in vitro*) and in short-term cultures (1–3 days), suggesting that the hypoxia-sensing mechanism was expressed *in vivo*, before the cells were brought into culture.

To allow comparisons among cells of different sizes, current density (pA pF^{-1}) was determined by dividing the steady-state outward current (at 45 ms; step from −60 mV) by the whole cell capacitance (range, 4–10 pF; see Methods). Under voltage clamp, the mean (± S.E.M.) outward current density during normoxia ($P_{O_2} = 150$ mmHg) was 115 ± 8·24 pA pF^{-1}

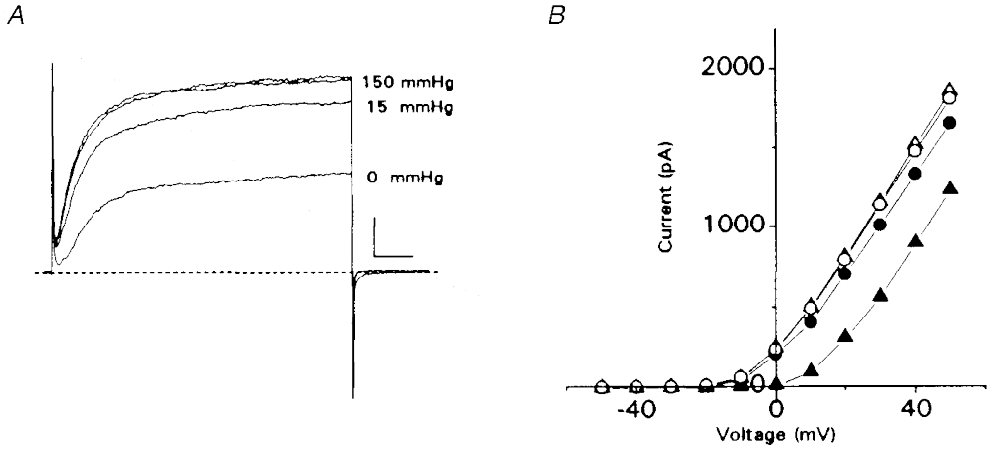

Figure 1. P_{O_2}-dependent suppression of outward current in neonatal rat adrenal chromaffin cells (AMC)

A, leak-unsubtracted outward currents recorded from a cell exposed sequentially to normoxia ($P_{O_2} = 150$ mmHg), hypoxia (P_{O_2} ~15 mmHg), normoxia again (not shown), anoxia ($P_{O_2} = 0$ mmHg) and finally normoxia (wash). All traces were obtained during voltage steps from −60 to +30 mV, and each trace is the average of 4 records; the 2 top traces are superimposed initial (control) and final (wash) recordings in normoxia. Horizontal scale represents 10 ms and vertical scale 200 pA. *B*, current *vs.* voltage plots for the cell in *A*. Records were taken at 10 mV voltage increments from a holding potential of −60 mV. Symbols are as follows: control, ○; hypoxia, ●; anoxia, ▲; wash, △. The anoxic stimulus was applied after the cell had recovered from the hypoxic stimulus (not shown). Wash represents recovery after anoxia. Note the magnitude of outward current suppression is P_{O_2} dependent. Similar results were obtained in 4 other cells exposed to normoxia, hypoxia and anoxia.

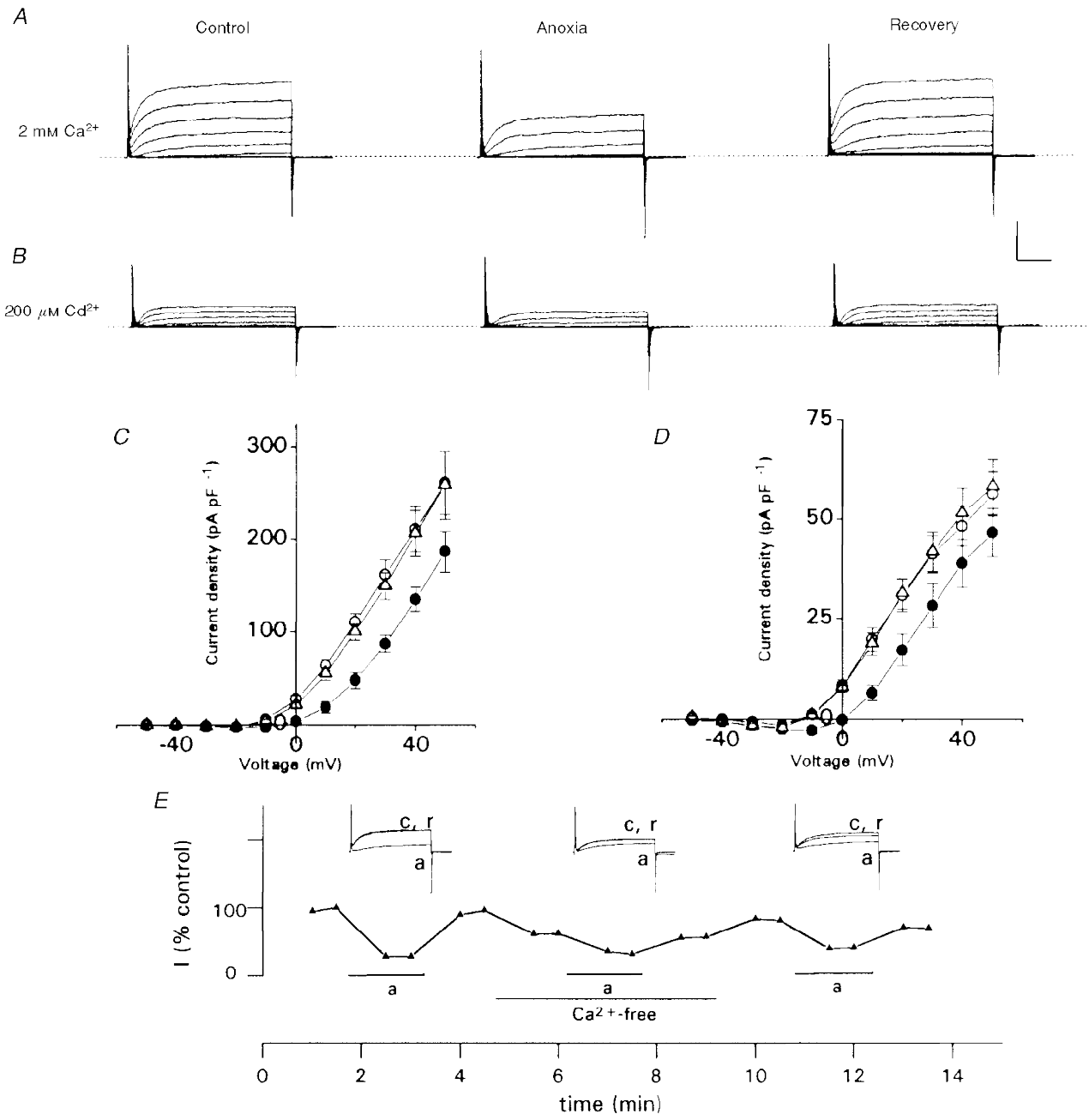

Figure 2. Effect of anoxia on Ca^{2+}-dependent and Ca^{2+}-independent outward currents in neonatal rat AMC

A, leak-unsubtracted outward current traces recorded from a cell in normal (2 mM) Ca^{2+}, before (left; control), during (middle) and after (right; recovery) exposure to anoxia. Traces shown are for voltage steps from a holding potential of −60 to +50 mV in 10 mV increments; the top trace in each record represents the step to +50 mV. Note anoxia reversibly suppresses outward current. *B*, the same cell (as in *A*) exposed to a similar protocol, except that Ca^{2+}-dependent currents were blocked by inclusion of 200 μM Cd^{2+} in the bathing solution. Note anoxia still had a suppressive effect on the residual Ca^{2+}-independent outward current. In *A* and *B*, the vertical scale represents 500 pA and horizontal scale represents 10 ms. *C* and *D*, current density *vs.* voltage plots for 10 representative cells, including the one in *A*. Mean current density (± S.E.M.) is shown for cells recorded in 2 mM Ca^{2+} (*C*) and 200 μM Cd^{2+} (*D*). The current density measured during anoxia was significantly different from control ($P < 0{\cdot}05$) at all voltage steps between −10 and +50 mV in *C*, or −10 and +30 mV in *D*, and recovery was complete at each step. Control, ○; anoxia, ●; recovery, △. *E*, time course of anoxic inhibition of outward currents (step to +40 mV) in the presence and absence (Ca^{2+}-free) of extracellular Ca^{2+}. Note that the cell responded to a third anoxic stimulus after return to normal Ca^{2+}-containing medium. Outward current is expressed as a percentage of the maximum recorded in normal Ca^{2+}. c, a and r refer to control, anoxia and recovery, respectively.

($n = 48$) for a voltage step from −60 to +30 mV. Exposure to anoxia caused a significant reduction in outward current density to 74·25 ± 5·96 pA pF^{-1} ($n = 48$; $P < 0·01$); after reperfusion with control (normoxic) solution, the current density returned to 100·8 ± 8·8 pA pF^{-1} ($n = 48$), a value not significantly different from control ($P > 0·2$). The anoxia-sensitive component of outward current ($I_{K(O_2)}$) comprised a mean of 34·5 ± 0·03% ($n = 48$) of the total outward K^+ current recorded in normoxia for the voltage step from −60 to +30 mV. By first testing for the presence of this anoxia-sensitive K^+ current, O_2-sensitive AMC were identified, thereby allowing the K^+ current subtype(s) that mediate O_2 chemosensitivity to be investigated in greater detail. In the voltage clamp experiments reported below, cells that failed to show > 90% recovery of the control current, after exposure to anoxia or pharmacological agents (with the exception of the poorly reversible, Ca^{2+}-dependent K^+ channel blocker, iberiotoxin), were excluded.

Anoxia suppresses both Ca^{2+}-dependent and Ca^{2+}-independent K^+ currents in neonatal AMC

Closure of a variety of K^+ channels, including both Ca^{2+}-dependent and Ca^{2+}-independent subtypes, mediates hypoxic chemosensitivity in carotid body type 1 cells (Lopez-Lopez *et al.* 1989; Peers, 1990; Gonzalez *et al.* 1994; Buckler, 1997) and in PC12 cells (Conforti & Millhorn, 1997). To test whether both subtypes participate in the anoxic suppression of K^+ current in neonatal AMC, cells were studied in either Ca^{2+}-free or Cd^{2+}-containing (200 μM) bathing solutions. These conditions have been shown to inhibit Ca^{2+}-dependent K^+ currents in adult rat AMC (Neely & Lingle, 1992) and neonatal rat type 1 cells (Peers, 1990). Blockade of Ca^{2+} entry by addition of 200 μM Cd^{2+} to the bathing solution resulted in a reduction in outward current by 64·3 ± 0·06% relative to control ($n = 10$; step from −60 to +30 mV); an example is shown in Fig. 2*A* and *B* (compare left traces). This reduction was not significantly different from that seen in Ca^{2+}-free medium, where the corresponding suppression was 60·1 ± 0·08% ($n = 12$). Thus, as in adult rat AMC (Neely & Lingle, 1992), the majority (~62%) of the outward current in neonatal, O_2-sensitive AMC is Ca^{2+} dependent.

Since anoxia suppressed the outward current by ~35% when Ca^{2+} currents were present (see above and Fig. 2*A*), it was of interest to determine whether the same stimulus had any effect on the residual K^+ current recorded in Ca^{2+}-free or Cd^{2+}-containing solutions. As shown in Fig. 2*B* and *D*, anoxia caused a small but significant suppression in outward current in the presence of 200 μM Cd^{2+} and the effect was reversible. Also, cells exposed to Ca^{2+}-free solutions were still capable of responding normally to another anoxic stimulus, after return to normal Ca^{2+} ($n = 3$; Fig. 2*E*). Experiments similar to those shown in Fig. 2*B*, *D* and *E* allowed a quantitative estimate of $I_{K(VO_2)}$, i.e. the magnitude of the Ca^{2+}-independent component of the total anoxia-sensitive current, $I_{K(O_2)}$. This current, $I_{K(VO_2)}$, is shown as difference current traces for the cell in Fig. 3*A*, lower traces, and compared with $I_{K(O_2)}$ for the same cell in Fig. 3*A*, upper traces. Comparisons of current densities (pA pF^{-1}) for groups of cells exposed to anoxia in Ca^{2+}-free or Cd^{2+}-

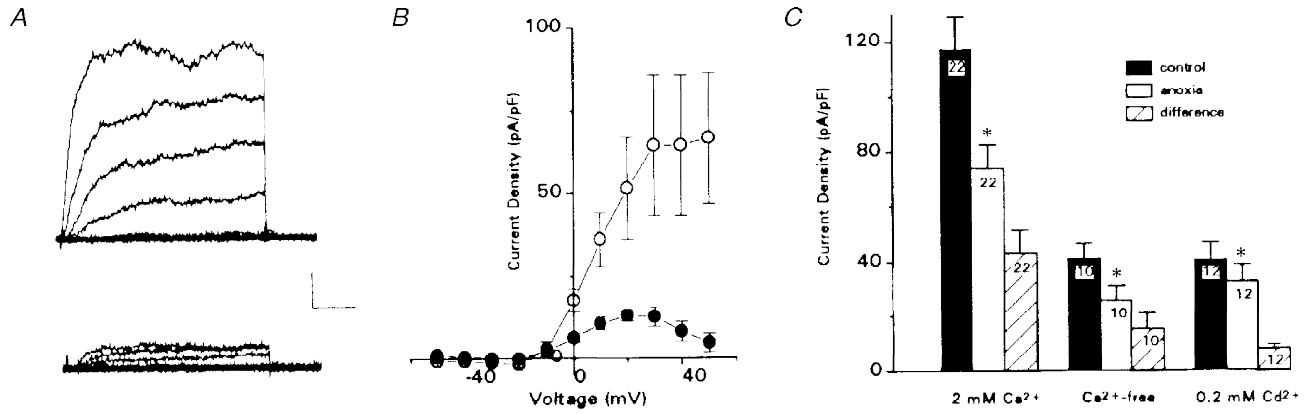

Figure 3. Comparison of anoxia-sensitive difference currents in neonatal chromaffin cells

A, anoxia-sensitive difference currents, obtained by subtracting current traces recorded during anoxia from corresponding ones in normoxia, shown in normal Ca^{2+}-containing (upper traces) and Ca^{2+}- free (lower traces) solutions. Subtracted traces are for 10 mV incremental steps between −50 and +30 mV; holding potential was −60 mV. Note the larger component of the anoxia-sensitive difference current is Ca^{2+} sensitive; lower traces represent the Ca^{2+}-*independent* O_2-sensitive current, $I_{K(VO_2)}$. Vertical scale bar represents 100 pA, horizontal scale bar represents 10 ms. *B*, current density *vs.* voltage plots for 6 representative cells, showing the total (mean ± S.E.M.) anoxia-sensitive component, $I_{K(O_2)}$ (○) and $I_{K(VO_2)}$ (●; recorded in 200 μM Cd^{2+}). *C*, comparison of mean (± S.E.M.) outward current density at +30 mV, for all cells investigated in normal Ca^{2+} (2 mM), Ca^{2+}-free and Cd^{2+}-containing (200 μM) bathing solutions. * Significantly different from control group ($P < 0·05$). The mean current density (at +30 mV) during anoxia in the presence of Cd^{2+}, or in Ca^{2+}-free solutions, was significantly different from that in the presence of 2 mM Ca^{2+} ($P < 0·01$).

containing solutions are shown in Fig. 3*B* and *C*. The difference current traces shown in Fig. 3*A*, where currents recorded in anoxia are subtracted from control (normoxic) currents, allow the anoxia-sensitive component to be isolated in both normal Ca^{2+} (upper traces) and Ca^{2+}-free (lower traces) solutions. It is evident from these traces, as well as from the current density *vs.* voltage plot in Fig. 3*B*, that the Ca^{2+}-independent component, $I_{K(VO_2)}$, represents only a small fraction of the total $I_{K(O_2)}$. For a voltage step from −60 to +30 mV, $I_{K(VO_2)}$ represents 39·1 ± 0·1 % ($n = 10$) of $I_{K(O_2)}$ in Ca^{2+}-free, and 26·1 ± 0·05 % ($n = 12$) in Cd^{2+}-containing solutions. This implies that the remaining ~65 % of $I_{K(O_2)}$ must be Ca^{2+} dependent, i.e. $I_{K(CaO_2)}$. Both of these O_2-sensitive K^+ currents were observed in the majority of cells tested ($n = 21/22$). A comparison of the absolute K^+ current densities in normoxia and anoxia, as well as the difference K^+ current density, is shown in Fig. 3*C* for cells recorded in normal Ca^{2+}, Ca^{2+}-free and Cd^{2+}-containing solutions. Note that the anoxia-sensitive, or difference K^+ current density, is similar in Ca^{2+}-free and Cd^{2+}-containing solutions (i.e. $I_{K(VO_2)}$).

Neonatal AMC possess anoxia-sensitive K_{ATP} currents

K_{ATP} channels have been implicated in hypoxic chemosensitivity of *adult* rat AMC, based on the observation that openers of these channels prevented the hypoxia-induced rise in intracellular Ca^{2+} (Mochizuki-Oda *et al.* 1997). In addition, activation of K_{ATP} channels during hypoxia is hypothesized to play a protective role in some central neurons (Jiang *et al.* 1994). If similar channels were present in *neonatal* rat AMC, then their activation by anoxia would tend to oppose or reduce the inhibition of outward current resulting from closure of the Ca^{2+}-dependent and Ca^{2+}-independent channels described above. To determine if O_2-sensitive AMC possess K_{ATP} channels we used both pinacidil (a K_{ATP} activator) and glibenclamide (a K_{ATP} blocker; Ashcroft & Ashcroft, 1990). In Fig. 4*Aa* and *Ab*, exposure of AMC to 100 μM pinacidil resulted in a reversible *augmentation* of outward current and current density. For a voltage step from −60 to +30 mV, the outward current density was increased from the control value of 128·3 ± 19·5 pA pF^{-1} to 173·3 ± 16·6 pA pF^{-1} in the presence of 100 μM pinacidil ($n = 5$; $P < 0·05$); after washout of pinacidil the current density recovered to 132·7 ± 25·8 pA pF^{-1}. These results suggest that O_2-sensitive AMC possess K_{ATP} channels.

We then tested whether activity of these K_{ATP} channels was regulated by O_2 tension, using the specific K_{ATP} channel blocker, glibenclamide (50 μM). Figure 4*Ba* and *Bb* shows the effect of glibenclamide on outward K^+ currents recorded during normoxia ($P_{O_2} = 150$ mmHg). In most (6/9) cells there was no detectable effect of glibenclamide (Fig. 4*Ba*), though in the remaining (3/9) cells, a small, reversible suppression in K^+ current was observed (not shown). Pooled data from the nine cells revealed that for a voltage step to +30 mV, the mean current density for the glibenclamide-sensitive component was 6·3 ± 4·0 pA pF^{-1} (range, 0 to 25·2 pA pF^{-1}), corresponding to 4·9 ± 0·8 % of the total outward current density. Plots of current density *vs.* voltage under normoxic conditions are shown in Fig. 4*Bb* for these nine cells before, during and after glibenclamide; overall, the latter had negligible effect over the voltage range −30 to +50 mV.

In contrast, exposure to anoxia had a profound effect on glibenclamide sensitivity. For example, in Fig. 4*Ca*, anoxia alone produced the usual suppression in K^+ current, but when combined with glibenclamide the suppression was much more dramatic (compare *same* cell in Fig. 4*Ba*), and the effect was completely reversible. Pooled data from nine cells revealed that for a voltage step from −60 to +30 mV, the outward current density was reduced from 80·7 ± 17·8 pA pF^{-1} in normoxia to 57·0 ± 11·6 pA pF^{-1} in anoxia alone, and this was further reduced to 32·0 ± 6·1 pA pF^{-1} in anoxia plus glibenclamide (significantly different from anoxia alone; $P < 0·01$). The mean outward current density after reperfusion with control (normoxic) solution was 75·5 ± 16·7 pA pF^{-1}, a value not significantly different from the initial control response. Current density *vs.* voltage plots are shown in Fig. 4*Cb* for these cells, which were exposed sequentially to normoxia, anoxia, anoxia plus glibenclamide, and then returned to normoxia. These data indicate that for a step from −60 to +30 mV, the anoxia-sensitive component of outward current $I_{K(O_2)}$ comprised 29·4 ± 0·1 % ($n = 9$) of the control (normoxic) K^+ current in the absence of glibenclamide, compared with 60·3 ± 0·1 % ($n = 9$) in its presence (difference significant; $P < 0·05$). Measurements of difference current density allowed a direct comparison of the magnitude of the glibenclamide-sensitive component in normoxia and anoxia. For a step to +30 mV, the glibenclamide-sensitive component during anoxia was 24·9 ± 7·9 pA pF^{-1} ($n = 9$), a value significantly different ($P < 0·05$) from that seen in normoxia (6·3 ± 4·0 pA pF^{-1}; $n = 9$).

Are anoxia-sensitive K_{ATP} channels in neonatal AMC also Ca^{2+} sensitive?

In central neurons, the O_2-sensitive K_{ATP} channels are Ca^{2+} sensitive (Jiang *et al.* 1994). To investigate whether the same is true for K_{ATP} channels in neonatal AMC, the effect of glibenclamide on O_2 sensitivity was tested in Cd^{2+}-containing solutions, which block Ca^{2+} entry. As shown in Fig. 4*Da* and *b*, the presence of glibenclamide (50 μM) had no *additional* effect on the outward current recorded during anoxia over the voltage range −30 to +30 mV when Cd^{2+} was present. Under these conditions, the glibenclamide-sensitive component was 6·6 ± 4·9 pA pF^{-1} ($n = 7$) in normoxia *vs.* 10·0 ± 3·3 pA pF^{-1} ($n = 7$) in anoxia for a voltage step from −60 to +30 mV (difference not significant; $P > 0·1$). This contrasts with the results reported above in normal Ca^{2+} (Cd^{2+}-free) solutions (Fig. 4*Ca* and *Cb*), where glibenclamide significantly reduced the K^+ current recorded in anoxia. Current density *vs.* voltage plots are compared in Fig. 4*Db* for these seven cells in the continuous presence of Cd^{2+}-containing solutions, during exposure to normoxia (○),

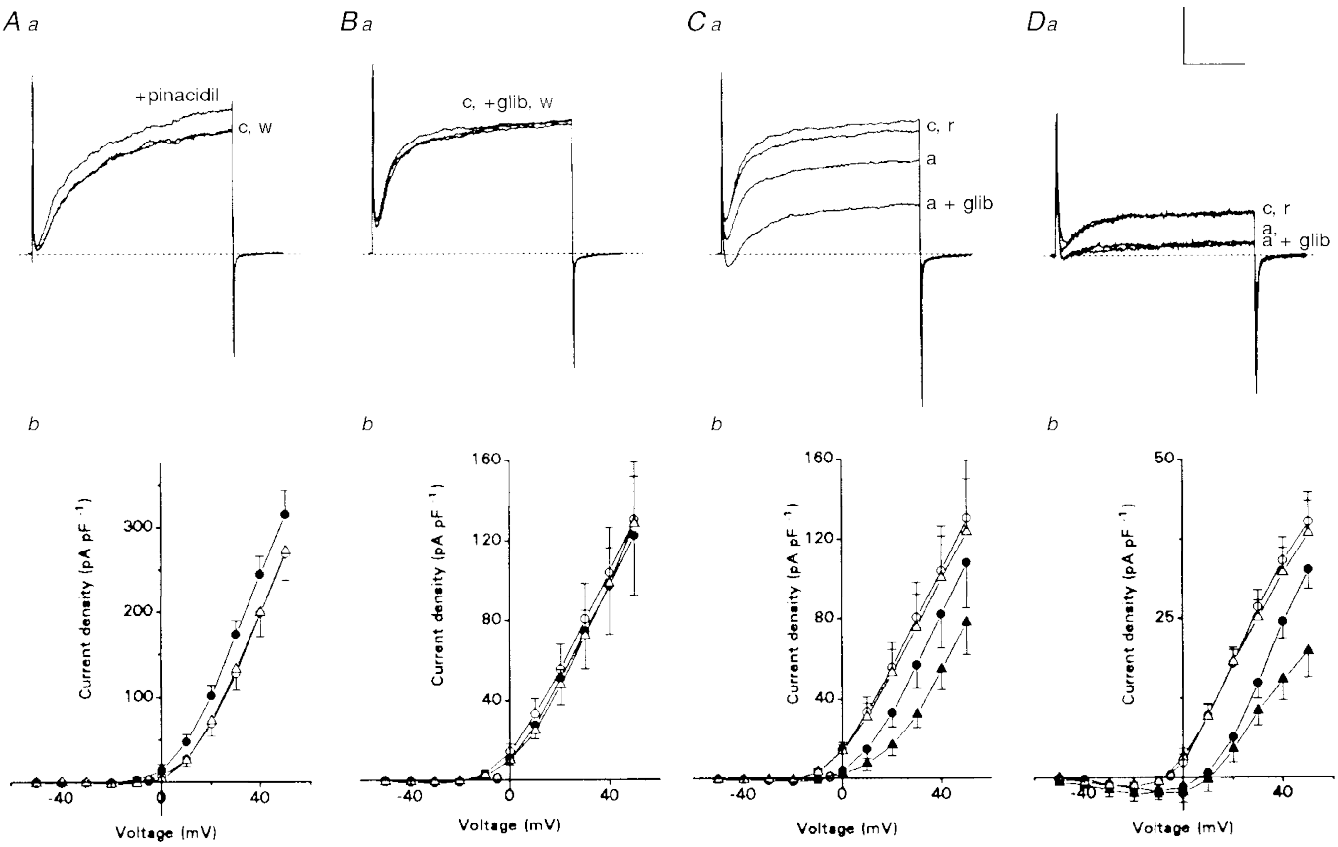

Figure 4. Comparison of the effects of K_{ATP} channel modulators on K^+ current and K^+ current density recorded during normoxia or anoxia in neonatal AMC

All current records are shown for cells studied under voltage clamp, and representative traces are shown for the voltage step from −60 to +30 mV. *Aa* and *b*: in normoxia, effects of pinacidil (100 μM), an activator of $I_{K(ATP)}$, on outward currents and current density. Note that sequential application of pinacidil (+pinacidil, ●), increases outward current above control (c, ○), and the effect was reversible after washout (w, △). Mean (± S.E.M.) K^+ current density is shown for a group of 5 cells, indicating that pinacidil significantly increased K^+ current density relative to control at all voltage steps between 0 and +50 mV ($P < 0{\cdot}05$). *Ba* and *b*: current records in normoxia for cells ($n = 9$) treated with control saline (c, ○), 50 μM glibenclamide (+glib, ●), a blocker of $I_{K(ATP)}$, and after washout (w, △) of this drug. Mean (± S.E.M.) K^+ current density *vs.* voltage plot for 9 cells shows that glibenclamide did not significantly affect outward current density. *Ca* and *b*: effect of simultaneous exposure to anoxia and glibenclamide on outward current for the same 9 cells as in *B*. Cells were sequentially exposed to normoxia/control (c, ○), anoxia (a, ●), anoxia plus glibenclamide (a + glib, ▲) and recovery (r, △). Note that the inhibitory effect of anoxia (a) on the outward current was greatly exaggerated in the presence of glibenclamide. Current density in anoxia was significantly less than normoxic control at all voltage steps between 0 and +50 mV ($P < 0{\cdot}01$); also, current density in anoxia plus glibenclamide was significantly less than anoxia alone, between −20 and +30 mV ($P < 0{\cdot}05$). *Da* and *b*: outward current and current density recorded in the presence of Cd^{2+} (200 μM), to block Ca^{2+}-dependent K^+ currents. Note that anoxia (a) reversibly suppressed the outward current, but addition of 50 μM glibenclamide (a + glib) had no *additional* suppressive effect during anoxia (compare *Ca*), suggesting that $I_{K(ATP)}$ is Ca^{2+} dependent. Mean (± S.E.M.) K^+ current density *vs.* voltage plot is for a group of 7 cells and shows that anoxia significantly ($P < 0{\cdot}01$) suppressed outward currents in the presence of Cd^{2+} at all potentials between −10 and +50 mV. In the presence of glibenclamide, the additional suppression of outward current by anoxia was absent at all test potentials below +40 mV ($P > 0{\cdot}05$). Symbols are the same as in *C*. This suggests $I_{K(ATP)}$ is Ca^{2+} sensitive. Vertical scale represents 400 pA in *A*, *B* and *C* and 100 pA in *D*. Horizontal scale represents 30 ms.

anoxia alone (●), anoxia plus glibenclamide (▲) and finally normoxia again (△). However, at voltage steps > +40 mV there is an apparent activation of $I_{K(ATP)}$ by anoxia (Fig. 4*Db*), even in the presence of Cd^{2+}. The underlying mechanism is unclear, but may involve interactions of divalent cations (eg. Cd^{2+} or Mg^{2+}) with the K_{ATP} channel (Ashcroft & Ashcroft, 1990).

Pharmacology of $I_{K(CaO_2)}$ and $I_{K(VO_2)}$

Since the predominant Ca^{2+}-dependent outward current in adult AMC is carried by large conductance maxi-K^+ or BK potassium channels (Neely & Lingle, 1992), which are known to be inhibited by hypoxia in carotid body type 1 cells (Wyatt & Peers, 1995), it was of interest to determine if $I_{K(CaO_2)}$ was also carried by BK channels. To test this we used the specific blocker, iberiotoxin (IbTx), to inhibit the large conductance BK channels (Galvez *et al.* 1990). Exposure of neonatal AMC to IbTx (50–100 nM) resulted in a suppression of the outward current, suggesting the presence of BK channels (Fig. 5*A*). The IbTx-sensitive component consisted of 47·6 ± 0·04 % ($n = 4$) of the overall outward current, for a voltage step from −60 to +30 mV. This is slightly less than the values (~62 %) reported above for the magnitude of the Ca^{2+}-dependent K^+ currents recorded in Ca^{2+}-free or Cd^{2+}-containing solutions, and is probably due to the persistence of small-conductance Ca^{2+}-dependent K^+ channels, which are insensitive to IbTx (Park, 1994). Exposure of neonatal AMC to anoxia in the presence

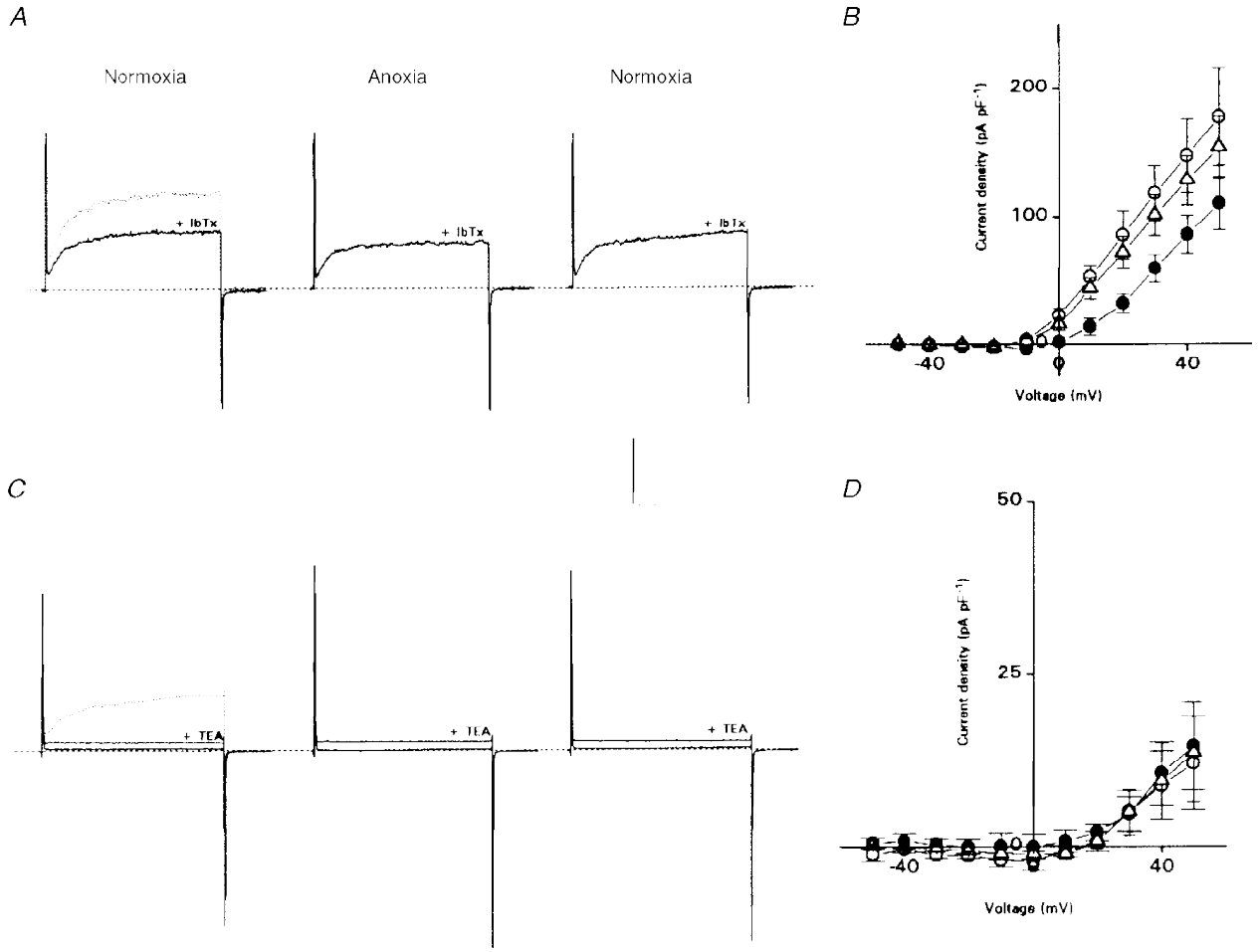

Figure 5. Effects of iberiotoxin (IbTx) and tetraethylammonium (TEA) on anoxia-sensitive currents in neonatal AMC

A, in normoxic conditions, IbTx (100 nM) suppressed outward current at +30 mV (left traces); upper trace is control record before IbTx. In the presence of IbTx, anoxia still suppressed outward current reversibly, though washout of the effects of IbTx was incomplete (not shown). *B*, mean current density (± S.E.M.) *vs.* voltage plots for 4 cells, in the presence of IbTx. Symbols are same as in Fig. 1. Anoxia significantly suppressed outward current at all voltage steps between 0 and +50 mV ($P < 0·05$). *C*, effects of TEA on outward currents. Addition of TEA (20 mM) suppressed ~96 % of outward current (left traces; upper dotted trace is control current for step to +30 mV). Anoxia had no *additional* effect on outward current in the presence of TEA (middle traces; only steps to +30 and +50 mV shown). *D*, mean current density *vs.* voltage plots for a group of 6 cells with similar initial densities; TEA was present throughout. Vertical and horizontal scale bars represent 200 pA and 20 ms, respectively, for both *A* and *C*.

of IbTx (Fig. 5*A*, middle trace) caused a further suppression in outward current. Plots of current density *vs.* voltage indicate that the suppression in IbTx-containing medium occurred at all voltage steps between −10 and +50 mV, and the effect of anoxia was reversible (Fig. 5*B*). Generally, washout of the effects of IbTx on the total K^+ current was incomplete, even after reperfusion for 10–15 min with control solution. The contribution of both the IbTx-sensitive and IbTx-insensitive K^+ currents to the total anoxia-sensitive $I_{K(O_2)}$ was determined from the difference current measurements for the voltage step to +30 mV. The IbTx-insensitive portion of $I_{K(O_2)}$ represented 42·2 ± 0·2% ($n = 4$) of the total $I_{K(O_2)}$, and the remaining 57·8 ± 0·2% was IbTx sensitive. Since the IbTx-insensitive component of $I_{K(O_2)}$ is similar to that obtained above for the Ca^{2+}-independent portion of $I_{K(O_2)}$, i.e. $I_{K(VO_2)}$, obtained in Ca^{2+}-free (~39%) and Cd^{2+}-containing (~28%) solutions, it appears that $I_{K(CaO_2)}$ is carried almost exclusively by IbTx-sensitive BK channels. To investigate further the pharmacology of anoxia-sensitive K^+ currents, the non-specific K^+ channel blocker, TEA, was used.

Figure 5*C* (left traces) shows that 20 mM TEA reduced substantially the outward current in a cell that was previously identified as anoxia sensitive. In nine such cells, 10–20 mM TEA caused a mean suppression of outward current by 95·7 ± 1·4% of control, as measured during a voltage step from −60 to +30 mV. However, in the presence of 10–20 mM TEA, anoxia had no additional effect on the residual K^+ currents in eight out of nine cells tested (e.g. Fig. 5*C* (middle traces) and *D*). Since the cell shown in Fig. 5*C* contained both $I_{K(CaO_2)}$ and $I_{K(VO_2)}$ components of outward current (not shown), it appears that both components are sensitive to 10–20 mM TEA. Figure 5*D* also indicates that the remaining outward current seen at more depolarized potentials (> 20 mV) is anoxia insensitive. Since anoxia activates $I_{K(ATP)}$ in these cells, the data also suggest that $I_{K(ATP)}$ is TEA sensitive.

O_2 sensitivity in AMC with different types of BK currents

In adult rat adrenal chromaffin cells two types of BK currents have been described. One is a non-inactivating current ($I_{BK(s)}$; in ~9% of adult AMC) and the other is a slowly inactivating current ($I_{BK(i)}$; in ~75% of adult AMC); the remaining ~15% of cells express both currents (Solaro *et al.* 1995). Since hypoxic suppression of outward currents was not observed in all neonatal AMC (~80% respond), the question arises whether O_2 chemosensitivity was restricted to a specific population of BK-expressing cells (i.e. BK_i or

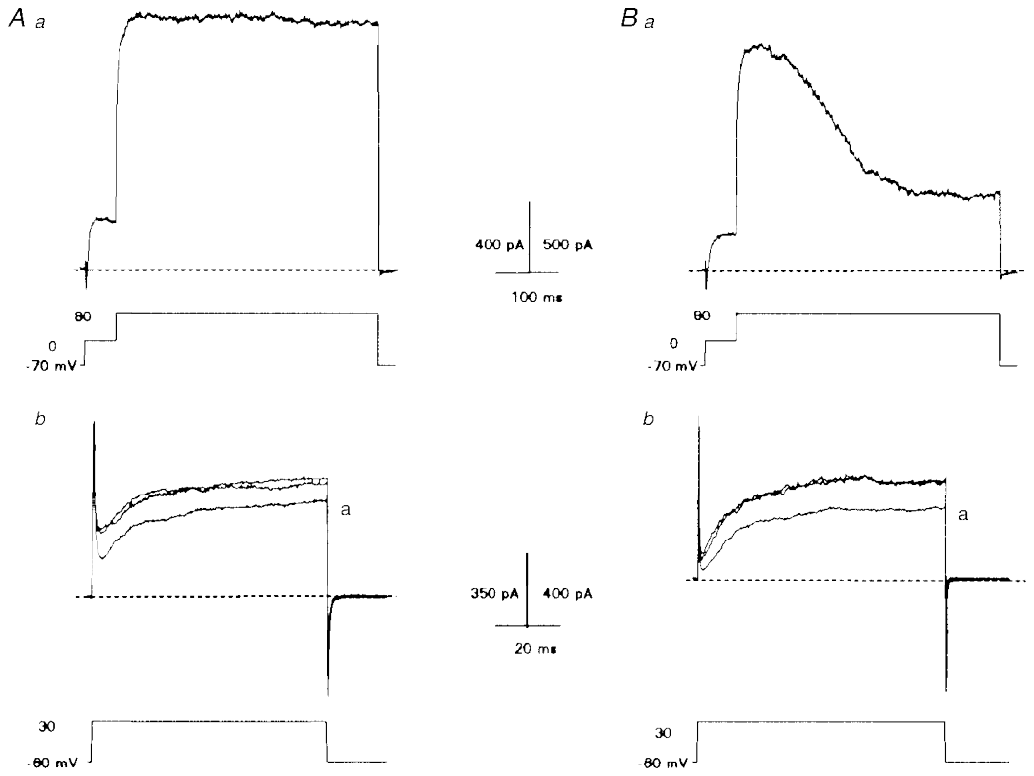

Figure 6. Chromaffin cells expressing either sustained or inactivating BK currents are anoxia sensitive

Aa and *Ba*, protocol used to identify AMC that expressed a sustained BK (*A*) or inactivating BK (*B*) current. Cells were initially held at −70 mV and briefly stepped to 0 mV for 50 ms (lower traces), before a final step to +80 mV for 500 ms. Both cells contained outward current that was reversibly suppressed by anoxia (a) as shown in *Ab* (for cell in *Aa*) and *Bb* (for cell in *Ba*). Traces shown for voltage step from −60 to +30 mV.

BK_s). To identify BK_i- and BK_s-expressing cells, a voltage-clamp protocol reported to enhance BK currents in these cells was applied (Solaro *et al.* 1995). Cells were held at −70 mV and stepped to 0 mV for 50 ms to load them with Ca^{2+}, and then immediately stepped to +80 mV for 500 ms to identify the type of BK current (see Fig. 6). Out of seventeen cells examined, twelve contained predominantly BK_s currents, i.e. they showed no inactivation of outward current during the 500 ms step to +80 mV (e.g. Fig. 6*Aa*, upper trace). The remaining five cells contained a slowly inactivating component of outward current, presumably due to the presence of $I_{BK(i)}$ (e.g. Fig. 6*Ba*, upper trace). It was found that both cells containing BK_s (10/12 cells) and BK_i (4/5 cells) responded to hypoxia with a suppression of outward current (Fig. 6*Ab* and *Bb*, respectively). These data suggest that O_2 sensitivity can occur in neonatal rat AMC which express either $I_{BK(s)}$ or $I_{BK(i)}$ currents.

Do $I_{K(CaO_2)}$ or $I_{K(VO_2)}$ mediate receptor potential in AMC during anoxia?

We previously reported that neonatal AMC depolarize during hypoxia, and that this depolarization was associated with a conductance *decrease* (Thompson *et al.* 1997). These observations are consistent with the closure of, for example, K^+ channels as a general mechanism for hypoxia-induced depolarization in O_2-chemoreceptive cells (Gonzalez *et al.* 1994). However, given a resting potential in neonatal AMC of ∼−60 mV, it is unclear whether inhibition of the O_2-sensitive currents described above, i.e. $I_{K(CaO_2)}$ and $I_{K(VO_2)}$, forms the basis of the initial membrane depolarization or receptor potential during hypoxia. To test whether inhibition of $I_{K(CaO_2)}$ and/or $I_{K(VO_2)}$ may contribute to the initial depolarization, neonatal AMC were exposed to solutions containing Cd^{2+} (200 μM) and/or TEA (20 mM), while the membrane potential was monitored using perforated-patch recording in current clamp mode.

As illustrated in Fig. 7*A* and *B* (left portion of trace), exposure of neonatal AMC to anoxia caused a significant (and reversible) depolarization from a mean resting potential of −57·6 ± 1·9 to −46·3 ± 1·8 mV ($n = 11$; $P < 0·01$). However, subsequent exposure to 200 μM Cd^{2+} had no effect on membrane potential (−58·3 ± 2·6 mV after Cd^{2+}, $n = 6$; see Fig. 7*A*, right portion of trace), nor did it prevent the anoxia-induced depolarization (mean membrane potential during anoxia in the presence of $Cd^{2+} = -49·0 \pm 2·5$ mV, $n = 6$; difference significant from Cd^{2+} control, $P < 0·01$). Since voltage-dependent Na^+ channels were blocked in these experiments with 0·5 μM TTX, the recorded spike activity in Fig. 7 was probably due to Ca^{2+} entry, and this was abolished in the presence of Cd^{2+} (see Fig. 7*A*; note spike activity in left but not right portions of the trace). These results suggest that $I_{K(CaO_2)}$ does not contribute significantly to the initial depolarization (or receptor potential) in neonatal AMC during anoxia, nor to the resting membrane potential. Confirmation of this point was obtained using 50 nM charybdotoxin (ChTx) to block the large conductance BK channels that mediate $I_{K(CaO_2)}$. For these experiments ChTx was used (instead of IbTx) since it is a much faster blocker of BK channels (Galvez *et al.* 1990), and therefore the effects of direct BK channel inhibition could be more easily assessed. In Fig. 7*B*, 50 nM ChTx failed to depolarize neonatal AMC, and also failed to prevent the anoxia-induced depolarization, even in this cell which had a relatively low initial resting potential (−40 mV). This result was seen in all cells tested ($n = 3$).

In contrast to the above results, the more general K^+ channel blocker TEA (10–20 mM) depolarized neonatal AMC from −57·6 ± 1·9 to −48·1 ± 5·2 mV ($n = 5$; difference significant, $P < 0·01$; see Fig. 7*C*). However, even in the presence of 20 mM TEA, exposure to anoxia caused a further significant depolarization to −40·8 ± 4·5 mV ($P < 0·01$), and the effect was reversible (Fig. 7*C*; right portion of trace). Additionally, when TEA and Cd^{2+} were applied together, the membrane depolarized, but the depolarizing effects of anoxia still persisted in the presence of these drugs ($n = 3$; not shown). These data suggest that though some TEA-sensitive K^+ channels are open at rest, they are not responsible for the anoxia-induced depolarization.

Blockade of other K^+ channel subtypes with 2 mM 4-AP did not alter resting membrane potential of neonatal AMC ($n = 4$; mean membrane potential was −59·7 ± 6·4 before, and −57·7 ± 5·6 mV after 4-AP). Further, in the presence of 2 mM 4-AP, anoxia still depolarized AMC from −59·7 ± 6·4 to −46·0 ± 5·4 mV ($n = 4$), an effect similar to that seen in the absence of 4-AP, where the membrane potential depolarized to −47·7 ± 6·1 mV ($n = 4$; see Fig. 7*D*). These observations suggest there is a distinct O_2-sensing mechanism in neonatal AMC that controls at least part of the initial depolarization or receptor potential during anoxia, and that is insensitive to conventional blockers of voltage-dependent K^+ channels.

Does a cationic current contribute to O_2 sensitivity in rat AMC?

In guinea-pig AMC, a cationic current that was activated by hypoxia was recently described (Inoue *et al.* 1998). Since during voltage-clamp studies in Cd^{2+}-containing (or Ca^{2+}-free) solutions we often observed a slight enhancement of inward current during hypoxia (e.g. Fig. 2*D* and 4*Db*), we wondered whether a similar cationic current may contribute to the receptor potential in rat AMC. To test this possibility we examined the effects of anoxia on AMC under current clamp, while perfusing a Na^+/Ca^{2+}-free bathing solution. Interestingly, under these conditions anoxia-sensitive AMC hyperpolarized to −63·1 ± 9·2 mV ($n = 4$) from the control membrane potential of −52·6 ± 6·5 mV ($P < 0·05$; Fig. 7*E*). Since this hyperpolarization was also observed in Na^+-free solutions that contained Ca^{2+} (not shown), it appears that a substantial Na^+ leak current contributes to the resting membrane potential of AMC. Application of an anoxic stimulus in the presence of a Na^+/Ca^{2+}-free bathing solution revealed that AMC depolarized from −63·1 ± 9·2

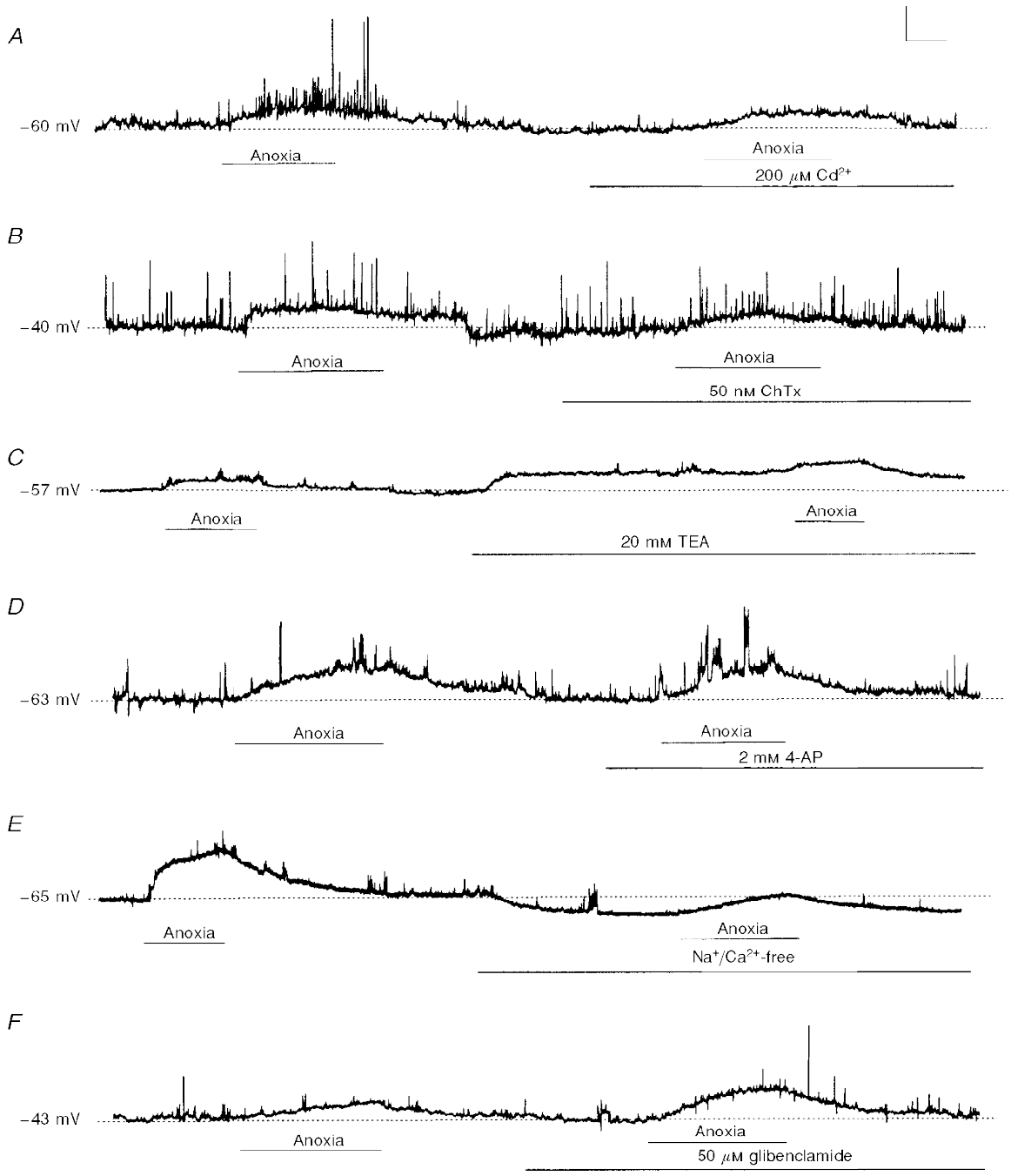

Figure 7. Effects of anoxia and manipulation of the extracellular fluid on membrane potential of neonatal AMC

Recordings under current clamp were obtained in the continuous presence of TTX (0·5–1 μM). *A*, anoxia depolarized AMC, though addition of Cd^{2+} alone did not; further, Cd^{2+} did not prevent the anoxia-induced depolarization, but abolished spike activity (compare right with left portions of trace). *B*, direct blockade of BK currents with 50 nM ChTx did not significantly alter membrane potential, nor prevent the anoxia-induced depolarization (compare left and right portions of trace). *C*, addition of TEA (20 mM) caused a slight membrane depolarization (right portion of trace), but did not block the anoxia-induced depolarization. *D*, addition of 4-AP did not depolarize nor prevent anoxia-induced depolarization of neonatal AMC. *E*, effects of perfusion with a Na^+/Ca^{2+}-free ($NMDG^+$ was substituted for Na^+; Ca^{2+} was replaced with 2 mM Mg^{2+} and 1 mM EGTA) bathing solution. Exposure to this solution hyperpolarized AMC and reduced, but did not block, the anoxia-induced depolarization. *F*, blockade of K_{ATP} channels with 50 μM glibenclamide did not affect resting membrane potential, nor did it prevent the anoxia-induced depolarization. However, in the presence of glibenclamide the anoxia-induced depolarization was significantly ($P < 0·05$) enhanced. Vertical scale bars represent 20 mV in *A*–*E* and 15 mV in *F*; horizontal bar represents 25 s.

to $-51{\cdot}6 \pm 9{\cdot}4$ mV ($n = 4$; Fig. 7*E*). Thus, the anoxia-induced depolarization was reduced, but not abolished, in the absence of extracellular Na^+ and Ca^{2+}. The mean depolarization in the control solution was $24{\cdot}2 \pm 2{\cdot}6$ mV ($n = 4$) compared with $11{\cdot}5 \pm 2{\cdot}5$ mV for the *same* cells studied in the absence of external Na^+ and Ca^{2+} (e.g. Fig. 7*E*; $P < 0{\cdot}01$).

Activation of K_{ATP} attenuates the anoxia-induced depolarization

We also investigated whether K_{ATP} channels were active at the resting potential of O_2-sensitive AMC and could therefore influence the magnitude of the receptor potential. Perfusion with extracellular solution containing the K_{ATP} channel blocker, glibenclamide (50 μM), had no significant effect ($P > 0{\cdot}2$) on membrane potential (Fig. 7*F*); the mean resting potential was $-52{\cdot}6 \pm 3{\cdot}2$ mV before, and $-54{\cdot}0 \pm 3{\cdot}5$ mV after glibenclamide for a group of eleven cells that were O_2 sensitive. Thus, K_{ATP} channels do not appear to contribute significantly to the resting potential of neonatal AMC during normoxia.

To determine if activation of $I_{K(ATP)}$ by anoxia (see above) functions to attenuate the receptor potential, the anoxia-induced depolarization was compared in the presence and absence of glibenclamide (50 μM). In the absence of glibenclamide, AMC depolarized by an average of $16{\cdot}6 \pm 2{\cdot}5$ mV ($n = 11$) during anoxia. However, this depolarization was significantly ($P < 0{\cdot}01$) enhanced when anoxia was applied in the presence of glibenclamide (mean $= 23{\cdot}3 \pm 3{\cdot}1$ mV; $n = 11$; e.g. Fig. 7*F*). Since $I_{K(ATP)}$ also appears to be TEA sensitive (see above), it was surprising that the anoxia-induced depolarization was not similarly enhanced in the presence of TEA. Although the reasons for this are unclear, the two drugs block $I_{K(ATP)}$ via different mechanisms and, unlike TEA, glibenclamide had no effect on resting membrane potential (compare Fig. 7*C* with *F*). These data suggest that, although $I_{K(ATP)}$ does not appear to contribute to the resting potential of AMC during normoxia, its activation by anoxia may serve to reduce the magnitude of the receptor potential.

DISCUSSION

The goal of the present study was to investigate the type(s) of voltage-activated K^+ currents that mediate O_2 chemosensitivity in neonatal rat adrenal chromaffin cells (AMC), and to determine whether these currents also contribute to the genesis of the hypoxia-induced depolarization or receptor potential (see Thompson *et al.* 1997). These hypoxia-sensing mechanisms appear to mediate the vital catecholamine surge that enables the neonate to survive the hypoxic stress associated with the transition to extrauterine life (Seidler & Slotkin, 1985; Slotkin & Seidler, 1988; Thompson *et al.* 1997). Our results from voltage-clamp studies indicated that the suppression of voltage-dependent outward K^+ current by anoxia is the net result of the differential modulation of several K^+ currents and comprises: (i) anoxic suppression of the large conductance Ca^{2+}-dependent K^+ current, $I_{K(CaO_2)}$; (ii) anoxic suppression of a Ca^{2+}-independent K^+ current, $I_{K(VO_2)}$; and (iii) anoxic *activation* of a glibenclamide-sensitive K^+ current, $I_{K(ATP)}$. Additionally, results from current clamp recordings indicated that alternative mechanisms to these voltage-dependent and anoxia-sensitive K^+ currents must be invoked to account for the genesis of the receptor potential seen during exposure of neonatal rat AMC to anoxia.

Anoxic suppression of outward current in neonatal AMC results primarily from the inhibition of large conductance, Ca^{2+}-dependent K^+ channels, i.e. BK or maxi-K^+ channels, which mediate $I_{K(CaO_2)}$ in this study. Though we cannot formally rule out an indirect effect due to anoxic suppression of Ca^{2+} currents (see Lopez-Barneo, 1996), in preliminary studies there was no consistent effect of anoxia on Ca^{2+} currents (R. J. Thompson & C. A. Nurse, unpublished observations), and we previously showed that entry of extracellular calcium and catecholamine secretion was enhanced in these cells by hypoxia (Thompson *et al.* 1997). When BK currents were eliminated, by using Ca^{2+}-free solutions or by the addition of either Cd^{2+} or iberiotoxin (IbTx), a smaller Ca^{2+}-independent component ($I_{K(VO_2)}$) amounting to ~35% of the total anoxia-sensitive outward current ($I_{K(O_2)}$) persisted. Since TEA (10–20 mM) abolished almost all of the outward current, and anoxia had a negligible effect on the residual currents when TEA was present, it appears that both $I_{K(CaO_2)}$ and $I_{K(VO_2)}$ are TEA sensitive. It is noteworthy that in PC12 cells, a cell line with an adrenomedullary chromaffin cell origin, hypoxia suppresses a Ca^{2+}-independent and TEA-sensitive outward current (Zhu *et al.* 1996), raising the possibility that it is similar to $I_{K(VO_2)}$ investigated in the present study. In contrast, however, hypoxia was reported to *stimulate* large conductance, Ca^{2+}-dependent K^+ channels in PC12 cells, though the pharmacology of this current was not reported (Conforti & Millhorn, 1997). It is possible that this anoxia-activated current is similar to $I_{K(ATP)}$ described here (see below) and in central neurons (Haddad & Jiang, 1997).

Anoxic activation of K_{ATP} currents

In addition to $I_{K(CaO_2)}$ and $I_{K(VO_2)}$, we observed a third voltage dependent, Ca^{2+}-sensitive K^+ current, $I_{K(ATP)}$, which was regulated by P_{O_2}. The presence of this current was inferred from experiments where the total outward current was enhanced by pinacidil (100 μM), a K_{ATP} channel activator, and where the anoxic suppression of outward current was augmented by glibenclamide (50 μM), a K_{ATP} blocker (see Ashcroft & Ashcroft, 1990). Unlike the suppressive effects of anoxia on the other two currents, $I_{K(ATP)}$ was *activated* by anoxia in these cells, suggesting more K_{ATP} channels became available or were opened at low P_{O_2}. Since this augmentation of K_{ATP} by anoxia was absent in a Cd^{2+}- or TEA-containing bathing solution (but persisted in the presence of the BK channel blocker, ChTx;

not shown), it appears that $I_{K(ATP)}$ is both Ca^{2+} and TEA sensitive. Similar results have been reported for O_2-regulated K_{ATP} channels described in central neurons (Jiang *et al.* 1994; Haddad & Jiang, 1997). Taken together, these results support the conclusion that anoxia augments K_{ATP} channels, but attenuates both Ca^{2+}-dependent ($I_{K(CaO_2)}$) and delayed rectifier type ($I_{K(VO_2)}$) K^+ currents in neonatal AMC.

Interestingly, the augmentation of K_{ATP} by anoxia observed in this study is in contrast with a recent suggestion that hypoxia inhibits K_{ATP} currents in adult rat chromaffin cells (Mochizuki-Oda *et al.* 1997). This interpretation was based on the finding that cromakalim, an activator of K_{ATP} channel currents, prevented the hypoxia-induced rise in intracellular Ca^{2+}; however, a direct inhibitory effect of hypoxia on K_{ATP} currents was not demonstrated. It is also noteworthy that Mochizuki-Oda *et al.* (1997) observed hypoxic chemosensitivity in adult rat chromaffin cells, whereas we did not (Thompson *et al.* 1997; see also Mojet *et al.* 1997). It is possible that use of longer-term cultures (up to 7 days) by Mochizuki-Oda *et al.* (1997), and the resulting longer period of denervation *in vitro*, resulted in recovery of hypoxic sensing in these cells (see Slotkin & Seidler, 1988).

Possible functional role of anoxic modulation of K^+ currents in neonatal AMC

In the present study, inhibition of Ca^{2+}-dependent K^+ (BK) channels was the major cause of the anoxic suppression of outward current in neonatal AMC. Similar BK channels are thought to participate in hypoxic chemosensitivity in rat carotid body type 1 cells (Wyatt & Peers, 1995; Jackson & Nurse, 1997), and are generally accepted to play important roles in action potential repolarization (Pancrazio *et al.* 1994), and in the ability of adult rat AMC to fire repetitive action potentials (Solaro *et al.* 1995). Thus, hypoxic inhibition of $I_{K(CaO_2)}$ may result in broadening of the action potential, leading to the observed rise in intracellular Ca^{2+} and catecholamine release (Thompson *et al.* 1997; see also Mojet *et al.* 1997). Additionally, hypoxic inhibition of the delayed rectifier type K^+ current, $I_{K(VO_2)}$ may also contribute to action potential broadening.

On the other hand, anoxia was found to *activate* a Ca^{2+}- and glibenclamide-sensitive K_{ATP} current in neonatal AMC. This activation tended to counteract or blunt the depolarizing effects of anoxia. Interestingly, in catecholaminergic neurons of the substantia nigra, hypoxic activation of Ca^{2+}- and glibenclamide-sensitive K_{ATP} channels leads to membrane hyperpolarization, which is presumed to serve a protective role during metabolic stress associated with hypoxia and ischaemia (Jiang *et al.* 1994; Haddad & Jiang, 1997). Conceivably, hypoxia-sensitive K_{ATP} channels in AMC may play a similar role during periods of prolonged neonatal hypoxia, e.g. during birth or repetitive apnoeic events. Activation of K_{ATP} channels by the concomitant fall in P_{O_2} and ATP, and the increase in intracellular Ca^{2+}, could limit membrane depolarization and prevent CA depletion from AMC, allowing them to maintain an influence on respiratory and cardiovascular physiology during subsequent hypoxic events (see Seidler & Slotkin, 1985; Slotkin & Seidler, 1988).

Origin of the receptor potential

In this study blockers of voltage-dependent, O_2-sensitive currents did not prevent the anoxia-induced depolarization in neonatal AMC. For example, direct inhibition of BK currents by charybdotoxin (50 nM) did not induce depolarization of neonatal AMC, suggesting that most of these channels are closed at the cell's resting potential (~−58 mV). Further, the presence of the relatively non-specific K^+ channel blocker TEA, which blocked all anoxia-sensitive K^+ channels in voltage clamp studies, also failed to prevent the anoxia-induced depolarization. However, TEA itself caused a slight depolarization, possibly due to closure of K^+ channels that were open at rest.

During exposure of AMC to anoxia, an apparent activation of a small inward current was frequently observed in voltage clamp studies when most of the outward current was blocked (e.g. Fig. 2*D*). This current was not studied in detail and its origin remains uncertain. On the one hand, it may result simply from inhibition of the residual outward current; alternatively, it may be due to activation of a cationic current, perhaps similar to the one recently described in hypoxia-sensitive guinea-pig AMC (Inoue *et al.* 1998). In the present study, the anoxia-induced depolarization of rat AMC persisted in the absence of extracellular Na^+ and Ca^{2+}, though the magnitude of the depolarization was decreased. Thus, activation of an inward cationic current by anoxia does not appear to be responsible for the receptor potential. Furthermore, these experiments suggest that the genesis of the receptor potential does not arise from the inhibition of a Na^+–K^+-ATPase, since under Na^+-free conditions this electrogenic pump is likely to be inactive.

In summary, it appears that chromaffin cells have evolved complex mechanisms for sensing O_2 and regulating catecholamine secretion in the perinatal period. These mechanisms involve the differential modulation of several K^+ channel subtypes. Additional work, aided by single channel analysis, is required to elucidate the molecular mechanisms by which these channels are regulated by low P_{O_2}, and the basis of the hypoxia-induced depolarization or receptor potential.

Ashcroft, S. J. H. & Ashcroft, F. M. (1990). Properties and functions of ATP-sensitive K- channels. *Cell Signalling* **2**, 197–214.

Buckler, K. J. (1997). A novel oxygen-sensitive potassium current in rat carotid body type 1 cells. *Journal of Physiology* **450**, 33–61.

Buckler, K. J. & Vaughan-Jones, R. D. (1994). Effects of hypoxia on membrane potential and intracellular calcium in rat neonatal carotid body type 1 cells. *Journal of Physiology* **476**, 423–428.

Conforti, L. & Millhorn, D. E. (1997). Selective inhibition of slow-inactivating voltage-dependent K^+ channel in rat PC12 cells by hypoxia. *Journal of Physiology* **502**, 293–305.

Galvez, A., Gimenez-Gallego, G., Reuben G., Roy-Contancin, L., Fingenbaum, P., Kaczorowski, G. J. & Garcia, M. L. (1990). Purification and characterization of a unique, potent, peptidyl probe for the high conductance calcium activated potassium channel from venom of the scorpion *Buthus tamulus*. *Journal of Biological Chemistry* **265**, 11083–11090.

Gonzalez, C., Almaraz, L., Obeso, A. & Rigual, R. (1994). Carotid body chemoreceptors: from natural stimuli to sensory discharges. *Physiological Reviews* **74**, 829–898.

Haddad, G. G. & Jiang, C. (1997). O_2-sensing mechanisms in excitable cells: role of plasma membrane K^+ channels. *Annual Review of Physiology* **59**, 23–43.

Inoue, M., Fujishiro, N. & Imanaga, I. (1998). Hypoxia and cyanide induce depolarization and catecholamine release in dispersed guinea-pig chromaffin cells. *Journal of Physiology* **507**, 807–818.

Jackson, A. & Nurse, C. A. (1997). Dopaminergic properties of cultured rat carotid body chemoreceptors grown in normoxic and hypoxic environments. *Journal of Neurochemistry* **69**, 645–654.

Jiang, C., Sigworth, F. J. & Haddad, G. G. (1994). Oxygen deprivation activates an ATP-inhibitable K^+ channel in substantia nigra neurons. *Journal of Neuroscience* **14**, 5590–5602.

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

Lopez-Lopez, J., Gonzalez, C., Urena, J. & Lopez-Barneo, J. (1989). Low P_{O_2} selectively inhibits K channel activity in chemoreceptor cells of the mammalian carotid body. *Journal of General Physiology* **93**, 1001–1015.

Mochizuki-Oda, N., Takeuchi, Y., Matsumura, K., Oosawa, Y. & Watanabe, Y. (1997). Hypoxia-induced catecholamine release and intracellular Ca^{2+} increase via suppression of K^+ channels in cultured rat adrenal chromaffin cells. *Journal of Neurochemistry* **69**, 377–387.

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

Neely, A. & Lingle, C. J. (1992). Two components of calcium-activated potassium current in rat adrenal chromaffin cells. *Journal of Physiology* **453**, 97–131.

Pancrazio, J. J., Johnson, P. A. & Lynch, C. III (1994). A major role for calcium-dependent potassium current in action potential repolarization in adrenal chromaffin cells. *Brain Research* **668**, 246–251.

Park, Y. (1994). Ion selectivity and gating of small conductance Ca^{2+}-activated K^+ channels in cultured rat adrenal chromaffin cells. *Journal of Physiology* **481**, 555–570.

Peers, C. (1990). Hypoxic suppression of K^+ currents in type 1 carotid body cells: selective effect on the Ca^{2+}-activated K^+ current. *Neuroscience Letters* **119**, 253–356.

Peers, C. & Buckler, K. J. (1995). Transduction of chemostimuli by the type 1 carotid body cell. *Journal of Membrane Biology* **144**, 1–9.

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

Seidler, F. J. & Slotkin, T. A. (1985). Adrenomedullary function in the neonatal rat: responses to acute hypoxia. *Journal of Physiology* **385**, 1–16.

Slotkin, T. A. & Seidler, F. J. (1988). Adrenomedullary catecholamine release in the fetus and newborn: secretory mechanisms and their role in stress and survival. *Journal of Developmental Physiology* **10**, 1–16.

Solaro, C. R., Prakriya, M., Ding, J. P. & Lingle, C. J. (1995). Inactivating and noninactivating Ca^{2+}- and voltage-dependent K^+ current in rat adrenal chromaffin cells. *Journal of Neuroscience* **15**, 6110–6123.

Thompson, R. J., Jackson, A. & Nurse, C. A. (1997). Developmental loss of hypoxic chemosensitivity in rat adrenomedullary chromaffin cells. *Journal of Physiology* **498**, 503–510.

Thompson, R. J. & Nurse, C. A. (1997). Hypoxia-sensitive K^+ channels in rat adrenal chromaffin cells. *Society for Neuroscience Abstracts* **23**, 1746.

Wyatt, C. N. & Peers, C. (1995). Ca^{2+}-activated K^+ channels in isolated type 1 cells of the neonatal rat carotid body. *Journal of Physiology* **483**, 599–565.

Youngson, C., Nurse, C. A., Yeger, H. & Cutz, E. (1993). Oxygen sensing in airway chemoreceptors. *Nature* **365**, 153–155.

Zhu, W. H., Conforti, L., Czyzyk-Kreska, M. F. & Millhorn, D. E. (1996). Membrane depolarization in PC-12 cells during hypoxia is regulated by an O_2-sensitive K^+ current. *American Journal of Physiology* **271**, C658–665.

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