Swelling- and cAMP-activated Cl⁻ currents in isolated rat carotid body type I cells

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1. In the whole-cell configuration of the patch clamp technique, isolated rat carotid body type I cells exhibited reversible activation of Cl⁻ currents during cell swelling effected by hypotonic extracellular solutions.

2. Hypotonic solutions evoked outwardly rectifying, non-inactivating currents which showed time-independent activation. The reversal potential (E_{rev}) for the hypotonically evoked current was 1.6 ± 0.6 mV ($n = 26$). Reduction of extracellular Cl⁻ from 133 to 65.5 mM caused a shift in E_{rev} of $+14.7 \pm 0.4$ mV ($n = 5$).

3. The swelling-activated Cl⁻ current could not activate when ATP was omitted from the patch pipette or when substituted for the non-hydrolysable ATP analogues 5′-adenylylimido-diphosphate, AMP-PNP (2 mM) or β,γ-methylene-adenosine 5′-triphosphate, AMP-PCP (2 mM). The current also failed to activate in the absence of free intracellular Ca^{2+}.

4. The swelling-activated Cl⁻ current was sensitive to blockade by the Cl⁻ channel blockers niflumic acid (300 μM) and 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS; 200 μM), although the blockade by DIDS was voltage dependent.

5. A similar, non-inactivating, outwardly rectifying Cl⁻ current was evoked by the inclusion of cAMP (200 μM) in the patch pipette. This current could be inhibited by niflumic acid (300 μM), DIDS (200 μM) and hypertonic solutions, and was virtually abolished in the absence of intracellular ATP.

6. In conclusion, carotid body type I cells possess Cl⁻ currents activated by cell swelling and rises in intracellular cAMP concentration. These currents may be involved in cell volume regulation, blood volume and osmolarity regulation and the response of the type I cell to chemostimuli.

The carotid body responds to changes in arterial P_{O_2}, P_{CO_2} and pH by increasing afferent chemosensory discharge of the carotid sinus nerve (CSN), which initiates corrective changes in ventilation (Fidone & Gonzalez, 1986; Gonzalez, Almaraz, Obeso & Rigual, 1994). The mechanisms underlying this chemotransductive process remain to be determined fully, but much evidence suggests that neurosecretory type I (glomus) cells in the carotid body depolarize in response to the above chemostimuli. This causes activation of voltage-gated Ca^{2+} channels and hence, the initiation of neurotransmitter release, which is associated with increased CSN discharge (Gonzalez *et al.* 1994; Peers & Buckler, 1995). Changes in CSN firing have also been reported to affect the hypothalamus, and stimulation of the carotid body has been shown to result in excitation of neurosecretory neurones in the supraoptic nucleus (Yamashita, 1977). This excitation leads to increased release of antidiuretic hormone (ADH) from the neurosecretory neurones (Share & Levy, 1966), ultimately resulting in decreased urinary flow. Hypertonic solutions have been reported to cause excitation of chemosensory

afferents and depolarization of carotid body type I cells (Gallego, Eyzagiurre & Monti-Bloch, 1979). Hypotonic solutions exert the opposite effect, causing carotid body type I cell hyperpolarization and decreased chemosensory discharge (Gallego *et al.* 1979). This would lead to a reduction of ADH secretion and an increase in urinary flow. Thus, the carotid body has been proposed as an 'osmoreceptor' whereby changes in blood tonicity are sensed, at least in part, at the level of the carotid body, resulting in the initiation of homeostatic mechanisms.

Hypotonic solutions are also well known for activating Cl⁻ currents in many cells (for example, Kubo & Okada, 1992; Nilius, Oike, Zahradnik & Droogmans, 1994; Vandenberg, Yoshida, Kirk & Powell, 1994; Gosling, Smith & Poyner, 1995) and these Cl⁻ currents are often termed swelling-activated or volume-sensitive Cl⁻ currents, since hypotonic solutions cause cell swelling and therefore cell volume changes. Another well-described activator of Cl⁻ currents is cAMP, the level of which has been reported to increase in carotid body type I cells during hypoxia (Wang, Cheng, Dinger & Fidone, 1989; Perez-Garcia, Almaraz & Gonzalez,

1990; Delpiano & Acker, 1991; Perez-Garcia, Almarez & Gonzalez, 1991; Wang, Cheng, Yoshizaki, Dinger & Fidone, 1991). Cl^- ions are not thought to be passively distributed across the carotid body type I cell membrane, and there is some evidence to suggest that Cl^- ions play an important role in determining the resting membrane potential of these cells (Oyama, Walker & Eyzaguirre, 1986).

Carotid body type I cells have been shown to possess large-conductance Cl^- channels, the activity of which is independent of voltage, Ca^{2+} and nucleotides, as determined in inside-out patch single-channel recordings (Stea & Nurse, 1989). These channels were unaffected by classical Cl^- channel blockers such as DIDS, furosemide and 5-nitro-2-(3-phenyl-propylamino)benzoic acid (NPPB), but were sensitive to blockade by anthracene-9-carboxylic acid (9-AC), all applied to the inside (cytosolic) face of the patch. However, effects of changing the tonicity of solutions were not investigated. Here, we demonstrate the presence of swelling- and cAMP-activated Cl^- currents in carotid body type I cells, using the whole-cell configuration of the patch clamp technique, and discuss the possible physiological role of these currents.

METHODS

The isolation and culture of carotid body type I cells was performed essentially as previously described (e.g. Wyatt & Peers, 1995). Briefly, 10-day-old Wistar rats (3–5 per preparation) were anaesthetized by breathing 5% halothane in oxygen through a face mask. Carotid bodies were removed and placed in ice-cold phosphate-buffered saline (PBS) containing 50 μM Ca^{2+}, collagenase (0.05% w/v) and trypsin (0.025% w/v) until the required number of organs had been collected. All donor animals were killed by decapitation whilst still deeply anaesthetized. To achieve enzymatic dissociation of the organs, they were incubated in the PBS at 37 °C for 15 min, after which they were teased apart using fine forceps and returned to 37 °C for a further 3–5 min. The digested tissue was then centrifuged at 200 g for 5 min (4 °C), after which the pellet was washed with Ham's F-12 medium containing insulin (84 u l^{-1}), penicillin (100 i.u. l^{-1}), streptomycin (100 μg ml^{-1}) and 10% heat-inactivated fetal bovine serum. The tissue was then centrifuged again using the same parameters and the pellet resuspended in Ham's F-12 medium. Following trituration, the dispersed cells were plated onto poly-D-lysine-coated coverslips and kept in a humidified incubator (5% CO_2, balance air) for up to 30 h before being used in experiments.

On each experimental day, pieces of fragmented coverslip were transferred to a recording chamber (volume, 80 μl) mounted on the stage of an inverted microscope (Olympus CK-2) and continuously superfused by gravity with a Cl^--rich isotonic solution (flow rate, 1–2 ml min^{-1}; see Table 1 for compositions of solutions). The whole-cell patch clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) was used to record membrane current (Axopatch 200A amplifier, Axon Instruments) with fire-polished patch pipettes of 4–9 MΩ resistance, manufactured using a two-stage vertical puller (Narishige PP-83). The solutions used were designed to minimize current flow through channels other than Cl^- channels and their osmolarity was measured using a Roebling Automatik osmometer (Camlab, Cambridge, UK). For whole-cell recordings, individual type I cells (identified as spherical, or near-

spherical, phase-bright and approximately 10 μm in diameter; see e.g. Peers & Green, 1991) were voltage clamped at 0 mV and 100 ms test potentials applied in 10 mV increments from −80 to +80 mV at a rate of 0.2 Hz (to obtain current–voltage (I–V) relationships). Alternatively, cells were subjected to successive 400 ms voltage ramps from −90 to +80 mV at a rate of 0.2 Hz from a holding potential of 0 mV to observe changes in the I–V relationships with time. Currents were filtered at 1 kHz with a 4-pole Bessel filter prior to digitization at 5 kHz and storage on computer disk. All voltage protocols and data acquisition were performed using the pCLAMP software suite (version 6.0.2, Axon Instruments) in combination with a DigiData 1200 interface board (Axon Instruments). All experiments were performed at room temperature (21–24 °C). Liquid junction potentials were measured using a freely flowing 3 M KCl junction (Fenwick, Marty & Neher, 1982) and ranged from 1 to 5 mV. Data have not been corrected for these.

Due to the non-inactivating nature of the currents described, leak currents were not subtracted and all currents were measured as absolute values with respect to the zero current level. Currents were measured for amplitude over the last 10–15 ms of the evoked current if measured from voltage steps, or at given voltages if measured from voltage ramps. Current inhibitions at given voltages were determined by expressing the current amplitude recorded under test conditions as a percentage of the current amplitude under control conditions. Data are expressed as means ± S.E.M. (n, number of observations), and tested for significance using Student's t test. Values of $P < 0.05$ were considered to be significant.

All culture materials were obtained from Sigma except Ham's F-12 medium, penicillin and streptomycin which were obtained from Gibco Laboratories. 4,4′-Diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS, Sigma) and niflumic acid (Sigma) were added to the bath solution from stock solutions in ethanol. This vehicle alone (0.1–0.2%) was without effect on whole-cell Cl^- currents. 8-Bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP, Na$^+$ salt; Sigma), adenosine-3′,5′-cyclic monophosphate (cAMP, Na$^+$ salt; Sigma), 5′-adenylylimidodiphosphate (AMP-PNP, Li$^+$ salt; Sigma), β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP, Na$^+$ salt; Sigma) and 1,2-bis(2-aminophenoxy)ethane-$N,N,N′,N′$-tetra-acetate (BAPTA, K$^+$ salt; Sigma) were directly dissolved in extra- and intracellular solutions when required. Any modifications to the solutions shown in Table 1 are indicated in the legends to the figures, where applicable.

RESULTS

Swelling-activated Cl^- currents

To investigate possible activation of Cl^- currents in carotid body type I cells, outward K$^+$ channels were blocked using extracellular TEA and Cs$^+$-filled patch pipettes (see Table 1 for solution composition). Inward Ca^{2+} currents were strongly suppressed using a low concentration of extracellular Ca^{2+} (0.1 mM) and a high concentration of extracellular Mg^{2+} (6 mM). Na$^+$ channels were not blocked as previous experiments have indicated that they are absent or non-functioning in these cells (e.g. see Peers & Green, 1991).

Immediately upon reaching the whole-cell configuration of the patch clamp technique, using isotonic solutions (300 mosmol l^{-1} inside/outside) with symmetrical Cl^- concentrations, only very small amplitude currents could be observed in carotid body type I cells when 100 ms test

Table 1. Composition of solutions used (mM) in electrophysiological recordings

	Extracellular bathing solutions (pH 7·4)						Intracellular solution (pH 7·2)
	Normal isotonic	Normal hypotonic	Normal hypertonic	Na⁺-free hypotonic	Low ($[Cl^-]_o$) hypotonic	NaCl-free hypotonic	Patch pipette *
NaCl	67·5	67·5	67·5	—	—	—	10
Choline chloride	—	—	—	67·5	—	—	—
Sodium gluconate	—	—	—	—	67·5	—	—
KCl	5	5	5	5	5	5	—
CsCl	—	—	—	—	—	—	117
TEA-Cl	48·3	48·3	48·3	48·3	48·3	48·3	—
$MgCl_2$	6	6	6	6	6	6	2
$CaCl_2$	0·1	0·1	0·1	0·1	0·1	0·1	1
EGTA	—	—	—	—	—	—	11
Hepes	5	5	5	5	5	5	11
D-Glucose	10	10	10	10	10	10	—
Na_2ATP	—	—	—	—	—	—	2
Osmolarity †	300	240	400	240	240	240	300

Solutions were made up using deionized, distilled water. The free Ca^{2+} ion concentration in the patch pipette was approximately 25 nM as determined using winMAXC version 1.7 software (Chris Patton, Stanford University, CA, USA). The pH of extracellular solutions was adjusted with NaOH, except for Na⁺-free and NaCl-free solutions, where TEA-OH was used. The pH of intracellular solutions was always adjusted with CsOH. * In some experiments where the patch pipette solution composition was altered, details are given in the relevant figure legends. † Osmolarities are shown in mosmol l⁻¹ and were adjusted (to within ± 1 mosmol l⁻¹ of the given osmolarity) using D-sucrose when required. When the osmolarity of the hypotonic solution was increased as a result of the inclusion of drugs (when ethanol was used as a vehicle), the hypotonicity of the hypotonic solution used to induce the current was adjusted to match. These solutions had an osmolarity ranging from approximately 250 to 260 mosmol l⁻¹.

potentials between −80 and +80 mV (in 10 mV increments at a rate of 0·2 Hz) were applied from a holding potential of 0 mV (−12 ± 7 pA at −80 mV, 45 ± 7 pA at +80 mV, $n = 11$; see Fig. 1A and D). After a 3–4 min hypotonic challenge (240 mosmol l⁻¹) the same voltage protocol revealed non-inactivating, outwardly rectifying currents (−144 ± 31 pA at −80 mV, 307 ± 71 pA at +80 mV, $n = 11$), which showed time-independent activation (see Fig. 1B and D). During a hypotonic challenge the cells were clearly seen to swell under the microscope, although this was not quantified. On return to isotonic conditions these current levels relaxed close to control values (−33 ± 7 pA at −80 mV, 71 ± 8 pA at +80 mV, $n = 11$; see Fig. 1C and D).

To obtain the E_{rev} for the evoked current, successive voltage ramps (400 ms, −90 to +80 mV) were applied at a rate of 0·2 Hz (holding potential, 0 mV). In addition to the determination of E_{rev}, this protocol also allowed us to observe how the swelling-activated current developed with time, and time-series plots of current amplitude at −80 and +80 mV were plotted to this end. The time taken in hypotonic solution before steady-state currents were obtained varied greatly and occurred between 3 and 7 min of exposure (see Fig. 1E, ○). The amplitudes of steady-state currents were −312 ± 40 pA at −80 mV and 522 ± 58 pA at +80 mV ($n = 26$). E_{rev} was measured when the current reached steady state and was 1·6 ± 0·6 mV ($n = 26$), which

is close to the theoretical Cl⁻ reversal potential of 0 mV under these conditions. Carotid body type I cells which did not receive a hypotonic challenge did not display activation of any current detectable under our recording conditions ($n = 5$; for example see Fig. 1E, ●).

When extracellular Na⁺ was removed (choline substitution, see Table 1), the E_{rev} of the swelling-activated current was 0·6 ± 1·5 mV ($n = 6$, see Fig. 2A), which was not significantly different from the E_{rev} measured under control conditions ($P > 0·9$). Similarly, the amplitude of the swelling-activated current was not affected by the removal of extracellular Na⁺ (−306 ± 85 pA at −80 mV, 503 ± 131 pA at +80 mV; $n = 6$, $P > 0·9$). These results indicate that it is unlikely that the current arose from non-specific cationic channels. To test whether changes in extracellular Cl⁻ ions would shift E_{rev} in a manner consistent with a Cl⁻-selective channel, the extracellular Cl⁻ ion concentration was reduced from 133 to 65·5 mM by replacing NaCl with sodium gluconate (see Fig. 2B). This reduction in extracellular Cl⁻ ion concentration produced a positive shift in E_{rev} of 14·7 ± 0·4 mV ($n = 5$), similar to the theoretical shift of +17·8 mV for a perfectly selective Cl⁻ channel. Directly removing NaCl (to achieve the same extracellular Cl⁻ ion concentration of 65·5 mM) produced an almost identical positive shift in E_{rev} of 14·5 ± 0·4 mV ($n = 3$, see Fig. 2C).

ATP- and Ca^{2+}-dependence of the swelling-activated Cl^- current

The above experiments were carried out with the patch pipette solution containing ATP (2 mM). To determine whether ATP was required for activation of the swelling-activated Cl^- current, experiments were carried out in randomly selected cells with an isotonic, ATP-deficient patch pipette solution (Fig. 3, upper graph). Under these conditions the current failed to activate in response to a hypotonic challenge (240 mosmol l^{-1}, $n = 4$), whereas cells exposed to ATP-containing patch pipette solutions always responded to a time-matched hypotonic challenge by activation of the current ($n = 7$). To investigate whether

ATP hydrolysis was required for activation of the current, randomly selected cells were exposed to an isotonic patch pipette solution in which ATP was substituted by AMP-PNP (2 mM), a non-hydrolysable ATP analogue. Under these conditions, using a time-matched experimental protocol, the current also failed to activate ($n = 5$), indicating that ATP hydrolysis was necessary for activation of the swelling-activated Cl^- current (see Fig. 3, upper graph). Similar results were obtained using another hydrolysis-resistant ATP analogue AMP-PCP (2 mM, $n = 5$, data not shown). Additionally, the use of Mg^{2+}-free, ATP-containing intracellular solution abolished the development of the swelling-activated Cl^- current in response to a hypotonic challenge ($n = 5$, data not shown).
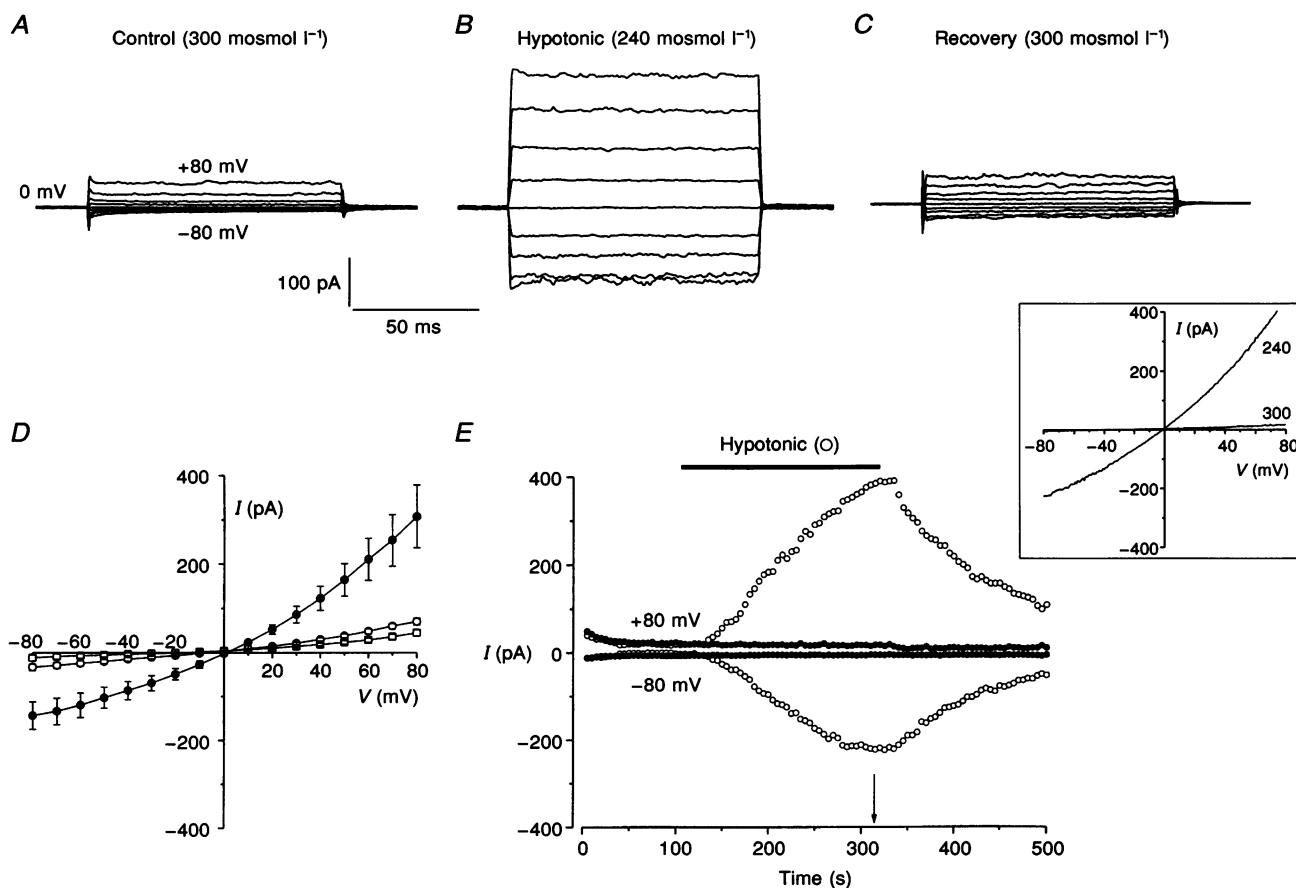

Figure 1. Swelling-activated membrane currents in carotid body type I cells

A, whole-cell currents measured from a carotid body type I cell elicited by 100 ms test potentials from −80 to +80 mV (20 mV increments shown, applied at 0·2 Hz) from a holding potential of 0 mV during isotonic conditions (300 mosmol l^{-1}, representative of 11 cells). *B*, whole-cell currents from the same cell and elicited by the same voltage protocol as in *A*, after a 4 min hypotonic challenge (240 mosmol l^{-1}, representative of 11 cells). *C*, whole-cell currents from the same cell and elicited by the same voltage protocol as in *A* and *B*, after return to isotonic conditions (representative of 11 cells). The currents shown in *C* were recorded when no further recovery was achieved after return to isotonic conditions. *D*, mean (± S.E.M., $n = 11$) *I-V* relationship for the swelling-activated current under control isotonic conditions (□), during a 3–4 min hypotonic challenge (●) and after return to isotonic conditions (○). *E*, time course of activation of the hypotonically evoked current (○; application indicated by the horizontal bar) recorded from a representative type I cell at two voltages (−80 and +80 mV) taken from repeatedly applied voltage ramps (from a holding potential of 0 mV). ●, time course of the current recorded in a different type I cell when no hypotonic challenge was given. The arrow indicates the time point at which the ramp *I-V* relationships from the two cells were recorded, shown in the inset.

In the above-described results, the cells were maintained in the whole-cell configuration with a free intracellular Ca^{2+} concentration of approximately 25 nM (see Methods). To test whether intracellular Ca^{2+} was required for activation of the swelling-activated Cl^- current, experiments were carried out using an isotonic zero Ca^{2+} patch pipette solution containing BAPTA (20 mM) in addition to the EGTA (11 mM) normally present. Using this patch pipette solution, a hypotonic challenge (240 mosmol l^{-1}) did not elicit any currents ($n = 7$, see Fig. 3, lower graph), suggesting that for activation of the swelling-activated Cl^- current, some free intracellular Ca^{2+} must be present.

Sensitivity of the swelling-activated Cl^- current to Cl^- channel blockers

The effects of the Cl^- channel blockers niflumic acid (300 μM) and DIDS (200 μM) were tested on the swelling-activated Cl^- current. These blockers were applied isotonically when the current had reached steady state and were left in contact with the cells until they had exerted their maximum effects before washout. DIDS was more effective on the outward current than the inward current, producing $15.5 \pm 4.9\%$ inhibition at -80 mV and $62.8 \pm 1.6\%$ inhibition at $+80$ mV ($n = 6$, see Fig. 4A and B) and current amplitudes recovered almost fully on washout. Niflumic acid was equally effective on both inward and outward currents, causing $81.7 \pm 4.8\%$ inhibition at -80 mV and 76.3% inhibition at $+80$ mV ($n = 5$, see Fig. 4C and D). The effect of niflumic acid on Cl^- currents only reversed slowly on washout (data not shown).

cAMP-activated Cl^- currents

Isotonic inclusion of cAMP (200 μM) in the patch pipette solution elicited a current which reached steady state within 4 min (-191 ± 29 pA at -80 mV, 369 ± 49 pA at $+80$ mV, measured from voltage ramps; $n = 14$; see Fig. 5A (●) and B). This current had an E_{rev} of 1.1 ± 0.9 mV ($n = 14$) in symmetrical Cl^-. The current showed outward rectification,

A

Figure 2. Cl⁻ selectivity of the swelling-activated current

A, ramp I–V relationships from a cell subjected to a hypotonic (240 mosmol l^{-1}) challenge until a steady-state current was obtained. Isotonically removing extracellular Na^+ ions (as indicated, by choline substitution) did not affect the E_{rev} or current amplitude of the hypotonically evoked current (representative of 6 cells, see text for quantitative data). *B*, ramp I–V relationships from another cell subjected to a hypotonic challenge until a steady-state current was obtained. Reducing the extracellular Cl^- concentration from 133 to 65.5 mM by isotonic replacement of NaCl with sodium gluconate (as indicated), shifted E_{rev} of the current to a more positive value, consistent with a Cl^--selective conductance (representative of 5 cells, see text for quantitative data). *C*, ramp I–V relationships from a different cell subjected to a hypotonic challenge until a steady-state current was observed. Reducing the extracellular Cl^- concentration to the same value as in *B* (this time by direct isotonic removal of NaCl as indicated), produced a positive shift in E_{rev} similar to that produced by the method described in *B* (representative of 3 cells, see text for quantitative data).
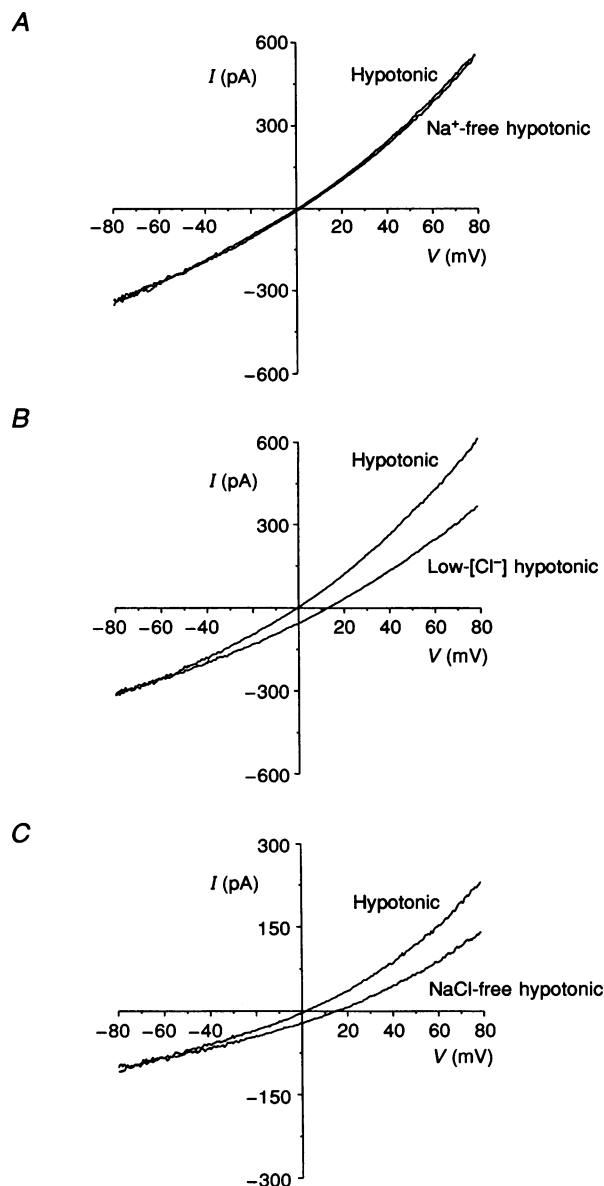
and when 100 ms test potentials between −80 and +80 mV (in 10 mV increments, at a rate of 0·2 Hz) were applied to the cells from a holding potential of 0 mV, the kinetics of the current were similar to those of the swelling-activated Cl⁻ current. That is, outward rectification, time-independent activation and lack of inactivation (-252 ± 46 pA at −80 mV, 416 ± 72 pA at +80 mV; $n = 5$; see Fig. 5C and D).

The cAMP-activated Cl⁻ current was virtually abolished if ATP was removed from the patch pipette solution (-16 ± 5 pA at −80 mV, 56 ± 7 pA at +80 mV, measured

at 4 min; $n = 5$; see Fig. 5A (■) and B). Variable, small levels of activation were sometimes seen prior to 4 min, but at 4 min when steady-state activation occurred in the cells dialysed with ATP, the ATP-deficient cells showed essentially no current activation.

The cAMP-activated current was sensitive to the isotonically applied Cl⁻ channel blockers niflumic acid (300 μM) and DIDS (200 μM), in a manner resembling the swelling-activated Cl⁻ current. The effect of DIDS was more pronounced on the outward current and produced $22 \cdot 1 \pm 1 \cdot 7\%$ inhibition at
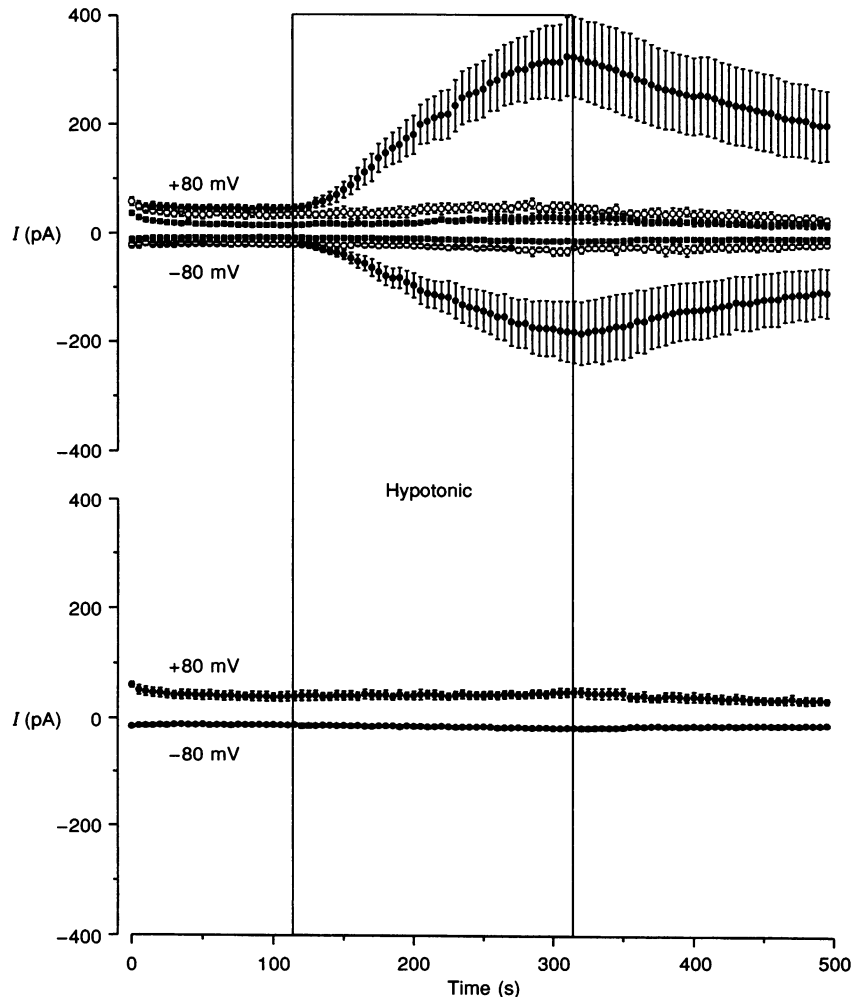

Figure 3. ATP and Ca²⁺ dependence of swelling-activated Cl⁻ currents

Upper graph, mean ($\pm$ s.e.m.) time-series plots of swelling-activated Cl⁻ currents from carotid body type I cells subjected to successive voltage ramps (−90 to +80 mV in 400 ms at a rate of 0·2 Hz) from a holding potential of 0 mV. The currents were evoked by a hypotonic shock (240 mosmol l⁻¹, from 300 mosmol l⁻¹; application period indicated by the box) and measured at −80 and +80 mV. ●, time course of activation and reversal of the Cl⁻ currents in response to a hypotonic shock under normal conditions with ATP (2 mM) and approximately 25 nM free Ca²⁺ in the patch pipette ($n = 7$). In randomly selected cells (O), using an isotonic, ATP-deficient patch pipette solution rendered the cells unable to generate currents in response to a hypotonic shock (240 mosmol l⁻¹, $n = 4$). Isotonic substitution of ATP with the non-hydrolysable ATP analogue AMP-PNP (2 mM) similarly prevented activation of the swelling-activated current in randomly selected cells (■, 240 mosmol l⁻¹, $n = 5$). Lower graph, mean ($\pm$ s.e.m.) time-series plot, obtained using the same voltage protocol as in the plot above. Data were obtained using normal patch pipette solution with ATP (2 mM), but without added Ca²⁺, and containing BAPTA (20 mM) in addition to EGTA (11 mM).

−80 mV and 87 ± 0·8 % inhibition at +80 mV (*n* = 5; see Fig. 6*A* and *B*). Niflumic acid was equally effective on both the inward and outward current, causing 90·2 ± 1·8 % inhibition at −80 mV and 87·3 ± 1·2 % inhibition at +80 mV (*n* = 5; see Fig. 6*C* and *D*).

Isotonic extracellular application of 8-Br-cAMP (2 mM) also caused the activation of an outwardly rectifying current which reached steady state after approximately 3 min (−38 ± 27 pA at −80 mV and 85 ± 27 pA at +80 mV; *n* = 5). This current had an E_{rev} of 2·3 ± 2·1 mV (*n* = 5), again close to the theorectical E_{rev} of a Cl⁻-selective conductance under our conditions (see Fig. 7*A* and *B*). This current was sensitive to blockade by niflumic acid (300 μM)

and both inward and outward currents were equally affected, with 73·0 ± 6·5 % inhibition at −80 mV and 68·0 ± 6·0 % inhibition at +80 mV (*n* = 5; see Fig. 7*C* and *D*).

Can the cAMP-activated Cl⁻ current be modulated by tonicity changes?

In order to investigate whether the swelling-activated Cl⁻ current arose from the same channel as the cAMP-activated current, experiments were carried out to reveal what effect hypertonic (400 mosmol l⁻¹) solutions had on the cAMP-activated current. Figure 8 shows that the cAMP-activated Cl⁻ current (evoked under isotonic conditions) was dramatically attenuated upon switching to a hypertonic bathing solution. The current was reduced from
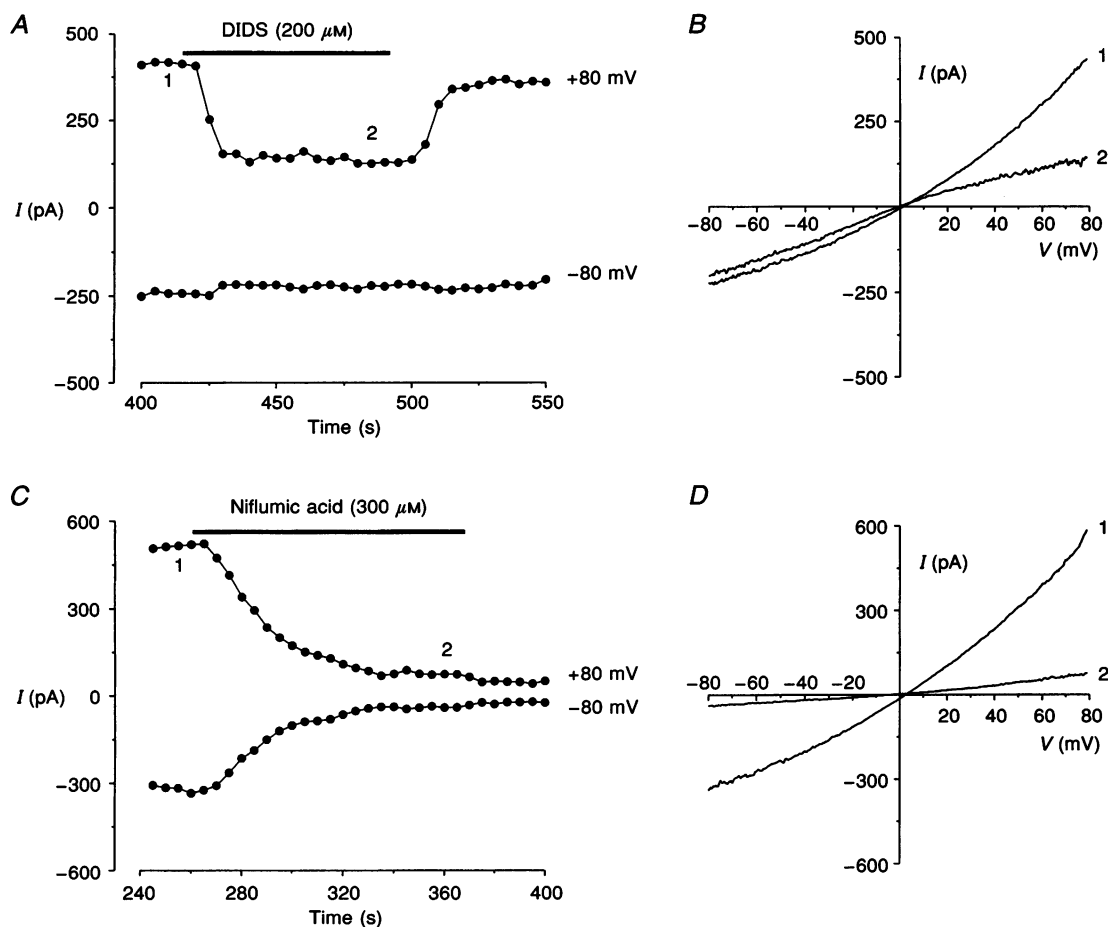

Figure 4. Pharmacology of swelling-activated Cl⁻ currents

A, time-series plot of swelling-activated Cl⁻ current from a representative type I cell subjected to successive voltage ramps (−90 to +80 mV in 400 ms at a rate of 0·2 Hz) from a holding potential of 0 mV. The current was evoked by a hypotonic shock (250 mosmol l⁻¹, from 300 mosmol l⁻¹) and measured at −80 and +80 mV. The plot shows evoked current upon reaching steady state and the effect of an isotonic bath application (indicated by the horizontal bar) of DIDS (200 μM) on the current. *B*, ramp *I–V* relationships from the same cell as in *A* from the time points indicated in *A* by 1 and 2 (representative of 6 cells, see text for quantitative data). *C*, time-series plot of swelling-activated Cl⁻ current from another type I cell subjected to the same voltage protocol as the cell in *A*. The plot shows the effect of an isotonic bath application (indicated by the horizontal bar) of niflumic acid (300 μM) on the current. *D*, ramp *I–V* relationships from the same cell as in *C* from the time points indicated in *C* by 1 and 2 (representative of 5 cells, see text for quantitative data).

−209 ± 64 pA at −80 mV and 421 ± 107 pA at +80 mV at 4 min to −47 ± 9 pA at −80 mV and 114 ± 22 pA at +80 mV after a 4 min perfusion with hypertonic solution (*n* = 5). It is possible that further current attenuation could be achieved by increasing the duration of the hypertonic challenge as the current levels did not appear to have reached steady state after 4 min of hypertonic perfusion (see Fig. 8*A*).

DISCUSSION

Properties of swelling-activated Cl⁻ channel currents

The results presented here demonstrate that carotid body type I cells possess a Cl⁻ current activated by cell swelling caused by a hypotonic extracellular environment. The

currents were large in amplitude compared with those measured under isotonic conditions and were outwardly rectifying. When voltage step commands were applied the currents showed time-independent (or 'instantaneous') activation and did not inactivate during 100 ms test potentials in the range −80 to +80 mV (see Fig. 1). This is consistent with swelling-activated Cl⁻ currents recorded from pancreatic *β*-cells (Best, Miley & Yates, 1996*a*; Best, Sheader & Brown, 1996*b*) which were non-inactivating during 500 ms test depolarizations to potentials in the range similar to our own. However, many other swelling-activated Cl⁻ currents in several cell types show inactivation during large depolarizations (over +50 mV), becoming more rapid as the membrane potential reaches more depolarized levels (Kubo & Okada, 1992; Valverde, Díaz, Sepúlveda, Gill,
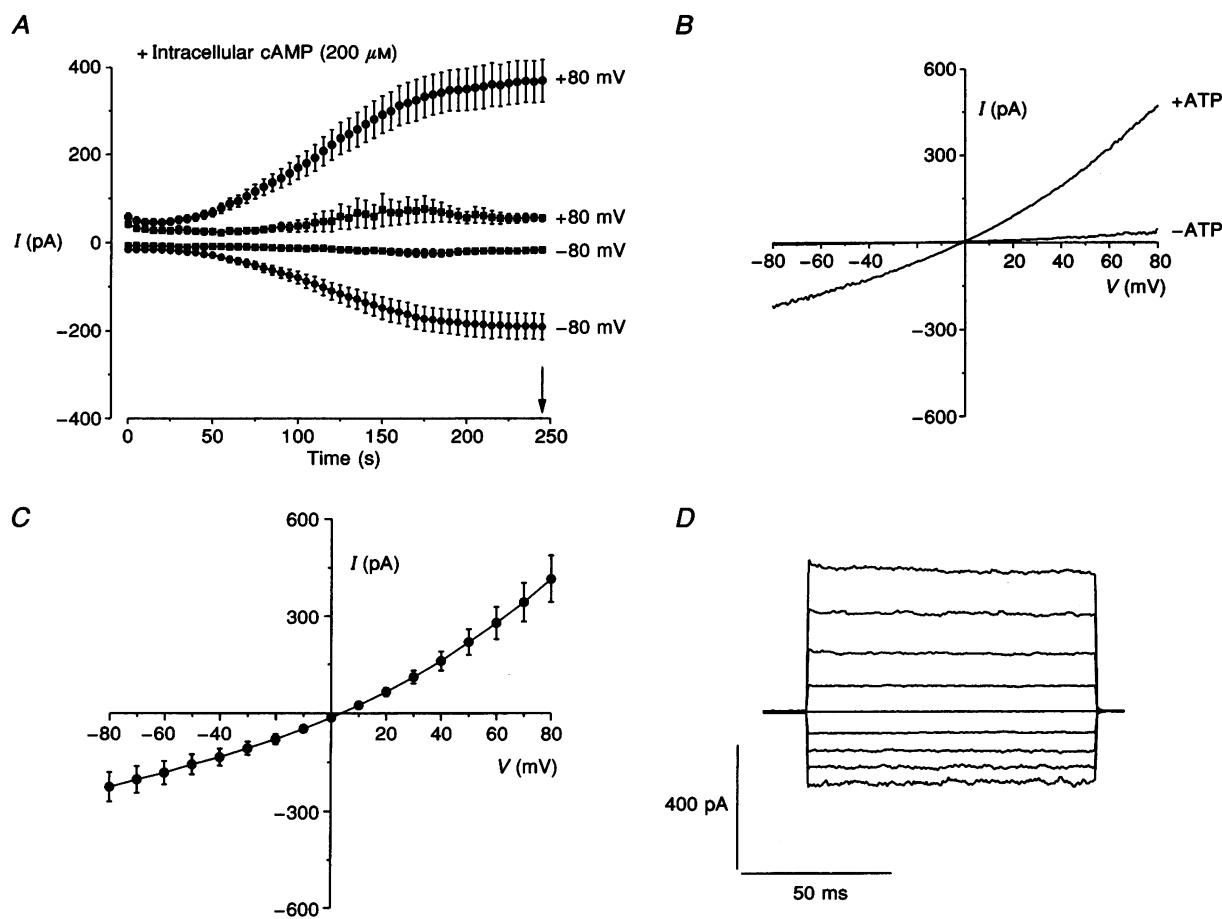

Figure 5. cAMP-activated membrane currents

A, mean (± S.E.M.) time-series plot of cAMP-activated membrane currents from carotid body type I cells subjected to successive voltage ramps (−90 to +80 mV in 400 ms at a rate of 0·2 Hz) from a holding potential of 0 mV, using normal isotonic extracellular solution (300 mosmol l⁻¹, *n* = 14). Currents were evoked by isotonic inclusion of cAMP in patch pipette solution (●, 200 μM, 300 mosmol l⁻¹) and were measured at −80 and +80 mV. ■, mean (± S.E.M.) current, evoked as for ●, except ATP was isotonically omitted from the patch pipette (*n* = 5). *B*, ramp *I–V* relationships from representative cells after 4 min of dialysis with cAMP using either ATP-containing intracellular solution (+ATP) or ATP-deficient intracellular solution (−ATP) as indicated (see text for quantitative data). *C*, mean (± S.E.M.) *I–V* relationship for the cAMP-activated Cl⁻ current, obtained by applying 100 ms test potentials from −80 to +80 mV (10 mV increments, at a rate of 0·2 Hz) from a holding potential of 0 mV after the currents had reached steady state (usually after 4 min; *n* = 5). *D*, a family of cAMP-activated Cl⁻ currents from a representative type I cell, evoked by the same voltage protocol as in *C* (only 20 mV increments shown).

Hyde & Higgins, 1992; Jirsch, Loe, Cole, Deely & Fedida, 1994; Gosling *et al.* 1995). However, in common with all the above-mentioned studies is the outwardly rectifying and 'instantaneously' activating nature of the present Cl⁻ current. Additionally, consonant with a Cl⁻-selective conductance, the E_{rev} was close to 0 mV in solutions with symmetrical Cl⁻ concentrations and upon reduction of the extracellular Cl⁻ concentration, the expected positive shift in E_{rev} was observed (see Fig. 2*B* and *C*). Furthermore, changing the concentration of extracellular Na⁺ did not affect E_{rev} or current amplitudes, supportive of the presence of an anion-selective, swelling-activated current in carotid body type I cells. The swelling-activated Cl⁻ current recorded in carotid body type I cells was sensitive to two structurally unrelated Cl⁻ channel blockers (see Fig. 4). The stilbene derivative DIDS (200 μM) produced a voltage-dependent block, being more effective at positive potentials. This effect has been

attributed to a reversible open channel block and DIDS, being negatively charged, would enter Cl⁻ channels more easily during depolarized potentials (Gosling *et al.* 1995). In contrast, niflumic acid (300 μM) showed a voltage-independent block, affecting both inward and outward currents equally. A potent blocker of Ca²⁺-activated Cl⁻ channels in vascular smooth muscle cells (Pacaud, Loirand, Lavie, Mironneau & Mironneau, 1989), niflumic acid has been demonstrated to produce a similar voltage-independent block of cAMP-activated Cl⁻ currents (Hughes & Segawa, 1993). However, in bovine pulmonary artery endothelial cells, niflumic acid has been shown to block a Ca²⁺-activated Cl⁻ channel in a voltage-dependent manner, being more effective at positive potentials (Nilius *et al.* 1997). The same workers also reported a weak block by niflumic acid on swelling-activated Cl⁻ currents in the same cells, albeit at a lower concentration (100 μM) than the one
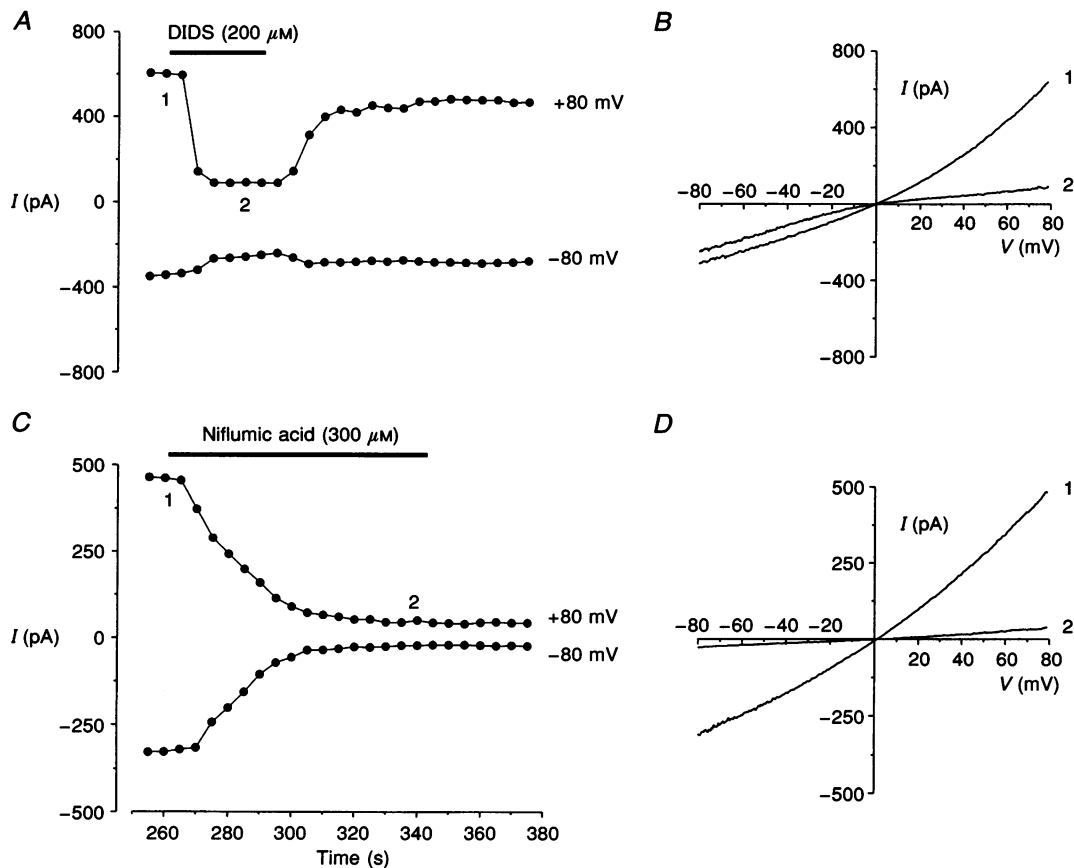

Figure 6. Pharmacology of cAMP-activated Cl⁻ currents

A, time-series plot of cAMP-activated Cl⁻ current from a representative type I cell subjected to successive voltage ramps (−90 to +80 mV in 400 ms at a rate of 0·2 Hz) from a holding potential of 0 mV. The current was measured at +80 and −80 mV after reaching steady state and the plot shows the effect of an isotonic bath application (indicated by the horizontal bar) of DIDS (200 μM) on the current. *B*, ramp *I–V* relationships from the same cell as in *A* from the time points indicated in *A* by 1 and 2 (representative of 5 cells, see text for quantitative data). *C*, time-series plot of cAMP-activated Cl⁻ current from another type I cell subjected to the same voltage protocol as the cell in *A*. The current was measured at −80 and +80 mV. The plot shows the effect of an isotonic bath application (indicated by the horizontal bar) of niflumic acid (300 μM) on the current. *D*, ramp *I–V* relationships from the same cell as in *C* from the time points indicated in *C* by 1 and 2 (representative of 5 cells, see text for quantitative data). *A* and *C* share the same *x*-axis.

used in this study (300 μM). Taken together with our findings, this indicates that niflumic acid is an effective blocker of swelling-activated Cl^- currents, in addition to its effects on cAMP- and Ca^{2+}-activated Cl^- currents.

The development of the swelling-activated Cl^- current in response to a hypotonic shock investigated here showed dependence on ATP hydrolysis, as the current failed to develop in the absence of intracellular ATP (or in the absence of intracellular Mg^{2+}), or when ATP was substituted for the hydrolysis-resistant ATP analogues AMP-PNP (see Fig. 3) or AMP-PCP. This is suggestive of the swelling-activated Cl^- current in carotid body type I cells belonging to either the ATP-binding cassette (ABC) superfamily of proteins which can function as drug efflux pumps, to which

the cystic fibrosis transmembrane regulator (CFTR) and P-glycoprotein belong (Valverde et al. 1992), or the structurally unrelated class of Cl^- channels which have ATP-binding sites and show DIDS-sensitive, outwardly rectifying currents (Paulmichl, Li, Wickman, Ackerman, Peralta & Clapham, 1992). Although a requirement of ATP hydrolysis for development of the swelling-activated Cl^- current in response to a hypotonic challenge has been demonstrated in our cells, some controversy exists in terms of whether ATP is an absolute requirement for swelling-regulated channel function in lung cancer cells (see Jirsch et al. 1994). However, a dependence on ATP hydrolysis for activation of an outwardly rectifying, DIDS-sensitive chloride conductance in frog proximal tubule cells has recently been reported (Robson & Hunter, 1997).
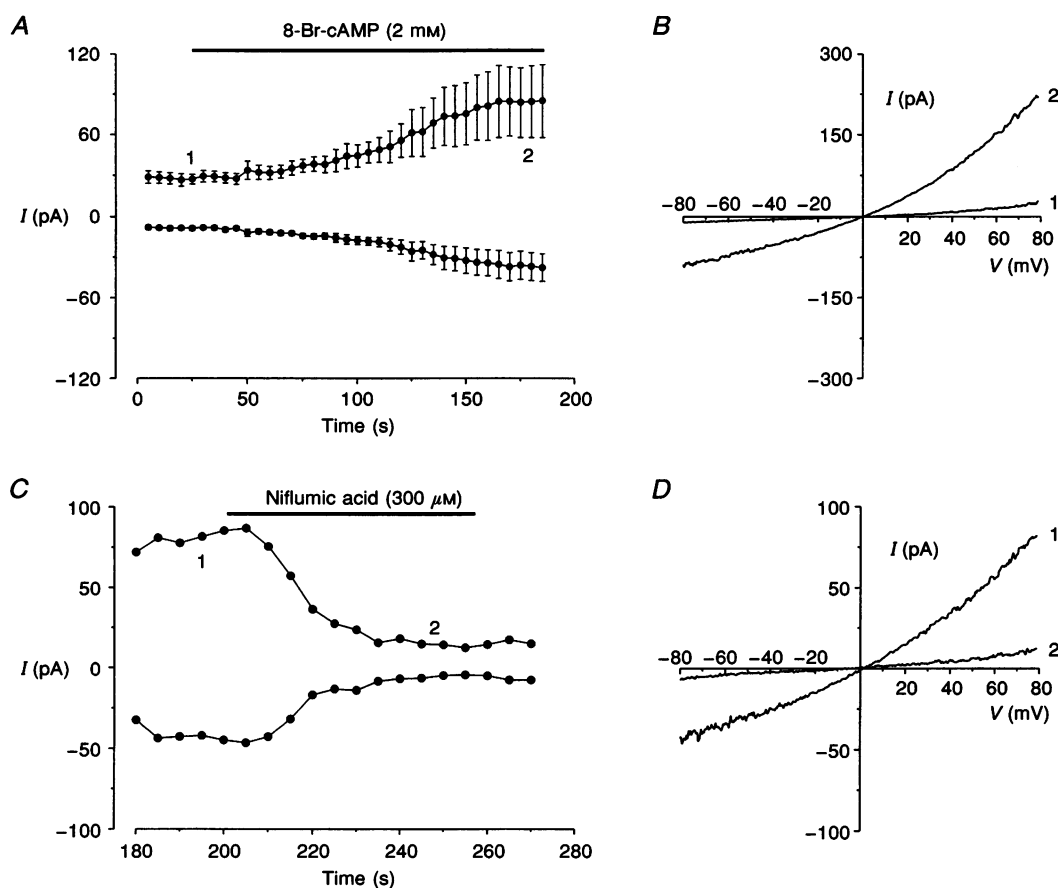

Figure 7. Membrane-permeable cAMP analogue 8-Br-cAMP also activates membrane currents

A, mean ($\pm$ S.E.M.) time-series plot of the effect of isotonic bath applications of the membrane-permeable cAMP analogue 8-Br-cAMP (2 mM; indicated by the horizontal bar) on currents recorded in carotid body type I cells evoked by successive voltage ramps (-90 to $+80$ mV in 400 ms at a rate of 0·2 Hz) from a holding potential of 0 mV. Currents were measured at -80 and $+80$ mV. ●, time course of development of the currents in response to 8-Br-cAMP ($n = 5$; see text for quantitative data). B, ramp I–V relationships from a representative cell at the time points indicated by 1 and 2 in A. C, time-series plot of 8-Br-cAMP-activated Cl^- current from another type I cell subjected to the same voltage protocol as the cell in A. The current was allowed to reach steady state and was measured at -80 and $+80$ mV. The plot shows the effect of an isotonic bath application (indicated by the horizontal bar) of niflumic acid (300 μM) on the current. D, ramp I–V relationships from the same cell as in C from the time points indicated in C by 1 and 2 (representative of 5 cells; see text for quantitative data).

The observation that low concentrations of free intracellular Ca^{2+} were necessary for the development of the swelling-activated Cl^- current in carotid body type I cells (see Fig. 3) has been described previously in vascular endothelial cells (Szücs, Heinke, Droogmans & Nilius, 1996), where a low basal concentration of free intracellular Ca^{2+} was required for activation of a swelling-regulated current. This so-called 'permissive' Ca^{2+} concentration (the authors found that increases further to 50 nM did not affect the current) was hypothesized to facilitate translocation of unavailable channels in the cytosol (in an 'off-configuration') to the cell membrane as available channels (in the 'on-configuration') during a hypotonic challenge (Szücs *et al.* 1996).

Of course, the possibility remains that the use of more severely hypotonic solutions might overcome the effects of using ATP- and Ca^{2+}-deficient patch pipette solutions. That is, a shift in the activation curve of the swelling-activated Cl^- current could have occurred as a result of the above-described manipulations of the intracellular environment,

rather than an absolute dependence on ATP or Ca^{2+} for activation. Unfortunately, further reductions in extracellular tonicity were not well tolerated by the carotid body type I cells and posed methodological problems due to inevitable ionic composition changes that would have to be made to achieve more severe extracellular hypotonicity. However, in spite of the uncertainty which therefore surrounds the question of absolute dependence on ATP hydrolysis and permissive levels of free intracellular Ca^{2+} for activation of swelling-activated Cl^- currents in carotid body type I cells, it is reasonable to state that during physiological tonicity changes a preference for ATP hydrolysis and a permissive level of free intracellular Ca^{2+} as a means for activation of the channel exists.

Properties of cAMP-activated Cl^- currents

The cAMP-activated current found in carotid body type I cells showed a very similar kinetic (see Fig. 5) and pharmacological (see Fig. 6) profile to the swelling-activated Cl^- current. It was activated by inclusion of cAMP in the

Figure 8. The cAMP-activated Cl^- current is inhibited by a hypertonic challenge

A, mean (± s.e.m.) time-series plot of cAMP-activated membrane currents from carotid body type I cells subjected to successive voltage ramps (−90 to +80 mV in 400 ms at a rate of 0·2 Hz) from a holding potential of 0 mV, using normal isotonic extracellular solution (300 mosmol l⁻¹; *n* = 5). Currents were evoked by isotonic inclusion of cAMP in patch pipette solution (200 μM, 300 mosmol l⁻¹) and were measured at −80 and +80 mV. After 4 min of cAMP dialysis the isotonic extracellular perfusate was switched to a hypertonic perfusate (400 mosmol l⁻¹, duration of challenge indicated by the box, see text for quantitative data). *B*, ramp *I–V* relationships from a carotid body type I cell taken at the time points indicated in *A* by 1 and 2 (representative of 5 cells).
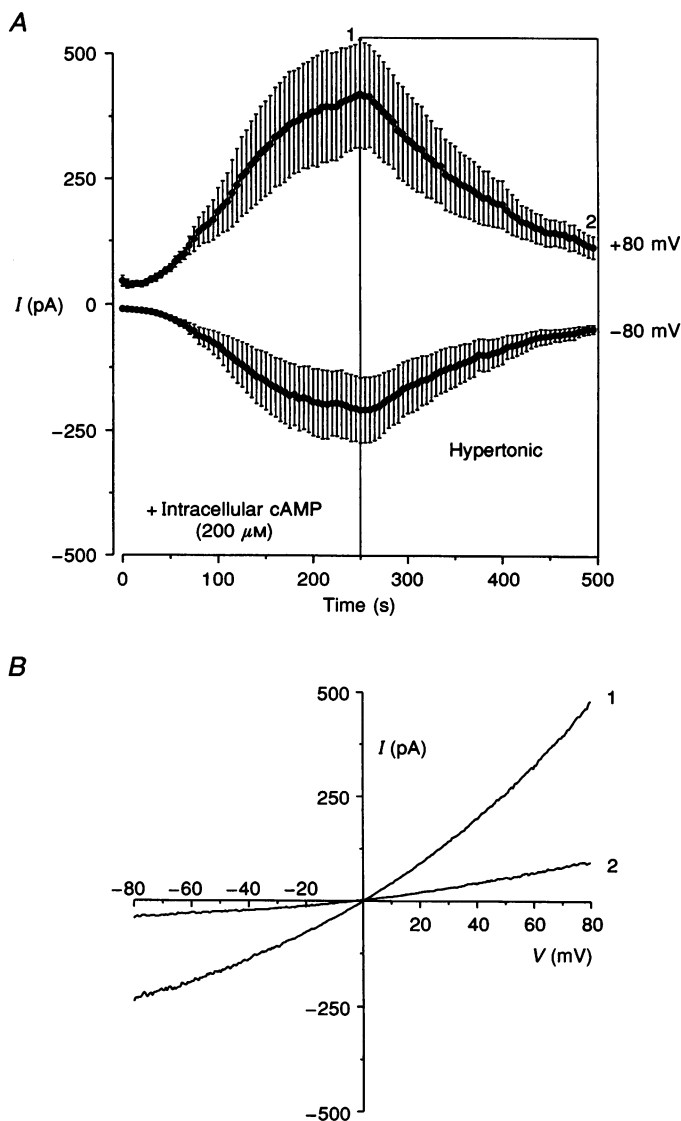
patch pipette (200 μM) and was discernible after the cells had been in the whole-cell configuration for only 1 min and reached steady state within 4 min. Carotid body type I cells are small and spherical and the patch pipettes were of a relatively low resistance (see Methods), so therefore conditions for effective cell dialysis with the patch pipette contents were favourable, as demonstrated by the fairly rapid activation of the cAMP-activated current after achieving the whole-cell configuration. The cAMP-activated current also showed a dependence on intracellular ATP for activation in a similar manner to the swelling-activated Cl$^-$ current. The small and variable activation seen early during recording in some of the cells subjected to the ATP-deficient patch pipette solution (see Fig. 5A) probably reflects the time course for diffusional exchange of cAMP and ATP between patch pipette and cell. However, by the time steady-state current was reached in the cells which were dialysed with ATP-containing patch pipette solution (at 4 min), this variable activation had subsided in the cells diffused with ATP-free solutions.

The current could also be elicited by extracellular application of the membrane-permeable cAMP analogue 8-Br-cAMP (2 mM), although the evoked currents were smaller than those seen when cAMP was included in the pipette. This would perhaps account for the apparent difference in niflumic acid sensitivity of these two currents, as the smaller 8-Br-cAMP-activated current may have been contaminated by a larger proportion of niflumic acid-insensitive leak current, resulting in a lower percentage inhibition for the 8-Br-cAMP-activated current. Both currents, however, showed outward rectification and an E_{rev} close to 0 mV in symmetrical Cl$^-$ concentrations, with Cl$^-$ as the major anion, suggestive of a Cl$^-$-selective conductance.

Although CFTR is activated by intracellular cAMP, the channel responsible for the cAMP-activated Cl$^-$ current in carotid body type I cells is unlikely to be CFTR as this channel gives rise to currents that have been described as having a linear I–V relationship in addition to being DIDS insensitive (Meng & Weinman, 1996). The cAMP-activated current in our cells was both outwardly rectifying and sensitive to blockade by DIDS. However, non-CFTR, outwardly rectifying cAMP-activated Cl$^-$ currents exist in airway epithelial cells (Schwiebert, Flotte, Cutting & Guggino, 1994) and in rat hepatocytes (Meng & Weinman, 1996).

Do swelling-activated Cl$^-$ currents and cAMP-activated Cl$^-$ currents arise from the same channel?

In the above-mentioned study by Meng & Weinman (1996) on rat hepatocytes, the cAMP-activated current was present alongside a swelling-activated Cl$^-$ current, as in the carotid body type I cells. While these two types of conductance have been shown to arise from physically distinct channel types in airway epithelial cells (Chan, Goldstein & Nelson, 1992), in rat hepatocytes the effect of

cAMP was to change the volume set-point of the swelling-activated Cl$^-$ channel, rather than a direct activation of a separate Cl$^-$ channel. This may also be the case in carotid body type I cells. This is supported by the fact that the swelling-activated Cl$^-$ current and cAMP-activated Cl$^-$ current share the same kinetics, pharmacology and dependence on intracellular ATP for activation. Additionally, the cAMP-activated current can be inhibited by hypertonic solutions (see Fig. 8). Of course, it is possible that two distinct channel types which share common modulation could be responsible for the two Cl$^-$ currents, although it is probably more likely that the same channel is responsible for the cAMP- and swelling-activated Cl$^-$ currents.

Possible functional importance in carotid body type I cells

In order to properly assign roles for the Cl$^-$ currents described in this report, it is important to know where the Cl$^-$ equilibrium potential (E_{Cl}) lies with respect to carotid body type I cell resting membrane potential under normal physiological conditions. Although Cl$^-$ ions have been described as contributing significantly to the resting membrane potential of rabbit carotid body type I cells (Oyama et al. 1986), E_{Cl} has not been determined. It is therefore difficult to know whether activation of these Cl$^-$ channels would produce type I cell depolarization or hyperpolarization. Changes in type I cell membrane potential would have significant implications for the chemotransductive process in these cells, as the activation of Ca^{2+} channels during type I cell depolarization to effect neurotransmitter release is a crucial step in type I cell stimulus–secretion pathways (Gonzalez et al. 1994; Peers & Buckler, 1995).

In the light of early evidence to suggest that hypotonic solutions caused carotid body type I cell hyperpolarization (Gallego et al. 1979), it is tempting to conclude that E_{Cl} lies negative to the resting membrane potential, based on the swelling-activated Cl$^-$ current described here. However, methodological problems associated with cell impalement by sharp microelectrodes containing very high Cl$^-$ concentrations used in the study by Gallego et al. (1979), must be taken into consideration when interpreting their data with regard to Cl$^-$ channel activity. Nevertheless, if it were the case that activation of the swelling-activated Cl$^-$ current described in the present report contributed to the type I cell hyperpolarization described in the earlier study, then the hypothesis that carotid body type I cells function as 'osmoreceptors' may well be valid. Supportive of the role as 'osmoreceptor' is also the very sensitive nature of the response of type I cells to changes in extracellular osmolarity as reported by Gallego et al. (1979). Thus, a change in blood osmolarity would be reflected in the membrane potential of carotid body type I cells, such that, for example, an increase in blood osmolarity would decrease the level of activation of the swelling-activated Cl$^-$ channel, leading to type I cell depolarization. It is not impossible to

imagine that there is a certain level of activation of this current during normal blood osmolarity, as we have shown that cAMP can activate a tonicity-sensitive channel. The basal levels of cAMP are not known in type I cells of the rat carotid body, but they may be sufficient to cause some activation of the tonicity-sensitive channel. This is particularly true if the cells are under the influence of neurotransmitters which stimulate adenylate cyclase, for which type I cells do possess receptors (Gonzalez *et al.* 1994). The membrane potential of rat carotid body type I cells ranges between approximately −40 and −50 mV (He, Wei & Eyzaguirre, 1993; Buckler & Vaughan-Jones, 1994; Wyatt, Wright, Bee & Peers, 1995) and very small current changes have been shown to have profound effects on type I cell membrane potential. For example, a 2 pA inward current injection has been shown to cause a depolarization of approximately 15 mV (Buckler & Vaughan-Jones, 1994). Therefore, assuming that the depolarization seen in type I cells during a hypertonic challenge (Gallego *et al.* 1979) would be sufficient to activate voltage-gated Ca^{2+} channels, the resultant Ca^{2+} influx would trigger neurotransmitter release to effect an increase in CSN firing. Increases in CSN firing have been demonstrated to cause release of ADH (Share & Levy, 1966) due to excitation of neurosecretory neurones in the supraoptic nucleus of the hypothalamus (Yamashita, 1977). ADH serves to decrease urinary flow, causing blood osmolarity to decrease and in this way carotid body type I cells may play an important role in blood volume homeostasis.

In addition to a possible role of the swelling-activated Cl⁻ current in blood volume homeostasis, these currents are often involved in cell volume regulation (see Robson & Hunter, 1994). During a period in hypotonic solutions, cells respond initially by swelling due to rapid entry of water, but then can be seen to revert slowly back to the original cell size. This is termed a volume regulatory decrease (VRD) and can be blocked by the stilbene derivative DIDS (Best *et al.* 1996*b*). In fact, the initial swelling phase during a hypotonic shock activates swelling-activated Cl⁻ and K⁺ channels causing a loss of KCl and osmotically obligated water from the cell, causing VRD (Robson & Hunter, 1994). VRD is a feature of many cell types and is particularly important for cell types which have high metabolic rates, such as carotid body type I cells (Gonzalez *et al.* 1994).

Levels of cAMP have been reported to rise in carotid body type I cells of the rabbit and cat during a hypoxic challenge (Wang *et al.* 1989; Perez-Garcia *et al.* 1990; Delpiano & Acker, 1991) and cAMP, its analogues and compounds which increase intracellular cAMP levels potentiate hypoxia-induced dopamine release (Perez-Garcia *et al.* 1991; Wang *et al.* 1991). In rabbit carotid body type I cells, the increase in cAMP during a hypoxic challenge is partly through a Ca^{2+}-dependent process (Perez-Garcia *et al.* 1990). This Ca^{2+}-dependent increase in cAMP is thought to be due

to released neurotransmitters (during hypoxia) acting on cell autoreceptors which are positively coupled to adenylate cyclase (Cachero, Rigual, Rocher & Gonzalez, 1996). One function of cAMP in rabbit type I cells has been shown to be inhibition of an O_2-sensitive K⁺ channel (causing cell depolarization) thus potentiating the low P_{O_2} inhibition of the same channel (López-López, De Luis & Gonzalez, 1993). However, in rat carotid body type I cells, cAMP did not modulate K⁺ or Ca^{2+} currents (Hatton & Peers, 1996). Now, a possible candidate for cAMP-mediated modulation has been demonstated in rat carotid body type I cells: the cAMP-activated Cl⁻ current. The role of this current will, as previously described, depend on where E_{Cl} lies with respect to the resting membrane potential, but most probably it will act to stabilize the membrane potential during a hypoxic challenge to aid negative feedback processes.

At present, it is not known whether the high-conductance Cl⁻ channels present in rat type I cells, as reported by Stea & Nurse (1989), underlie the macroscopic Cl⁻ currents investigated here. Certainly, major differences are apparent; the Cl⁻ channels described previously (Stea & Nurse, 1989) were insensitive to Ca^{2+}, DIDS, ATP and cAMP, and did not display rectification. However, these studies were all conducted using excised, inside-out patches in which regulatory cytosolic factors might be lost. Therefore, we cannot categorically state that the channels observed by Stea & Nurse (1989) are distinct from those underlying the whole-cell Cl⁻ currents reported here.

In conclusion, we have demonstrated the presence of Cl⁻ currents in rat carotid body type I cells activated by cell swelling and cAMP, which may be carried by the same channel. When interpreting the roles for these currents it must be remembered that both tonicity and cAMP (and ATP availability) will influence the level of activation of this channel, if indeed one channel type is responsible for the currents described in this report. The swelling-activated Cl⁻ current may mediate, at least in part, the physiological response to altered blood osmolarity and may also contribute to VRD at the level of individual cells. The cAMP-activated Cl⁻ current is likely to play an important modulatory role in the chemotransductive process connected to changes in arterial P_{O_2}, by regulating carotid body type I cell excitability.

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