

Presynaptic action of adenosine on a 4-aminopyridine-sensitive current in the rat carotid body

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1. Plasma adenosine concentration increases during hypoxia to a level that excites carotid body chemoreceptors by an undetermined mechanism. We have examined this further by determining the electrophysiological responses to exogenous adenosine of sinus nerve chemoafferents *in vitro* and of whole-cell currents in isolated type I cells.
2. Steady-state, single-fibre chemoafferent discharge was increased approximately 5-fold above basal levels by 100 μM adenosine. This adenosine-stimulated discharge was reversibly and increasingly reduced by methoxyverapamil (D600, 100 μM), by application of nickel chloride (Ni^{2+} , 2 mM) and by removal of extracellular Ca^{2+} . These effects strongly suggest a presynaptic, excitatory action of adenosine on type I cells of the carotid body.
3. Adenosine decreased whole-cell outward currents at membrane potentials above -40 mV in isolated type I cells recorded during superfusion with bicarbonate-buffered saline solution at $34\text{--}36$ °C. This effect was reversible and concentration dependent with a maximal effect at 10 μM .
4. The degree of current inhibition induced by 10 μM adenosine was voltage independent ($45.39 \pm 2.55\%$ (mean \pm s.e.m.) between -40 and $+30$ mV) and largely ($\sim 75\%$), but not entirely, Ca^{2+} independent. 4-Aminopyridine (4-AP, 5 mM) decreased the amplitude of the control outward current by $80.60 \pm 3.67\%$ and abolished the effect of adenosine.
5. Adenosine was without effect upon currents near the resting membrane potential of approximately -55 mV and did not induce depolarization in current-clamp experiments.
6. We conclude that adenosine acts to inhibit a 4-AP-sensitive current in isolated type I cells of the rat carotid body and suggest that this mechanism contributes to the chemoexcitatory effect of adenosine in the whole carotid body.

The purine nucleoside adenosine is present in all cells as an intermediary of metabolism and is derived predominantly from the cleavage of 5'-adenosine monophosphate (AMP) by 5'-nucleotidase. Its intracellular production can be increased rapidly during hypoxia (Winn *et al.* 1981) leading to its increased facilitated diffusion into the extracellular space where, through activation of specific G-protein coupled membrane bound (P_1) purinoceptors, it can act in an auto- or paracrine fashion or even at a more systemic level via the circulation. This link with metabolism makes adenosine an attractive candidate when investigating the cellular response to oxygen lack and activation of adenosine receptors tends towards either increasing oxygen delivery or reducing oxygen demand. A similar 'protective' function has been ascribed for adenosine at a systemic level, as a stimulatory action of the nucleoside upon carotid body chemoreceptor afferent discharge has been recorded *in vivo* (McQueen & Ribeiro, 1981) which is sufficient to increase ventilation in

rats (Monteiro & Ribeiro, 1987) and humans (Watt *et al.* 1987). This excitatory effect is retained *in vitro* (Runold *et al.* 1990) and is thus independent of the well-established effects of adenosine upon blood flow.

Pharmacological and histochemical evidence suggests that the receptor activated at the carotid body is most likely the $\text{A}_{2\text{A}}$ subtype (McQueen & Ribeiro, 1986; Weaver, 1993; Sebastiao & Ribeiro, 1996) but the location of these receptors and the mechanism by which their activation initiates afferent chemoreceptor discharge is unknown. The carotid body is a highly vascular, composite receptor made up of many glomeruli containing clusters of type I cells surrounded by glial-like type II cells with an efferent and afferent innervation, in which the neural crest-derived, type I cell is now widely believed to act as the primary transducer element. Although some differences exist in the precise details, there is now a general agreement that hypoxia induces inhibition of a component of the total outward K^+ current in these cells

that contributes to the resting membrane potential, thus leading to membrane depolarization, voltage-gated Ca^{2+} entry and Ca^{2+} -dependent neurosecretion onto (*in vivo*) closely apposed afferent nerve endings (Gonzalez *et al.* 1994; Peers & Buckler, 1995). The aim of the present study was to determine if the cellular action of adenosine mimicked that of hypoxia by firstly determining the Ca^{2+} dependence of its excitatory action in an *in vitro* whole carotid body preparation and secondly to determine the effect of adenosine upon whole-cell currents recorded in isolated type I cells.

A preliminary report of part of this study has been published in abstract form (Vandier *et al.* 1998).

METHODS

Anaesthesia in adult Wistar rats (> 3 weeks old; 120–200 g) was induced with 3–4% halothane in O_2 and maintained at 1.5–2.5% halothane, whilst left and right carotid bifurcations were removed as described previously (Pepper *et al.* 1995). The level of halothane was then increased to 4% and animals were killed by decapitation. Excised bifurcations were prepared either for single chemoafferent fibre recording or for patch-clamp recording of isolated type I cells.

Single chemoafferent fibre recording

Each carotid bifurcation was pinned on Sylgard (Dow Corning) in a small volume (~0.2 ml) tissue bath and superfused at 3 ml min^{-1} with warmed (36.7–37 °C), gassed (95% O_2 –5% CO_2), bicarbonate-buffered saline solution (composition (mM): 125 NaCl, 3 KCl, 1.25 NaH_2PO_4 , 5 Na_2SO_4 , 1.3 MgSO_4 , 24 NaHCO_3 , 2.4 CaCl_2 , 10 glucose, pH ~7.38) whilst the excess connective tissue around the bifurcation was removed to expose the carotid body and the sinus nerve. The preparation was then partially digested in a gassed enzyme solution (0.06% collagenase (Sigma Type II), 0.02% protease (Sigma Type IX)) for 25 min at 37 °C to facilitate the recording of neuronal activity.

Extracellular recordings of afferent single-fibre activity were made from the cut end of the carotid sinus nerve using glass suction electrodes and standard Neurolog modules (Digitimer Ltd, Welwyn Garden City, Hertfordshire, UK). Chemoreceptor discharge was discriminated as activity exceeding a level which was at least 50% of the amplitude of the baseline noise above the noise and which responded to a decrease in superfusate P_{O_2} with a reversible increase in discharge frequency. Single fibres were discriminated on the basis of amplitude and shape. Action potentials were sampled digitally after conversion to TTL pulses via a window discriminator (Neurolog NL200) by a computer (Macintosh IIci with NB-MIO-16 DA and NB-MIO-8G DMA cards, National Instruments Co., Austin, TX, USA) running customized LabVIEW 2 (National Instruments Co.) software. TTL pulses were counted and binned into 10 s periods.

The P_{O_2} and temperature of the superfusate were continually monitored in the superfusion line immediately before entering the bath by a membrane oxygen electrode (No. 733, Diamond General, Ann Arbor, MI, USA) and meter (O_2/CO_2 Analyser, Cameron Instrument Company, Port Aransas, TX, USA) and thermocouple (871A, Tegan Inc., Madison, OH, USA). The oxygen electrode was calibrated against, and P_{CO_2} was determined from, superfusate samples collected just distal to the carotid body and measured on a blood gas analyser (IL 1640, Instrumentation Laboratory,

Warrington, UK). Afferent spike activity and superfusate P_{O_2} were monitored on an oscilloscope (Gould 1604) and recorded throughout each experiment on a VHS video recorder via a DC modified PCM digital unit (Applegarth Electronics).

Patch-clamp recording of isolated type I cells

Carotid bodies were removed from excised bifurcations and incubated for 10 min in magnesium-free, low Ca^{2+} (50 μM), bicarbonate-buffered saline solution. Collagenase (0.4 mg ml^{-1} , Type I, Worthington) and trypsin (0.2 mg ml^{-1} , Sigma) were then added and the solution held at 37 °C for 25 min. The digested tissue was centrifuged (1000 r.p.m. for 10 min) and the enzyme solution removed and replaced by Ham's F-12 culture medium (Sigma) supplemented with 10% fetal calf serum, 100 i.u. ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, 80 u l^{-1} insulin and 2 mg glutamine. Following trituration with fire-polished Pasteur pipettes, dispersed cells were plated onto glass coverslips and kept in a humidified incubator (5% CO_2 –95% air at 37 °C) overnight. Cells were used within 14–24 h following isolation.

Solutions and drugs

The bicarbonate-buffered, extracellular saline solution contained (mM): 117 NaCl, 4.5 KCl, 23 NaHCO_3 , 1 MgCl_2 , 2.5 CaCl_2 and 11 glucose and was equilibrated with 5% CO_2 and 95% air (pH ~7.4). For Ca^{2+} -free solutions, CaCl_2 was removed and 1 mM EGTA and 3.5 mM MgCl_2 were added. Adenosine (Sigma) and 4-AP (Sigma) were added directly in saline solution. The pipette filling intracellular solution used for whole-cell recordings contained (mM): 6 NaCl, 107 KCl, 2 Na_2ATP , 11 Hepes, 5 EGTA, 2 MgCl_2 , 2 CaCl_2 (pH 7.2 adjusted with KOH). $p\text{Ca} \approx 7$ was calculated using CAMG software (W. H. Martin, Yale University, CT, USA).

Extracellular solutions were superfused at ~2 ml min^{-1} in a 200 μl bath chamber. Given a dead space of 600 μl and assuming that a total change of bath solution occurs with 800 μl of perfused solution (i.e. 4 × bath chamber volume), bath concentrations would begin to change at ~18 s and new solution equilibria would be reached ~42 s after switching to the new solution. All experiments were performed at 34–36 °C and at a P_{O_2} ~150 mmHg (confirmed on IL 1640 blood gas analyser).

Electrophysiology

Isolated type I cells were identified as spherical in shape and approximately 10 μm in diameter and in preliminary studies (data not shown) by amine fluorescence (de la Torre & Surgeon, 1976). The whole-cell patch-clamp configuration was used to record currents. Electrodes of 5–7 M Ω were made using Clarke GC150F-10 borosilicate glass capillaries (Clarke Electromedical, Reading, UK) and were fire-polished before use. The junction potentials between the electrode and the bath were cancelled using the voltage pipette offset control of the amplifier. Electrode capacitances were electronically compensated (~4 pF). Cell capacitance was calculated by integration of the current obtained during pulses between –60 and –70 mV, divided by the pulse amplitude. To take account of cell size, current amplitudes were normalized by dividing by cell capacitance. Mean cell capacitance was calculated as 2.74 ± 0.09 pF ($n = 29$).

Data acquisition and analysis

Experiments were conducted using an Axopatch-1D (Axon Instruments Inc.) amplifier and currents were filtered with a Bessel low-pass filter. Records were simultaneously displayed on an oscilloscope (Gould 1602), digitized with Digidata-1200 A/D converter (Axon Instruments Inc.) and stored on disk in an PC computer using pCLAMP version 6.02 software (Axon Instruments

Inc.). The membrane potentials of type I cells were held at -70 mV and the current–voltage (I – V) relation between -90 and 30 mV was obtained using ramp protocols (average of 5 ramps at 0.24 mV ms^{-1} every 10 s) and 10 mV increment pulse protocols (average of the last 20 ms of 300 ms duration pulses every 6 s). Currents were sampled at 5 kHz and filtered at 1 kHz. The membrane potential was recorded using the amplifier in current-clamp mode ($I = 0$) and stored on standard VHS videocassette via a DC modified PCM digital unit (Applegarth Electronics). Analyses of the data were performed using CLAMPFIT and FETCHAN (pCLAMP software) and ORIGIN 4.1 software (Microcal Inc., Northampton, MA, USA).

All data are expressed as means \pm s.e.m. and tested for significant differences ($P < 0.05$) with regression analysis or one factor ANOVA with *post hoc* tests as indicated, using Statview II (Abacus Concepts, Berkeley, CA, USA) software.

RESULTS

Ca^{2+} dependence of adenosine in the *in vitro* carotid body

Chemoreceptor discharge was recorded from 24 single-fibre chemoafferents from 14 carotid body preparations. In all cases, spontaneous basal discharge (0.14 ± 0.02 impulses s^{-1}) was obtained during hyperoxic ($P_{\text{O}_2} > 400$ mmHg) normocapnia ($P_{\text{CO}_2} = 34.8 \pm 0.4$ mmHg) and was increased significantly by reducing superfusate P_{O_2} . No protocol lasted longer than 60 min.

The chemoreceptor discharge response to adenosine ($1 \mu\text{M}$ – 1 mM) was recorded in six preparations as the mean

discharge during the final 2 min of a 10 min exposure to each concentration, at which time discharge was steady. Adenosine significantly increased steady-state chemodischarge ($P < 0.01$, ANOVA). Basal discharge in the absence of adenosine was 0.12 ± 0.03 impulses s^{-1} and was 0.11 ± 0.02 and 0.17 ± 0.06 impulses s^{-1} at 1 and $10 \mu\text{M}$ adenosine, respectively. Discharge was elevated almost 5-fold to 0.55 ± 0.16 impulses s^{-1} at $100 \mu\text{M}$ adenosine and fell slightly but not significantly at 1 mM to 0.40 ± 0.13 impulses s^{-1} . *Post hoc* testing (Scheffé's F test) revealed that this effect of adenosine was significant only at $100 \mu\text{M}$ and 1 mM.

The effect of adenosine in the absence of extracellular Ca^{2+} was determined in five preparations (Fig. 1). Chemoreceptor discharge was reduced considerably, but never totally abolished, to 0.03 ± 0.02 impulses s^{-1} ($P < 0.01$, ANOVA), in the continued presence of $100 \mu\text{M}$ adenosine, by the exclusion of CaCl_2 from the superfusate and addition of 4 mM MgSO_4 and 1 mM EGTA. This discharge level was significantly less than the adenosine stimulated (0.95 ± 0.28 impulses s^{-1} ; $P < 0.05$ *post hoc* Student's paired t test) and basal (0.23 ± 0.07 impulses s^{-1} ; $P < 0.05$ *post hoc* Student's paired t test) discharge levels in this series of experiments and was fully reversible upon return to normal Ca^{2+} .

The source of Ca^{2+} entry in adenosine-mediated chemoreceptor discharge was further determined by use of the non-selective Ca^{2+} channel antagonist, nickel chloride (Ni^{2+} , 2 mM; $n = 5$)

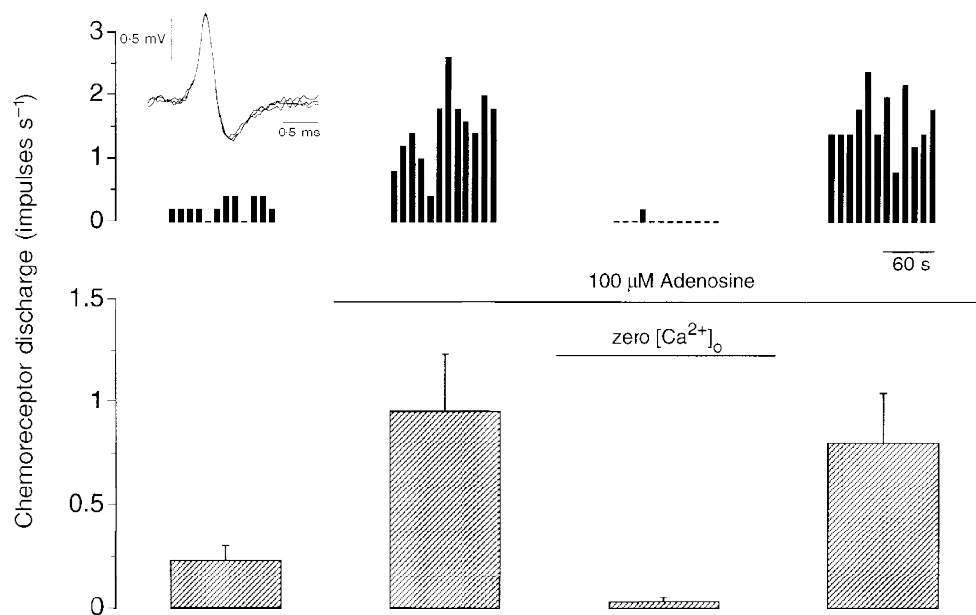


Figure 1. The stimulatory effect of adenosine is Ca^{2+} dependent

Single-fibre chemoreceptor activity recorded during steady-state control (left panel) and during exposure to $100 \mu\text{M}$ adenosine in the presence and absence of extracellular Ca^{2+} (middle panels) and after re-addition of Ca^{2+} (right panel). An example from one preparation is shown at the top, binned every 10 s and expressed in impulses s^{-1} . The inset at top left shows three superimposed action potentials. The mean \pm s.e.m. of five preparations is shown below. Superfusate $P_{\text{O}_2} > 400$ mmHg and $P_{\text{CO}_2} \sim 35$ mmHg throughout.

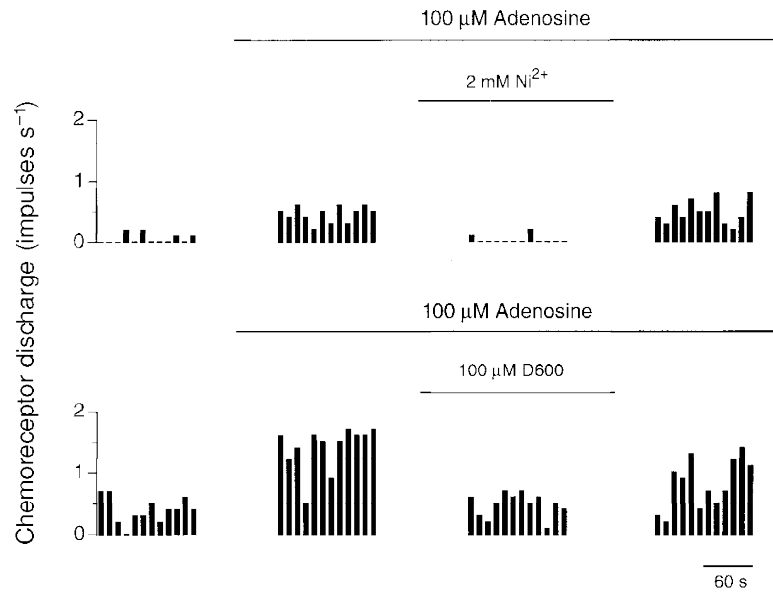


Figure 2. Effect of Ni²⁺ and D600 upon adenosine stimulated chemoreceptor discharge

Single-fibre chemoreceptor activity recorded from two preparations during steady-state control (left panel) and during exposure to 100 μM adenosine in the presence and absence of either 2 mM Ni²⁺ or 100 μM D600 (middle panels) and after removal of antagonist (right panel). Discharge was binned every 10 s.

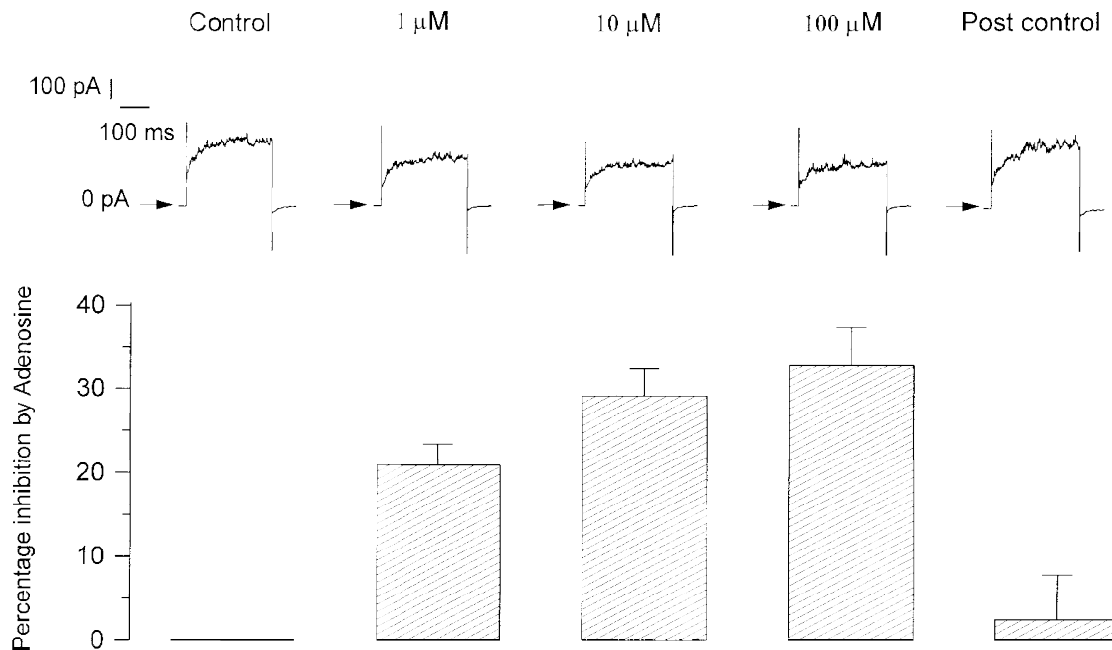


Figure 3. Dose-dependent effect of adenosine on outward current

Top, example of outward currents elicited in one type I cell during 300 ms pulses to +30 mV from a holding potential of -70 mV in control conditions, at three different concentrations of adenosine and during post control conditions subsequent to the removal of adenosine. Currents during adenosine infusion were recorded 1 min after the calculated new solution equilibria would have been reached and 4 min after post control wash of adenosine. Bottom, means + S.E.M. ($n = 6$) of the percentage inhibition of outward currents at +30 mV induced by the three concentrations of adenosine.

and the L-type Ca^{2+} channel blocker, methoxyverapamil (D600, $100 \mu\text{M}$; $n = 8$). D600 was used in preference to dihydropyridines, such as nifedipine, as these compounds have been shown to decrease the uptake of adenosine through the nucleoside transporter (Bartrup & Stone, 1990). Both Ni^{2+} and D600 significantly ($P < 0.001$, ANOVA) reduced chemodischarge in the presence of $100 \mu\text{M}$ adenosine (Fig. 2) with Ni^{2+} reducing chemodischarge by $97 \pm 1\%$ from 0.27 ± 0.04 to 0.01 ± 0.004 impulses s^{-1} ($P < 0.05$, Scheffé's F test) and D600 reducing it by $68 \pm 7\%$ from 0.70 ± 0.17 to 0.23 ± 0.07 impulses s^{-1} ($P < 0.05$, Scheffé's F test). Whilst the adenosine-stimulated absolute discharges, in the absence of antagonists, were not significantly different between both groups of experiments ($P > 0.05$, unpaired t test), the absolute discharge in the presence of Ni^{2+} and D600 was significantly different ($P < 0.05$, unpaired t test) as was the normalized percentage inhibition between both groups ($P < 0.02$, unpaired t test).

Dose-dependent effect of adenosine in isolated cells

Step depolarizations to $+30$ mV from a holding potential of -70 mV were applied for 300 ms every 6 s, firstly in the absence and then in the presence of various concentrations of adenosine in six type I cells. Example currents are shown in Fig. 3 where it can be seen that these currents were outward and sustained with inactivation not apparent within the pulse period. Increasing adenosine concentration decreased the amplitude of the outward current by 21.00 ± 2.40 , 29.17 ± 3.26 and $32.92 \pm 4.43\%$ of the control current at 1, 10 and $100 \mu\text{M}$ respectively ($P < 0.05$, ANOVA) and in a reversible manner (Fig. 3). *Post hoc* testing (paired t test) revealed that this effect of adenosine was significantly different between $1 \mu\text{M}$ and $10 \mu\text{M}$ adenosine ($P < 0.001$) and between $1 \mu\text{M}$ and $100 \mu\text{M}$ ($P < 0.001$) but not between $10 \mu\text{M}$ and $100 \mu\text{M}$ adenosine ($P > 0.2$) suggesting that the maximal effect of adenosine was obtained between 10 and $100 \mu\text{M}$. Further experiments

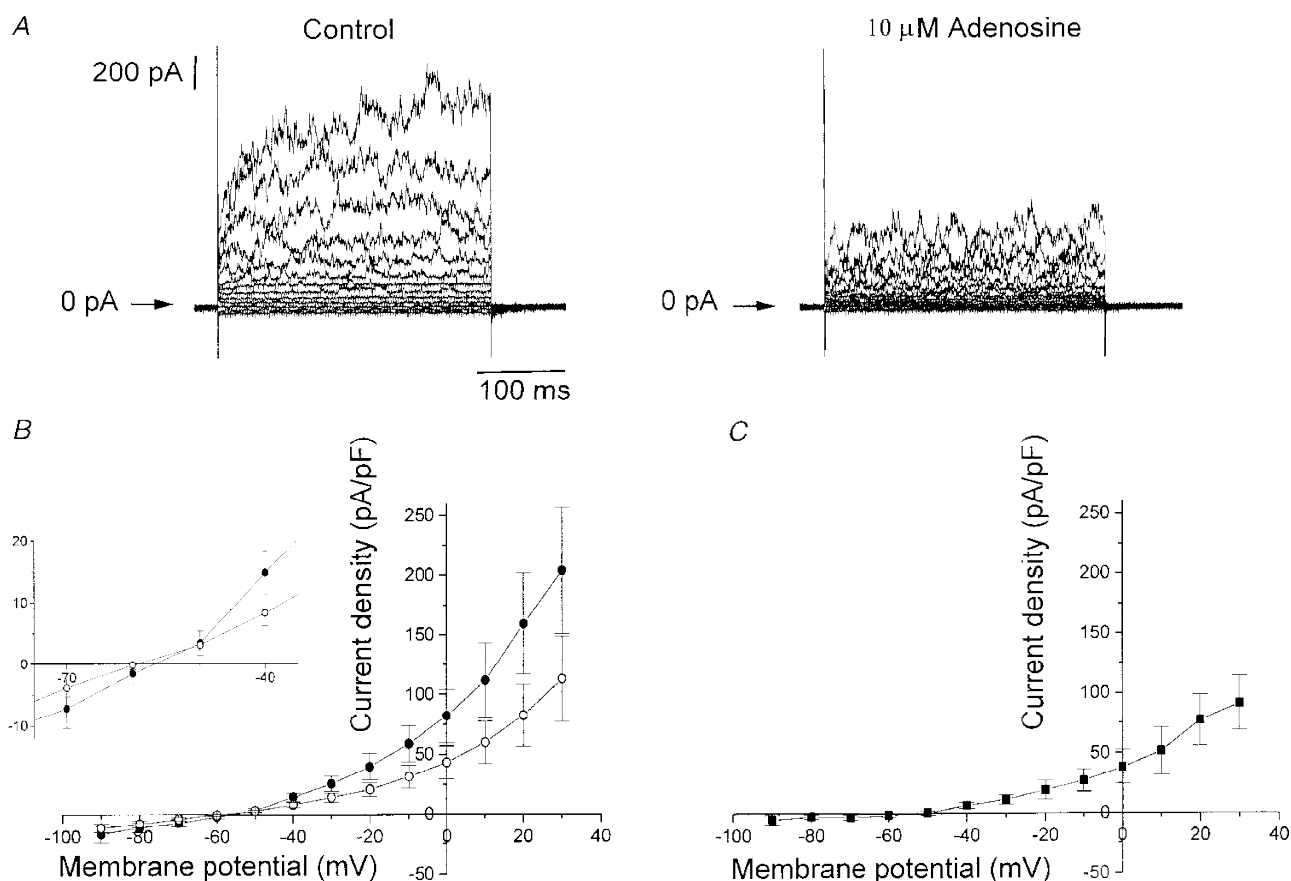


Figure 4. Adenosine decreases outward currents above -40 mV

A, example of outward currents obtained during 300 ms pulses to membrane potentials between -90 and $+30$ mV from a holding potential of -70 mV in control conditions and with $10 \mu\text{M}$ adenosine in the bath. B, mean \pm s.e.m. ($n = 7$) current density–voltage relation obtained in control conditions (●) and in the presence of $10 \mu\text{M}$ adenosine (○). The inset shows an expanded scale of the same current density–voltage relation between -75 and -45 mV which highlights the negligible effect of adenosine at membrane potentials where current density is equal to 0 pA pF^{-1} . C, means \pm s.e.m. ($n = 7$) of the adenosine-sensitive component of the total current density–voltage relation determined as the difference between the current density amplitudes in control conditions and in the presence of $10 \mu\text{M}$ adenosine.

were therefore performed with $10 \mu\text{M}$ adenosine as the minimum concentration able to induce the maximal effect.

Adenosine decreases the amplitude of outward current above -40 mV

Depolarization of the membrane between -90 and $+30 \text{ mV}$, from a holding potential of -70 mV , elicited currents which were outward above -50 mV and which increased in amplitude with increasing depolarization. The application of $10 \mu\text{M}$ adenosine decreased the amplitude of these outward currents. Example currents from one cell are shown in Fig. 4A. The current density–voltage relations of this cell averaged with six other cells are shown in Fig. 4B. The current density–voltage relation was linear between -90 and -50 mV before adenosine and between -90 and -40 mV in the presence of adenosine and showed outward rectification at more positive potentials. Adenosine significantly decreased the amplitude of the current ($P < 0.002$, ANOVA). This effect of adenosine was significant only above -40 mV ($P < 0.05$, *post hoc* paired *t* test). Near the resting membrane potential of $\sim -55 \text{ mV}$ (corresponding to the membrane potential where the current density was 0 pA pF^{-1}), adenosine was without effect on currents (Fig. 4B, inset) with no significant differences observed at -60 mV ($P > 0.40$) or

-50 mV ($P > 0.80$). The current density–voltage relation of this adenosine-sensitive current (given as the difference between control and adenosine) is represented in Fig. 4C and shows an activation threshold near -50 mV .

The effect of adenosine is voltage independent and largely Ca^{2+} independent

To investigate the role of external Ca^{2+} in the effect of adenosine, the bathing solution was changed to a Ca^{2+} -free solution for 2 min (to attain a steady state) in seven cells. In four of these cells we then applied $10 \mu\text{M}$ adenosine. Figure 5A shows, in one cell, the effect of removal of external Ca^{2+} and the effect of adenosine upon this residual current. The mean current density–voltage relation obtained in these three conditions is shown in Fig. 5B. Removal of external Ca^{2+} significantly decreased the current density ($P < 0.001$, ANOVA) and, in this condition, adenosine could still significantly decrease the amplitude of the remaining current ($P < 0.001$, ANOVA).

The inhibition produced by adenosine in the presence, or absence, of external Ca^{2+} (Figs 4B and 5B) did not appear to be voltage dependent at potentials greater than -40 mV . This was confirmed by determining the degree of inhibition (difference in current density/control current density)

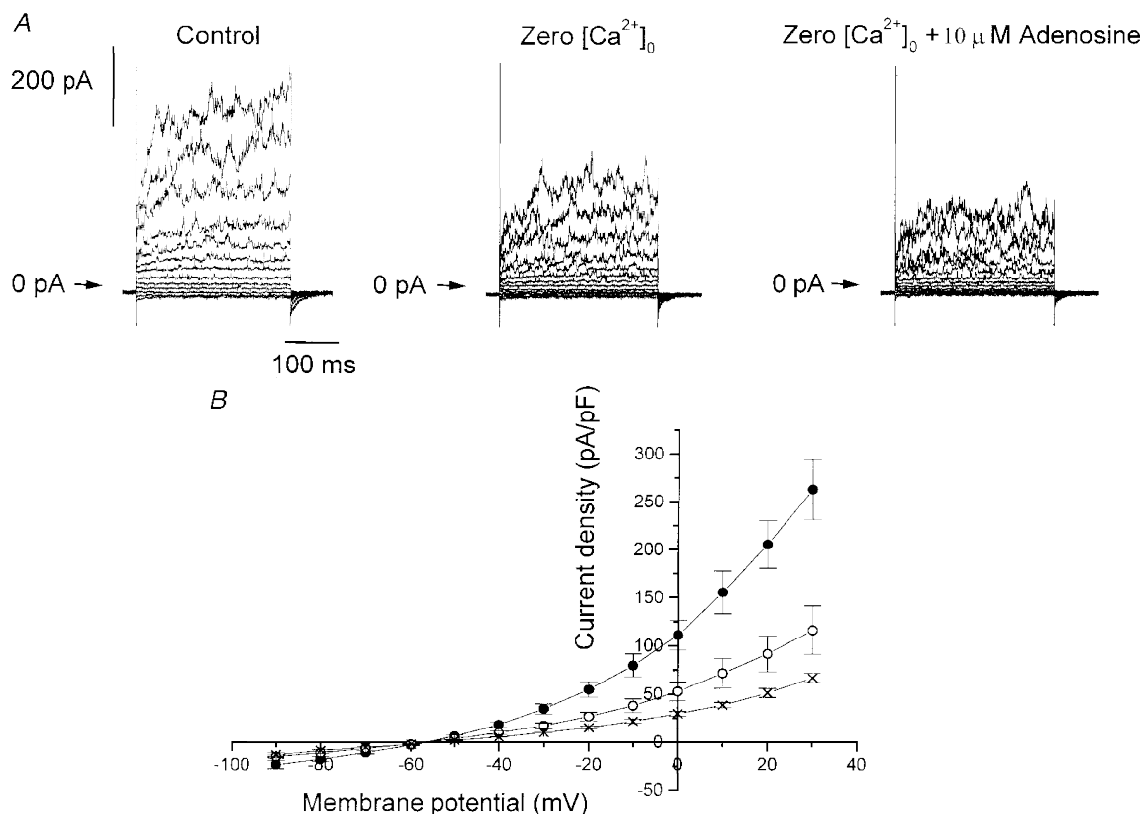


Figure 5. Adenosine decreases outward currents in the absence of external Ca^{2+}

A, example of outward currents obtained during 300 ms pulses to membrane potentials between -90 and $+30 \text{ mV}$ from a holding potential of -70 mV in control conditions, in the absence of external Ca^{2+} (Zero $[\text{Ca}^{2+}]_o$) and in the presence of $10 \mu\text{M}$ adenosine. B, means $\pm \text{s.e.m.}$ of the current density–voltage relation obtained in control conditions (\bullet , $n = 7$), in the absence of external Ca^{2+} (\circ , $n = 7$) and in the presence of $10 \mu\text{M}$ adenosine (\times , $n = 4$).

produced by adenosine in both conditions. In the presence of Ca^{2+} , adenosine decreased the amplitude of the control current by $42.68 \pm 8.77\%$ at -40 mV and by $47.60 \pm 5.88\%$ at $+30$ mV. This percentage of inhibition, induced by adenosine, was not significantly different across this potential range ($P > 0.90$, ANOVA) with a mean inhibition of $45.39 \pm 2.55\%$. In the absence of external Ca^{2+} , the inhibition of current by adenosine remained voltage independent ($P > 0.90$, ANOVA) with a mean inhibition of $34.20 \pm 2.91\%$ across the voltage range examined ($P < 0.01$, ANOVA compared with that when Ca^{2+} is present), suggesting that only approximately one-quarter ($1 - (34.20/45.39)$) of the inhibitory effect of adenosine was Ca^{2+} dependent.

4-AP abolishes the effect of adenosine

Figure 6A shows a family of outward currents obtained in one cell and from a holding potential of -70 mV to -90 mV and up to $+30$ mV and Fig. 6B represents the mean current density–voltage relation in control conditions, in the presence of 5 mM 4-AP in the absence or presence of 10 μM adenosine. In 10 cells, 4-AP significantly decreased the amplitude of the current density below that of control ($P < 0.001$, ANOVA) with a decrease of $80.60 \pm 3.67\%$ at

$+30$ mV. In the continued presence of 5 mM 4-AP, adenosine had no effect on the remaining current ($P > 0.70$, ANOVA). At membrane potentials close to the reversal potential of the net whole-cell current, 4-AP did not decrease the current density. For example, at -60 mV, control current density was -2.84 ± 0.97 pA pF $^{-1}$ and was 0.37 ± 0.32 pA pF $^{-1}$ in the presence of 4-AP. This suggested, as was found for adenosine, that 4-AP would not depolarize isolated type I cells from their resting membrane potential.

In the presence of 4-AP, the degree of inhibition observed in the presence of adenosine remained voltage independent with no significant difference between -40 and $+30$ mV ($P > 0.90$, ANOVA) with a mean inhibition of $-2.29 \pm 3.21\%$. This was significantly different ($P < 0.0001$, ANOVA) from the degree of inhibition induced by adenosine in the absence of 4-AP and further demonstrates that the current inhibited by adenosine is 4-AP sensitive.

Adenosine has no effect at the resting membrane potential

As adenosine was without significant effect on the amplitude of currents near the resting membrane potential, this suggested that it would not be able to modify this potential

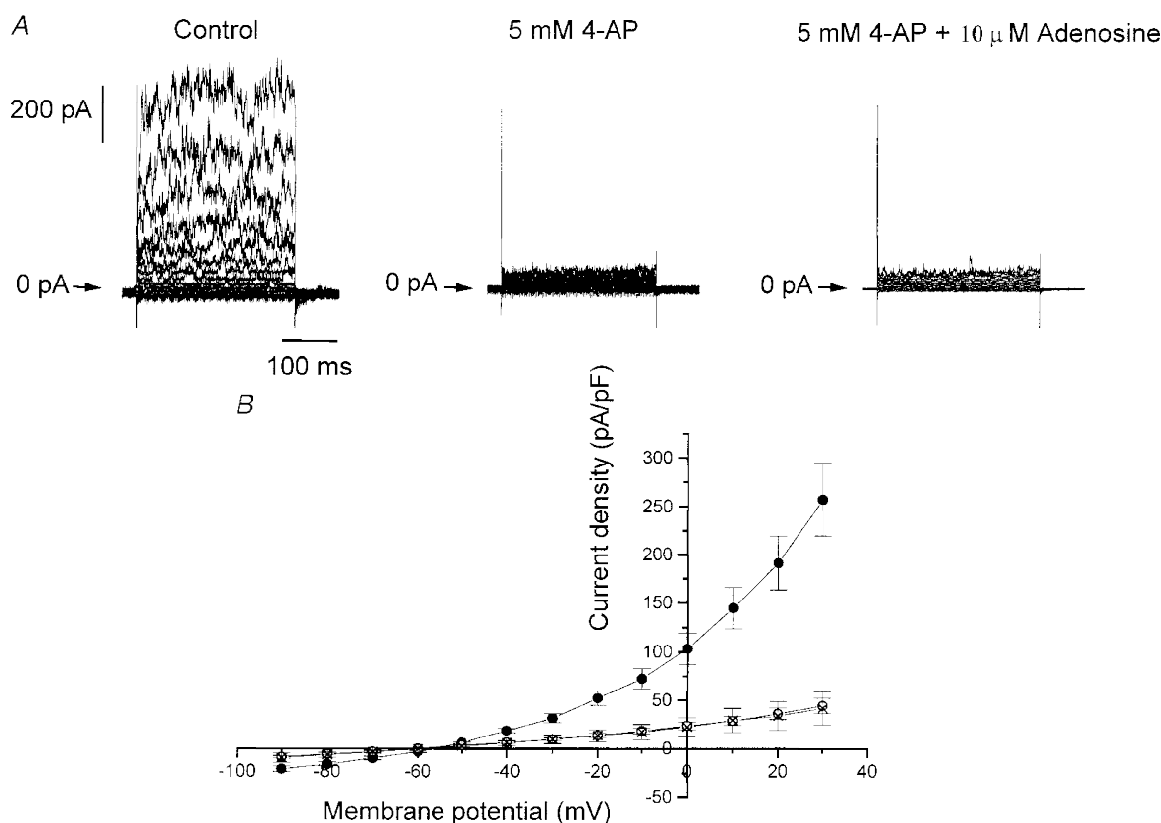


Figure 6. 4-AP decreases outward currents and abolishes the effect of adenosine

A, an example of outward currents obtained during 300 ms pulses to membrane potentials between -90 and $+30$ mV from a holding potential of -70 mV in control conditions, and in the presence of 5 mM 4-AP both in the absence and presence of 10 μM adenosine. B, mean \pm s.e.m. current density–voltage relations obtained in control conditions (\bullet , $n = 10$), in the presence of 5 mM 4-AP (\circ , $n = 10$) and in the last condition but with 10 μM adenosine (\times , $n = 3$).

in isolated type I cells. We have addressed this suggestion more directly by testing the action of adenosine on the resting membrane potential using current clamp. Figure 7A shows an example obtained in one cell in which the resting membrane potential of -55 mV was not modified by $10 \mu\text{M}$ adenosine applied for 2 min. In six cells studied, we found the resting membrane potential to be -57.86 ± 1.45 mV which is close to the value found from the current density–voltage relation of Fig. 4B. In the presence of $10 \mu\text{M}$ adenosine, the resting membrane potential was -57.17 ± 4.36 mV, corresponding to a depolarization of just 0.50 ± 0.55 mV. This new resting membrane potential was not significantly different from control conditions ($P > 0.05$, paired *t* test). To test whether these cells could show stimulus-induced depolarization, we tested the effect of external (respiratory) acidosis, a known inducer of depolarization (Buckler & Vaughan-Jones, 1994*b*), on the resting membrane potential. In five cells, during control conditions (P_{CO_2} , 32.0 ± 0.5 mmHg; pH_o , 7.39 ± 0.01) in which the resting membrane potential was -59.20 ± 1.68 mV, external acidosis (P_{CO_2} , 52.0 ± 2.6 mmHg; pH_o , 7.25 ± 0.02) induced a significant depolarization of 4.80 ± 0.66 to -54.40 ± 1.03 mV ($P < 0.002$, paired *t* test).

DISCUSSION

Our results show that rat carotid body chemoafferent discharge is increased by adenosine via a non-vascular mechanism and thus confirm previous reports from which recordings were made from multi-fibre or whole sinus nerve recordings from cat carotid bodies (McQueen & Ribeiro, 1981; Runold *et al.* 1990). As we have demonstrated this action of adenosine occurs at the level of single fibres we can, in addition, discount fibre recruitment as the mechanism underlying these previous reports of chemoexcitation. We cannot compare directly the concentrations of adenosine used in our study with those used in the *in vivo* study of McQueen & Ribeiro (1981) but do note that the concentrations required to cause significant chemoexcitation in our rat *in vitro* preparation do exceed those required in

the cat *in vitro* (Runold *et al.* 1990). At present we have no explanation for this difference which may reflect a genuine species difference or may be related to methodological differences.

The generation of chemoafferent discharge is now widely accepted to result from Ca^{2+} -dependent neurotransmission from the type I cell onto postsynaptically located receptors on the afferent nerve terminals. In accordance with this hypothesis, the excitatory actions of natural stimuli such as hypoxia and hypercapnia are considerably reduced by the absence of external Ca^{2+} from the superfusion/perfusion media of isolated carotid body preparations (Roy *et al.* 1997) or *in situ* preparations (Shirahata & Fitzgerald, 1991). In their series of studies on the cat carotid body, McQueen & Ribeiro (1981, 1983, 1986) suggested that adenosine most probably acted at the presynaptically located type I cell, basing their argument upon evidence from other cell types, but they did not test this more directly. In the present study, we therefore demonstrated that a Ca^{2+} -free superfusate could significantly reduce the chemodischarge induced by adenosine and take this to be more direct evidence of an action of adenosine on type I cells.

The Ca^{2+} dependence of the excitatory effect of adenosine implies an absolute requirement for receptor-mediated influx of Ca^{2+} into type I cells. This concept is supported by our finding that Ni^{2+} could abolish the adenosine-mediated discharge to almost the same degree as Ca^{2+} -free solutions. Part, but not all, of this Ca^{2+} influx appears to be through L-type Ca^{2+} channels as D600 was also able to reduce discharge significantly, suggesting that membrane depolarization is an essential step for the full organ response to adenosine. These data are in close agreement with those reported for intracellular Ca^{2+} measurements made in type I cells in response to anoxic (Buckler & Vaughan-Jones, 1994*a*) or hypercapnic (Buckler & Vaughan-Jones, 1994*b*) stimulation.

We have demonstrated that adenosine can decrease the amplitude of the rat type I cell outward current, at

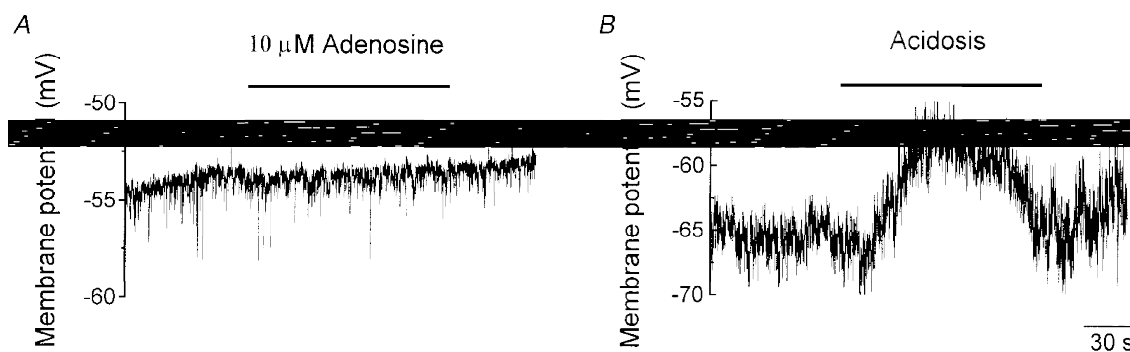


Figure 7. Adenosine has no effect at the resting membrane potential

A, an example of the negligible effect of $10 \mu\text{M}$ adenosine in one cell on the resting membrane potential during current clamp. *B*, an example of the depolarizing effect of acidosis (pH_o 7.30) in one cell on the resting membrane potential during current clamp.

potentials in excess of -40 mV and therefore having an effect similar to hypoxia in these cells (Peers, 1990). To our knowledge, this is the first time that this effect has been described in type I cells and is observable at a concentration known also to affect K^+ currents in smooth muscle, cardiac and PC12 cells (Dart & Standen, 1993; Hiraoka & Furakawa, 1998; Kobayashi *et al.* 1998). In smooth and cardiac muscle, hypoxia/ischaemia and adenosine both increase the amplitude of the K^+ current. In contrast, adenosine does not mimic hypoxia in PC12 cells (Kobayashi *et al.* 1998) as, in these cells, the increase in K^+ current induced by adenosine acts to attenuate the decrease in this current induced by hypoxia (Zhu *et al.* 1996). Thus, adenosine appears protective against hypoxia in PC12 cells, whilst it may mediate, in part, the action of hypoxia in smooth muscle, cardiac and type I cells of the carotid body.

The K^+ current of type I cells contains both Ca^{2+} -sensitive and -insensitive components (Peers & O'Donnell, 1990; Lopez-Lopez *et al.* 1997). As a large part of the effect of adenosine, in the present study, was still observed in the absence of external Ca^{2+} and 4-AP significantly reduced the amplitude of the outward current and prevented any subsequent action of adenosine, this suggests that adenosine acts primarily to decrease the amplitude of a delayed rectifier K^+ current in rat type I cells. A similar Ca^{2+} -insensitive effect has been reported for the action of hypoxia upon isolated type I cells in rabbits (Lopez-Barneo *et al.* 1988) and in PC12 cells (Zhu *et al.* 1996) and a similar preventative action of 4-AP upon adenosine-mediated effects has been described in other neuronal tissue (Scholfield & Steel, 1988). However, a small but significant component of the current blocked by adenosine was Ca^{2+} dependent. We cannot therefore exclude the existence of an additional action of adenosine upon Ca^{2+} -activated K^+ currents as has been described for the action of hypoxia in rat type I cells (Peers, 1990) and/or an adenosine-mediated increase in Ca^{2+} currents. The action of adenosine upon Ca^{2+} currents in the rat type I cell has not been reported, but it has recently been shown that adenosine can decrease the amplitude of L-type Ca^{2+} currents in PC12 cells (Kobayashi *et al.* 1998).

Pharmacological evidence using specific adenosine-receptor agonists suggested that the action of adenosine at the cat carotid body was mediated via activation of A_2 receptors (McQueen & Ribeiro, 1986) and given that *in situ* hybridization has revealed intense A_{2A} mRNA in rat carotid bodies (Weaver, 1993) and low doses of an A_{2A} agonist stimulated ventilation in rats (Sebastiao & Ribeiro, 1996) this appears the most likely receptor subtype expressed in this organ. This receptor is widely accepted as being coupled to stimulatory guanine nucleotide binding (G_s) proteins which stimulate adenylate cyclase in many cell types (Fredholm, 1995; Sebastiao & Ribeiro, 1996) including those of the carotid body (Chen *et al.* 1997). This action on adenylate cyclase activity could explain our observed responses, as elevations in cAMP levels have been shown to decrease the amplitude of 4-AP-sensitive outward currents

in isolated rabbit type I cells (Lopez-Lopez *et al.* 1993). Moreover, the decrease of the outward current by cAMP in type I cells is voltage independent (Lopez-Lopez *et al.* 1993) as we have also observed with the effect of adenosine. The suggestion that this occurred via a phosphorylation of the K^+ channels (Lopez-Lopez *et al.* 1993) was confirmed in PC12 cells in which adenosine A_{2A} receptor activation could activate the cAMP-PKA pathway (Kobayashi *et al.* 1998). As we have shown that the effect of adenosine on type I cells is largely Ca^{2+} independent, as is the hypoxia-induced elevation in cAMP in these cells (Perez-Garcia *et al.* 1990; Wang *et al.* 1991), the simplest explanation for the action of adenosine in type I cells would therefore be via an increase in cAMP. Our data could therefore support the contention of Chen *et al.* (1997) that the elevation of cAMP during hypoxia is mediated by the release of endogenous adenosine. However, in another study, no effects of cAMP or PKA were observed on currents recorded from neonatal rat type I cells (Hatton & Peers, 1996) and these authors argued against a role for cyclic nucleotides in chemotransduction. Whether this reflects differences in species, age, temperature or in the choice of pH buffers remains an issue to be resolved. We cannot, however, exclude an action of adenosine upon the $\beta\delta$ subunit of G_s protein acting to decrease K^+ currents (Peers & Carpenter, 1998) via activated PKC (Fredholm, 1995).

Hypoxia-induced neurosecretion from type I cells is Ca^{2+} -dependent (Gonzalez *et al.* 1994) with the most compelling evidence suggesting that this elevation in intracellular Ca^{2+} occurs via influx through L-type Ca^{2+} channels (Buckler & Vaughan-Jones, 1994a). This implies cell depolarization as a prerequisite for hypoxic chemotransduction which necessitates a component of the current contributing to the resting membrane potential to be hypoxia sensitive. Of the three types of K^+ currents inhibited by hypoxia that have been described (Ganforina & Lopez-Barneo, 1992; Wyatt & Peers, 1995; Buckler, 1997), a voltage-, Ca^{2+} - and 4-AP-insensitive background (leak) current (Buckler, 1997) appears the most likely to mediate cell depolarization as it is activated at resting membrane potentials. Additionally, pharmacological block of the other channel types does not increase chemoreceptor discharge (Doyle & Donnelly, 1994; Pepper *et al.* 1995). Not surprisingly therefore, our results showed that adenosine had no effect on the current near the resting membrane potential and was not able to depolarize isolated type I cells. The observation that a slight external acidosis of around 0.15 pH units could induce depolarization, as previously described (Buckler & Vaughan-Jones, 1994b), demonstrates that any small effect of adenosine would have been observed if it were present.

How then can we explain the excitatory effect of adenosine observed in the whole carotid body when we have no evidence for membrane depolarization in isolated type I cells? It is possible that the resting membrane potential of cells in the whole organ is held more depolarized than that of isolated cells and therefore at potentials at which a 4-AP-sensitive current is activated. Qualitative differences in the

electrophysiological responses to natural stimuli between single cells and small clusters of cells have been reported (Pang & Eyzaguirre, 1992) which may arise from inter-cell chemical and/or electrical synaptic contact between type I cells (McDonald & Mitchell, 1975). The membrane resistance of an individual cell could thus be affected by its neighbours either directly through gap junctions or through the release of neurotransmitters/modulators acting in an autocrine or paracrine fashion. Another possibility is that type I cell depolarization, subsequent to K⁺ channel inhibition, is not a requirement for chemoexcitation. Such a suggestion has previously been made as the membrane resistance of type I cells recorded from within the whole carotid body was not changed by a level of hypoxia which increased chemoafferent discharge and 4-AP was unable to excite chemoafferent discharge from the whole carotid body, even in the presence of hypoxia (Donnelly, 1995). A more recent study (Lahiri *et al.* 1998) has come to a similar conclusion, although a role for the leak K⁺ current could not be excluded due to the lack of specific blockers of this current.

In summary, we have confirmed an excitatory action of adenosine upon carotid body chemoafferent discharge *in vitro* and have demonstrated an inhibitory action of adenosine on a 4-AP-sensitive component of the total outward current in isolated type I cells. The causal link between these two actions remains to be established.

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