Behavior of Junction Channels Between Rat Glomus Cells During Normoxia and Hypoxia

VERÓNICA ABUDARA, R. G. JIANG, AND C. EYZAGUIRRE
1Department of Physiology, University of Utah School of Medicine, Salt Lake City, Utah 84108-1297; and 2Department of Physiology, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay

Received 14 November 2001; accepted in final form 22 March 2002.

Abudara, Verónica, R. G. Jiang, and C. Eyzaguirre. Behavior of junction channels between rat glomus cells during normoxia and hypoxia. J Neurophysiol 88: 639–649, 2002; 10.1152/jn.00939.2001. The activity of gap junction channels between cultured and clustered carotid body glomus cells of the rat was studied with dual voltage clamping during normoxia (P0₂ 300 Torr) and hypoxia induced by carotid body glomus cells of the rat was studied with dual voltage clamping during normoxia (P0₂ 300 Torr) and hypoxia induced by sodium dithionite (Na₂S₂O₄) or 100% N₂. Na₂S₂O₄ reduced the saline P0₂ to ~10 Torr, whereas 100% N₂ reduced ambient O₂ to ~60 Torr. The following observations were made. 1) In normoxia, the intercellular macroconductance (Gj = 3.0 ± 1.01 ns, mean ± SE) was changed unevenly (increased and decreased) under hypoxic conditions by either agent, although N₂ produced the largest changes. 2) The intercellular microconductances of the channels (gg = 104.44 ± 10.16 pS under normoxic conditions) significantly decreased in 100% N₂ but showed depressions and enhancements in Na₂S₂O₄. 3) The conductance of single-junctional channels (SChs), calculated as gg variance/mean gg, yielded a mean of ~17.6 pS. Larger values were obtained with manual measurements of the data (~34 pS). Hypoxic hypoxia (induced by 100% N₂) significantly depressed the conductance of SChs when calculated from digitized records or from manual measurements. Hypoxia induced by Na₂S₂O₄ did not significantly change junctional conductance. 4) The number of intercellular channels, calculated as gg/SCh gg, had a mean of ~452 (range 1 to 2,471). During N₂-induced hypoxia, this number significantly decreased to ~84 but remained unchanged during Na₂S₂O₄ hypoxia. 5) The open mean time of junction channels varied from 4 to 30 ms in different experiments, having an overall mean of µ = 11.33 ± 0.33 ms. This value was significantly reduced by 100% N₂, but was not changed by Na₂S₂O₄. 6) Intracellular calcium ([Ca²⁺]i), 46.2 ± 4.84 nM under normoxia, significantly increased to 77.32 ± 11.27 nM with Na₂S₂O₄ and to 66.39 ± 11.64 nM with 100% N₂. It is concluded that 100% N₂ uncouples glomus cells by significantly reducing intercellular macro- and microconductances. Hypoxia induced by Na₂S₂O₄ had variable effects. The coupling effects of hypoxia may depend on, or be added by, changes in [Ca²⁺]i, and/or intracellular pH changes. However, secreted transmitters and ATP plus the effects of hypoxia on second messengers and other cytoplasmic components may also play an important role in this phenomenon.

INTRODUCTION

The chemoreceptor (glomus) cells of the rat carotid body are electrically coupled because of the presence of gap junctions between them (Kondo and Iwasa 1996; McDonald 1981). During hypoxia or extracellular acidification, most cells (~70%) partially uncouple, whereas the rest show tighter coupling (Abudara and Eyzaguirre 1996b, 1998; Abudara et al. 2001; Monte-Bloch et al. 1993). It has been suggested that coupling and uncoupling are integral processes in the secretion of transmitters by the glomus cells because they regulate intercellular exchanges (Eyzaguirre and Abudara 1999).

In this study, we sought to explore the behavior of intercellular channels during normoxia and hypoxia induced by the superfusion of cultured and clustered glomus cells with sodium dithionite (Na₂S₂O₄) or saline equilibrated with 100% N₂. Furthermore, we tried to get an insight into possible mechanisms underlying the effects of decreased O₂ on intercellular coupling. Two possibilities are widely accepted as likely factors in triggering the effects of hypoxia on cells. One is intracellular acidification, and the other is an increase in intracellular Ca²⁺ concentration ([Ca²⁺]i). In cultured and clustered glomus cells, hypoxia induced by either Na₂S₂O₄ or 100% N₂ reduces intracellular pH (pHi) in ~60% of the cells, whereas it increases pHi in the others (He et al. 1991b; Pang and Eyzaguirre 1993a). Concerning [Ca²⁺]i, this ion also increases during hypoxia (Buckler and Vaughan-Jones 1994; Piotruschka 1985; Sato et al. 1991; Zhang and Eyzaguirre 1999) and this effect depends on the severity of the hypoxia (Dasso et al. 2000). Also, the coupling between glomus cells is sensitive to the levels of extracellular Ca²⁺ ([Ca²⁺]o), decreasing during superfusion with high [Ca²⁺]o, and tightening when [Ca²⁺]o is removed (Abudara and Eyzaguirre 1996a). Because [Ca²⁺]i follows the changes in [Ca²⁺]o, we needed to establish whether there was a correlation between hypoxia and changes in [Ca²⁺]i.

In the experiments described below, we established that 100% N₂-induced hypoxia was more effective than Na₂S₂O₄-induced hypoxia in uncoupling or coupling glomus cells. However, both agents had similar effects on [Ca²⁺]i. Consequently, hypoxic uncoupling may be influenced by factors other than those produced by pHi and/or [Ca²⁺]i, changes, as presented in DISCUSSION.

METHODS

The methods used have been described in detail in a recent publication (Abudara et al. 2001). For the convenience of the readers, the salient points are presented here. Two procedures were employed: 1)
voltage clamping of two adjoining glomus cells and 2) \([Ca^{2+}]_i\) measurements.

**Voltage clamping**

Cultures of glomus cell clusters were prepared as described previously (Abudara and Eyzaguirre 1998; Abudara et al. 2001). Briefly, carotid bodies were removed from Wistar rats (40–60 g) anesthetized with 50 mg/kg (ip) pentobarbital sodium. The organs were thoroughly rinsed with sterile Hank’s balanced salt solution and then immersed in serum-free growth medium for mechanical dissociation. After the cells settled at the bottom of collagen-coated Petri dishes, the cultures were incubated in a humid atmosphere (5% CO2-95% air at 37°C) for 1–7 days. The culture medium was a mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 (Sigma, St. Louis, MO), supplemented with 0.014 M NaHCO₃, 80 U/l of insulin, and 1% penicillin-streptomycin-fungizone ( Gibco). The pH was adjusted to 7.4 with HEPES-NaOH. dB-cAMP (1–3 mM) was added to the cultures to facilitate the formation of intercellular junctions (Chanson et al. 1996; Kessler et al. 1985; Matesic et al. 1996) as has been done in previous work (Abudara and Eyzaguirre 1998; Abudara et al. 1999, 2000).

The cells were transferred to a 1-ml chamber mounted on the stage of an inverted phase-contrast microscope. The preparation was superfused at room temperature with Ham’s F-12 equilibrated with 100% O₂ (pH 7.4), flowing at 1 ml/min. Po₂ in the bath was ~300 Torr. Two microelectrodes (filled with 3 M KCl, 10–20 MΩ), were mounted on the same micromanipulator and positioned on the tissue at ×450 magnification. The intracellular microelectrodes were independently connected to the input stages of feedback amplifiers (WPI S-7050A, Patch Clamp Systems) for current or voltage clamping. The output of each amplifier was low-pass filtered at 100 Hz to reduce noise. Hypoxia was produced by superfusing the preparations with 1 mM Na₂S₂O₄ or by equilibrating the superfusate with 100% N₂. NaOH was used to adjust the extracellular pH to 7.43 when superfusing with Na₂S₂O₄. pH and Po₂ were monitored in the bath with small specific electrodes. To monitor macrojunctional conductance (Gj) between glomus cells, both cells were voltage clamped at a value intermediate between their respective resting potentials, and command pulses were applied to one cell, inducing a voltage drop (ΔVj) across the intercellular junction. The current produced in the coupled cell was equal in amplitude to the junctional current (Ij); thus Gj = Ij/ΔVj (Abudara and Eyzaguirre 1998). To study channel conductances, a constant (DC) ΔVj was used, thus clamping each cell at a different level. The activity of intercellular channels (gating) appeared as simultaneous current step changes (i₁ and i₂) of opposite polarity (mirror images). Thus, intercellular channel activity could be distinguished from nonjunctional noise, which had the same polarity in both recordings. To calculate intercellular channel conductance (gj), we used one-half of the difference in activity of both coupled cells; thus gj = (Δi₁-Δi₂)/2 × ΔVj⁻¹. This method takes into account outward and inward currents and eliminates or sharply reduces nonjunctional noise, which can alter results if one uses only currents entering one of the coupled cells.

Two methods were employed to estimate the conductance of single channels. 1) The gj variance (pS²) was divided by the mean gj (Nicholls et al. 1992), and 2) current transitions from one level to the next (without obvious intermediate steps) were measured by hand from the Scope records. Channel conductance (gjd) was calculated by dividing the amplitudes of current steps (pA) by ΔVi (mV).

The mean open time of single-junction channels was calculated with noise (fluctuation) analysis. The variances of the conductances (σ² in pS²) of each experiment were transferred to the Scope program, plotting variance versus time. This program permitted calculation of the mean variance of all sweeps in the time domain. A cosine tapered fast Fourier transform gave the power density of these values (pS² · s) as a function of frequency (Hz). A log/log plot was constructed with a Cricket Graph program to obtain an exponential fit of the data. The corner frequency (fₘ), taken as one-half of the low-frequency asymptote, permitted the conversion of Hz to ms as τ = 1/[2πfₘ] (Anderson and Stevens 1973; Gold and Martin 1983; Hille 1992; Nicholls et al. 1992).

Voltage and current recordings were simultaneously stored on a Vetter videotape system and on-line in a Macintosh computer through a Mac Lab4 interface. Data were acquired by the Scope v. 3.2.8 program. Once stored in the computer, the information was retrieved in digital form and transferred to Statview spread sheets for quantification and analyses.

**Measurement of f[Ca²⁺]i**

Carotid bodies were removed and immersed in an ice-cold solution containing (in mM) 98 NaCl, 47 Na-glutamate, 4.6 KCl, 3 CaCl₂, 1.1 MgCl₂, 7 glucose, and 5 HEPES, pH 7.4. The organs were then placed in Ca²⁺- and Mg²⁺-free Hanks’ medium (Sigma) at room temperature for 30 min before they were moved to a Ca²⁺- and Mg²⁺-free Hanks’ solution containing 0.3% collagenase Type II (Sigma) for 40 min at 37°C. After being washed in Ham’s F-12, the carotid bodies were gently dissected in Ham’s F12 nutrient medium, which also contained 80 μl of insulin (Sigma), 10% bovine serum, and 1% antibiotic-antimycotic (Gibco) at pH 7.43. The cells were plated onto poly-L-lysine-coated glass coverslips and kept in an incubator at 37°C for 1–2 h for adhesion to the glass surface.

The cells were loaded with 1 μM Fura-2 AM and 0.02% pluronic F-127 (Molecular Probes, Eugene, OR) for 10–20 min at 37°C. The preparation was mounted in a superfusion chamber (100 °C, Kessler et al. 1985; Matesic et al. 1996) as has been done in previous work (Abudara and Eyzaguirre 1998; Abudara et al. 1999, 2000). The cells were superfused through intermittent 334- and 380-nm excitation and a 520-nm emission filter. Ratios were converted to [Ca²⁺]i by a two-point-calibration formula

\[ [Ca^{2+}]_i = \frac{K_d[(R - R_{Lo})/(R_{Hi} - R)]}{D_{Lo}} \]

where R is the measured fluorescence ratio Kd is the dissociation constant of the dye-Ca²⁺ complex, RLo is the fluorescence ratio given by low-Ca²⁺ solutions, RHi is the ratio obtained with high-Ca²⁺ standards, DenLo is the denominator intensity for low Ca²⁺, and DenHi the denominator intensity for high Ca²⁺. Calibrations were done with standard Ca²⁺ solutions as reported by Brown and Owen (1979). Results were stored in the computer and analyzed after transfer to Statview spread sheets and to the MacLab Scope program.

**RESULTS**

We studied 28 electrically coupled cell pairs with different degrees of coupling between them. The membrane potential (Em) of the cells varied from −79 to −14 mV [mean −28 ± 0.8 mV (SE)], with a mean input resistance 181.6 ± 18.74 MΩ. These values were lower than those previously obtained from single impalements in cultures (−34.7 ± 0.53 mV; n = 101) (He et al. 1991a,b) and those calculated by Fieber and McCleskey (1993) with noninvasive methods (approximately −32 mV). However, our impalements (even producing low Em) were characterized by sharp and stable negative shifts of

**J Neurophysiol • VOL. 88 • AUGUST 2002 • www.jn.org**
the baseline. In any case, and concerning the results presented below, there was no correlation between the \( E_m \) of the cells and intercellular coupling within 40 mV from the resting potential (Abudara and Eyzaguirre 1998) as is generally the case (Spray and Bennett 1985).

Macroconduction and channel activity during normoxia

The junctional macroconduction \( (G_j) \) varied from 0.04 to 24.36 ns, with a mean (±SE) of 3.0 ± 1.01 ns (see also Abudara et al. 2001). There was multiple-channel activity in our recordings because, as shown below, there are from very few to >1,000 junction channels between glomus cells. Channel activity (flickering) was seen only when a mean trans junctional voltage \( (\Delta V_j) \) of 100.1 ± 10.9 mV (range 40–190 mV) was applied (Abudara et al. 2001).

Figure 1 shows six double traces of a long recording of two coupled glomus cells (traces 1 and 2). Cell 1 was voltage clamped at −30 mV (its resting potential), whereas cell 2 was held at −180 mV, creating a trans junctional voltage of 150 mV. Intercellular activity was voltage dependent, but the threshold was high. Channel flickering appeared only during a DC \( \Delta V_j \) of from 40 to 190 mV, with no signs of channel desensitization or rectification for seconds or minutes. Intercellular channel currents appeared as deflections of similar amplitude and opposite polarity (mirror images) in both recordings. Cell 2 showed inward currents, whereas outward currents were recorded in cell 1. Thus when the traces separate, the channels opened. The largest and slowest deflections had superimposed multiple flickering, probably representing partial channel closing. The durations of the openings varied from <1 s to several seconds. These multiple channel recordings are similar to those obtained in coupled heart myocytes (Veenstra and DeHaan 1988).

In 17 control experiments, multiple intercellular channel conductances \( (g_j) \) were calculated as \( i/\Delta V_j \). The mean \( g_j \) was 104.44 ± 10.165 pS, and there was a significant \( (P < 0.04; r = 0.513) \) and direct correlation with intercellular macroconductance \( (G_j) \) as shown in Fig. 2A.

Effects of hypoxia on intercellular coupling

\( G_j \) changes. Short superfusions with 1 mM Na2S2O4 decreased \( P_O_2 \) in the bathing saline to ~10 Torr in 200 s (Abudara and Eyzaguirre 1998) and partially uncoupled (decreased \( G_j \) ~65% of glomus cell pairs, whereas \( G_j \) increased in the rest (solid surface in Fig. 2B). A less severe hypoxia (~60 Torr) occurred during superfusion with saline equilibrated with 100% \( N_2 \), and it took a bit longer (250 s) to reach this value (Abudara and Eyzaguirre 1998). Coupling between glomus
cells followed a similar pattern because the same proportion of cells uncoupled (65%) or coupling tightened (25%). However, both effects were more marked under 100% N2 (dotted surface in Fig. 2B (Abudara and Eyzaguirre 1996b).

**Intercellular channel activity during Na₂S₂O₄**

Figure 3 illustrates two different experiments in which this reducing agent induced opposite effects on coupling between glomus cells. The mean $g_j$ values (obtained from 6.4-s recordings) were plotted against the recording time. The depressant effect of Na₂S₂O₄ on intercellular coupling and the increased coupling produced by this agent are shown in Fig. 3, A and B, respectively.

Figure 4 shows an example of the depressant effect of 1 mM Na₂S₂O₄ on total intercellular channel activity. Cell 1 was voltage clamped at $-6$ mV ($E_{m1} = -24$ mV), whereas cell 2 ($E_{m2} = -27$ mV) was held at $-99$ mV, producing a $\Delta V_j$ of 105 mV. Thus current flowed from cell 1 to cell 2. A 38.4-s recording, split into six double traces, during the control period is shown in Fig. 4A. When the channels are shown open, the traces are separated. Figure 4B depicts channel activity during superfusion with 1 mM Na₂S₂O₄. Inspection of the left and right traces clearly shows that channel activities, seen in the control situation were blunted by Na₂S₂O₄. The control and Na₂S₂O₄ were measured from a baseline (lowest values) after digitizing all traces, and $g_j$ were calculated as $i_j/\Delta V_j$. As seen in the illustration, the mean $g_j$ in the controls ($102.6 \pm 4.6$ pS) significantly ($P < 0.035$ by Kolmogorov-Smirnov test) decreased to $65.9 \pm 4.67$ pS.

The channel conductances obtained in this experiment were grouped in amplitude histograms at 5-pS intervals, giving counts and percentage of occurrence for each interval. These analyses are shown in Fig. 4C. The thin line represents the distribution of conductances (in %) under normoxic conditions, and the thick trace is the conductance distribution during the severe hypoxia induced by 1 mM Na₂S₂O₄. It should be noted

**FIG. 3.** Different effects of hypoxia induced by 1 mM Na-DTN on 2 coupled pairs in the time domain. Intercellular channel conductance decreased (A) but increased in (B). Each point represents mean $g_j$ obtained during a 6.4-s interval.

**FIG. 4.** Example of the depressant effect of 1 mM Na-DTN on intercellular channel conductance. A: 6 double traces of conductances during control period. B: traces recorded during the effect of the reducing agent. Mean conductance went from $102.6 \pm 4.6$ pS in controls to $65.9 \pm 4.67$ pS during hypoxia. A and B, bottom, values for resting and holding potentials and $\Delta V_j$. C: same experiment shows an amplitude distribution histogram (% occurrence) of control conductances (thin line) and conductances obtained during hypoxia (thick line). Note that hypoxia shifted the distribution curve to the left. D: difference of histograms presented in C ($\Delta$% occurrence), showing a marked increase in smaller conductances and disappearance of larger ones.
that the peak of the distribution in the controls (~100 pS) shifted to the left (~60 pS) because the larger conductances decreased, and there was predominance of the smaller conductances. This is better seen in Fig. 4D, which is a differential histogram of these measurements showing the shift in the proportions of conductances before and after Na₂S₂O₄. Further evidence that the cells uncoupled was established by the fact that Gᵢ decreased from 2.3 to 1.4 nS.

The opposite effect, that is, increased intercellular channel activity induced by Na₂S₂O₄, is illustrated in Fig. 5. Figure 5A shows two superimposed 6.5-s traces in which currents have been converted to conductances in the control (bottom trace) and during superfusion with Na₂S₂O₄ (top trace). The intercellular channel conductance practically doubled, increasing from ~75 to 130 pS. The statistical significance between the two traces was high (P < 0.0002 by Kolmogorov-Smirnov test). Figure 5B presents histograms of conductance distribution (%) during normoxia (thin line) and during Na₂S₂O₄ superfusion (thick continuous trace). Differences in occurrence are shown by the broken thick line. The smaller conductances (round a peak of 50 pS) decreased and eventually disappeared during Na₂S₂O₄ hypoxia. Also, this reducing agent induced the appearance of larger conductances that were absent during normoxia. In this experiment, Gᵢ increased from 0.41 to 0.57 nS, denoting increased coupling.

Figure 6A is a composite histogram, grouping results from all experiments on the effects of Na₂S₂O₄ on intercellular conductance. There were no significant differences (Wilcoxon signed-rank test) between the controls and during Na₂S₂O₄ hypoxia for the simple reason that conductance decreased in some experiments but increased in others. This variability is well illustrated in Fig. 6B, which is the differential histogram of results presented in Fig. 6A.

Effects of 100% N₂ on intercellular junctions

When measuring intercellular macroconductances, it became clear that 100% N₂ was more effective than Na₂S₂O₄ in depressing or enhancing intercellular coupling (Fig. 2B). The conductance of intercellular channels was studied in six experiments where, in four cases, this parameter was depressed by 100% N₂. The areas in Fig. 7, A and B, show the results obtained from all six experiments. The mean conductances from each sweep (1,280 points) were plotted against the sweep number in the controls (A) and during hypoxic hypoxia (B). The amplitude distribution histograms of the conductances (percentage) are presented in Fig. 7C. The thin line is the gᵢ distribution under normoxic conditions, and the thick trace presents changes in this parameter during superfusion with 100% N₂. The distribution curve significantly (P < 0.002 by U-test and P < 0.007 by Kolmogorov-Smirnov test) shifted to the left, because the larger conductances decreased drastically, and there were more numerous smaller conductances. Figure 7D is the differential histogram depicting this effect: the increase in smaller conductances and decrease of larger ones.

Single-junction channel (gᵢ) in normoxia and hypoxia

As indicated in Methods, single intercellular channel gᵢ was calculated as gᵢ variance/mean gᵢ from digitized recordings.
(Nicholls et al. 1992) and by manual measurements in the controls and during hypoxia induced by Na$_2$S$_2$O$_4$ or 100% N$_2$. Different conductance values were obtained from digitized records and from manual measurements, as is the case when membrane channels are measured by fluctuation analysis and from patch recordings (Fenwick et al. 1983) (see also DISCUSSION). Nevertheless, in spite of these differences, the significance of the effects induced by Na$_2$S$_2$O$_4$ and 100% N$_2$ were similar.

For digitized measurements of the effects of hypoxia produced by Na$_2$S$_2$O$_4$, we used 145 control sweeps (1,280 points/sweep) and 190 sweeps during Na$_2$S$_2$O$_4$ superfusion. The mean $g_j$ control value (17.6 ± 0.94 pS) did not change significantly during superfusion with Na$_2$S$_2$O$_4$ ($g_j$ = 16.6 ± 0.78 pS). There were increases and decreases in conductance. For 100% N$_2$ studies, we computed 82 control sweeps and 55 sweeps during superfusion with 100% N$_2$. In this case, the control $g_j$ was similar (17.7 ± 1.68 pS), but N$_2$-induced hypoxia significantly decreased it to 10.34 ± 0.87 pS. Manual measurements made in physiological solutions before ($n$ = 422) and during ($n$ = 199) superfusion with 1 mM Na$_2$S$_2$O$_4$ gave a control $g_j$ of 38.9 ± 0.96 pS that was not changed significantly by the reducing agent ($g_j$ = 41.6 ± 1.29 pS). However, as in the case of digitized measurements, 100% N$_2$ significantly decreased single channel $g_j$, which went from a control value of 32.5 ± 1.29 pS ($n$ = 283) to 20.6 ± 1.39 pS ($n$ = 109). These results are presented in graphic form in Fig. 8.

**Mean open time of intercellular channels**

Noise analysis (see METHODS) was used to calculate the mean open time of junction channels. In a previous publication (Abudara et al. 2001), we reported that saline acidification to pH 6.3 did not change the open time of channels between glomus cells. Similar results were obtained in this study with Na$_2$S$_2$O$_4$ but not with 100% N$_2$. During normoxia, the mean open time measured in 17 junctions varied from 3.98 to 30 ms (mean 11.34 ± 1.34 ms). In 12 of these junctions, the preparation during hypoxia induced by Na$_2$S$_2$O$_4$. The mean was 57.6 ± 55.8 (range 6–3.292). During 100% N$_2$, there was a significant ($P < 0.001$, same test) decrease in the number of intercellular channels (83.6 ± 8.9 (range 4–262). These effects are illustrated in Fig. 9, as shown by the open bars.

**Number of intercellular channels under normoxic and hypoxic conditions**

In 17 experiments, the number of channels ($n$) was calculated as $G$/single channel $g_j$, following Hille’s (1992) suggestion for membrane currents. We estimated that in control solutions the mean was 452.2 ± 38 channels (range 1–2,471). This number did not significantly change ($P < 0.12$ by U-test) during hypoxia induced by Na$_2$S$_2$O$_4$. The mean was 575.6 ± 55.8 (range 6–3.292). During 100% N$_2$, there was a significant ($P < 0.014$ in both measurements).
rations were superfused with 1 mM Na$_2$S$_2$O$_4$, and the mean open time did not change because this parameter decreased in five instances, increased in another five, and there was no change in two. In other experiments, three preparations were superfused with saline equilibrated with 100% N$_2$. In all cases the mean open time decreased.

Figure 10 illustrates these experiments. Figure 10A shows a log/log plot of the $g_j$ variance versus Hz obtained from 12 coupled cell pairs. The exponential curves obtained during normoxia and during Na$_2$S$_2$O$_4$ hypoxia yielded mean open times of 11.9 ± 1.88 ms in the controls and 11.8 ± 1.83 ms during hypoxia. Figure 10B describes the mean open time, in milliseconds, obtained in each of the experiments in the controls and during superfusion with Na$_2$S$_2$O$_4$, showing the variability of the results. Figure 10C is another log/log plot of the $g_j$ variance versus Hz obtained from three coupled cell pairs during normoxia and during superfusion with 100% N$_2$. The mean open time during normoxia was 10.9 ± 0.54 ms; that was reduced to 9.5 ± 0.55 during hypoxic hypoxia. Figure 10D shows differences in the variances presented in Fig. 10C. In practically all cases, these values were smaller during hypoxia, resulting in a significant difference between control and hypoxic variance values ($P < 0.007$ by Wilcoxon signed-rank test).

Effects of hypoxia on [Ca$^{2+}$]$_i$,

([Ca$^{2+}$]$_i$) was measured in 48 cells from 18 experiments, resulting in a mean [Ca$^{2+}$]$_i$ of 46.2 ± 4.84 nM. During superfusion with 1 mM Na$_2$S$_2$O$_4$ and 100% N$_2$, the values were 77.32 ± 11.27 ($P < 0.001$ by Wilcoxon test) and 66.4 ± 11.64 nM ($P < 0.005$, same test), respectively. With both stimuli, ~60% of the cells showed an increase in [Ca$^{2+}$]$_i$, whereas the rest showed either little change or a decrease in [Ca$^{2+}$]$_i$. In most, but not all, cases nifedipine (10 µM) blocked or depressed the [Ca$^{2+}$]$_i$ changes induced by Na$_2$S$_2$O$_4$ or 100% N$_2$, suggesting that at least some of their effects were mediated
through voltage-gated L-type channels (R. G. Jiang and C. Eyzaguirre, unpublished observations). Thus we found similar effects of Na₂S₂O₄ and 100% N₂ on [Ca²⁺]ᵢ, in spite of the fact that Na₂S₂O₄ decreased Po₂ to much lower values. Dasso et al. (2000) found graded responses of [Ca²⁺]ᵢ to graded changes in Po₂. However, we did not conduct dose-response experiments with the same hypoxic stimulus. Therefore, future experiments with graded stimuli, using Na₂S₂O₄ or 100% N₂, may reveal similar or different properties (see DISCUSSION).

Figure 11, A and B, illustrates examples of two cells stimulated with Na₂S₂O₄ and 100% N₂. Figure 11C shows the mean [Ca²⁺]ᵢ in each of the controls, giving an overall mean (µ) of 46.2 ± 4.84 nM. Figure 11D presents a percentile distribution of ratios-test (hypoxia) values over control measurements. The effects of hypoxia induced by Na₂S₂O₄ and 100% N₂ were not statistically different. However, the curves show larger effects of Na₂S₂O₄ above the 60th percentile level, suggesting that this agent may be more effective than 100% N₂ in increasing [Ca²⁺]ᵢ.

DISCUSSION

It is important to discuss or justify the choice of techniques in this study. Intracellular microelectrodes were used instead of the more commonly employed patch-type pipettes. In intercellular junctions, the intracellular medium is important in regulation, and it could be argued that microelectrodes disturb this medium much less than do patch pipettes. Although we know the ionic composition of the cytosol in glomus cells (He et al. 1991a; Oyama et al. 1986a,b; Pang and Eyzaguirre 1993b; Zhang and Eyzaguirre 1999; Zhang et al. 1995), we only have limited qualitative knowledge concerning the second messengers, proteins, or peptides that are bound to influence the intercellular junctions (see Pérez-García and González 1997; Zapata 1997). Also, we used fluctuation (noise) analysis to establish the properties of single-junction channels, realizing it is an indirect method. However, we chose to use this analysis because it does not rely on artificial procedures to reduce the number of channels (e.g., long-chain alcohols or other junction blockers). However, because there is a discrepancy in cell membrane studies when channels are indirectly measured by fluctuation analyses and, directly, from membrane patches (Fenwick et al. 1982), we also measured channel currents and conductances by hand. As in a previous study (Abudara et al. 2001), manual measurement of single intercellular channels gave larger values than those detected by fluctuation analysis. In our case, manual measurements were likely biased toward larger values because it was difficult at times to be certain whether small deflections had partners of equal amplitude and opposite polarities. When there was uncertainty, those deflections were not considered in the analyses. Nevertheless, computerized and manual measurements gave similar results, showing that 100% N₂ was more effective than Na₂S₂O₄ in depressing intercellular conductances.

Hypoxic hypoxia (induced by 100% N₂) was considerably more effective than hypoxia elicited by Na₂S₂O₄ in affecting the junction channels between glomus cells. This happened in spite (or because) of the fact that 100% N₂ decreased saline Po₂ much less than Na₂S₂O₄. The gₑ changed more drastically during N₂-induced hypoxia than during Na₂S₂O₄ hypoxia (see also Abudara and Eyzaguirre 1998). Also, 100% N₂ significantly depressed intercellular microconductances and reduced the number of active intercellular channels and their mean open time, whereas Na₂S₂O₄ had variable effects. We do not know the reasons for the different effects of these two hypoxic agents. However, it is important to recall that, in the lung, Na₂S₂O₄ produces superoxide anions and hydrogen peroxide (Archer et al. 1995), which may also happen in the carotid
body. Applications of hydrogen peroxide to pairs of glomus cells increases intercellular coupling in >80% of the pairs (L. Monti-Bloch, V. Abudara, and C. Eyzaguirre, unpublished observations). Furthermore, Na$_2$S$_2$O$_4$ evokes Ca$^{2+}$ influx into glomus cells (see above), regardless of PO$_2$ levels (Carpenter et al. 2000), which could contribute to cell uncoupling (Abudara and Eyzaguirre 1996a). Consequently, the uncoupling effects induced by Na$_2$S$_2$O$_4$ hypoxia may have been blunted by release of hydrogen peroxide and enhanced by Ca$^{2+}$ influx. The effects of 100% N$_2$ were clearer, possibly because this agent had a more straightforward uncoupling action (e.g., Ca$^{2+}$ influx), with little or no release of coupling agents. However, concerning possible mechanisms of hypoxic coupling changes, the following information is pertinent.

It is generally agreed that intracellular acidity and increases in [Ca$^{2+}$], contribute to cell uncoupling in many tissues (Francis et al. 1999; Lazrak and Peracchia 1993; Obaid et al. 1983; White et al. 1990). Na$_2$S$_2$O$_4$ and 100% N$_2$ change the pH$_i$ of cultured and clustered glomus cells, inducing intracellular acidification in ~60% of the cases and alkalinization in the others (He et al. 1991b; Pang and Eyzaguirre 1993a). Therefore, it would be tempting to assume that hypoxia acts on intercellular coupling via pH$_i$ changes that uncouple most glomus cells during acidification (Abudara and Eyzaguirre 1998; Monti-Bloch et al. 1993). However, both hypoxia-inducing agents also increase [Ca$^{2+}$$_i$] (which uncouples glomus cells), an action blocked by cobalt (Abudara and Eyzaguirre 1996a, 1998). Therefore, both high [Ca$^{2+}$$_i$] and low pH$_i$ may act in synchrony. It is still puzzling why 100% N$_2$ is more effective than Na$_2$S$_2$O$_4$ in uncoupling glomus cells when it induces weaker pH$_i$ changes and similar increases in [Ca$^{2+}$$_i$]. Therefore, an explanation of mechanisms based only on changes in [Ca$^{2+}$$_i$] and/or pH$_i$ is not satisfactory because hypoxic uncoupling (or increased coupling) may also be influenced by other factors.

The carotid body glomus cells contain a number of chemicals, the concentration of which changes during hypoxia, and some of them are released from the cells. For instance, glomus cells contain ACh, catecholamines [especially dopamine concentration (DA), serotonin(5-HT), enkephalins, prostanlkins, ATP, substance P, and peptides] cholecystokins and atrial natriuretic peptide (ANP), as shown by many authors (see Delpiano and Yung-García 1990; Wang et al. 1989). Most of the substances influence intercellular coupling in other tissues, and they may also participate in the carotid body. For instance, ACh and DA are released from glomus cells during hypoxia, and when exogenously applied, uncouple most glomus cells (Monti-Bloch et al. 1993). This means that the released substances can affect intercellular coupling. In other tissues, exogenous applications of ACh and DA also uncouple cells (He et al. 2000; Piccolino et al. 1984; Randriamampionita et al. 1988).

Administration of dB-cAMP has long-term effects on coupling because it increases the number of gap junctions (Chanson et al. 1996; Romanello et al. 2001; van Rijen et al. 2000), a phenomenon that also occurs in junctions between glomus cells (Abudara et al. 1999, 2000). Also, acute administration of cAMP tightens coupling between glomus cells (Abudara and Eyzaguirre 1998), and this substance increases during hypoxia. Therefore it is possible that during hypoxia, an increase in cellular cAMP would tend to improve coupling between glomus cells. Likewise, a decrease in cGMP, a decoupler in other tissues (Kwak et al. 1995), would also tend to increase intercellular coupling. As a consequence, hypoxia would release two opposing forces on glomus cell coupling, one trying to decouple the cells (ACh and DA) and another having the opposite effect (increased cAMP and decreased cGMP). This may explain the variable effects of hypoxia on glomus cell coupling, depending on which one predominates at a given time.

Concerning the other agents within the glomus cell cytoplasm and their possible role in coupling, the following is pertinent: 1) ATP is contained in the dense-cored granules of glomus cells (also containing DA) and is released by exocytosis during hypoxia. Once released, part of it is converted to adenosine by ectonucleotidases. These purinergic agonists may activate the membranes of adjoining cells because of the presence of purinergic receptors for ATP (P$_1$) and adenosine (P$_2$) (see Zapata 1997). In other tissues, ATP release produces or increases Ca$^{2+}$ waves and increases junction permeability, acting extracellularly (Cotrina et al. 2000; Guthrie et al. 1999; Homolya et al. 2000; Iasakson et al. 2001; Sauer et al. 2000). A similar mechanism may be present in glomus cell junctions, leading to increased coupling during hypoxia. 2) Cyclooxygenases and prostaglandin E$_2$ (PGE$_2$) may also play a role in coupling between glomus cells and their changes during hypoxia, although this has not been studied. This stimulus increases the synthesis of endogenous PGE$_2$, and its exogenous application inhibits catecholamine release from glomus cells during hypoxia and inhibits inward Ca$^{2+}$ currents. In osteocytelike MLO-Y4 cells, PGE$_2$ seems to be essential for intercellular communication across gap junctions (when mechanically activated) by increasing connexin 43 (Cheng et al. 2001). 3) Exogenous applications of the secretagogue cholescytokinin octapeptide (CCK-8) increases chemosensory discharges in the carotid body after a period of depression. In pancreatic acini, CCK-8 induces electrical uncoupling (Ngzahayao and Kolb 1993). However, we still are not certain if changes in chemosensory discharges are related to intercellular coupling in the carotid body. 4) Applications of ANP to the cat and rabbit carotid bodies depress or inhibit the increased sensory discharge elicited by hypoxia. This effect appeared to be elicited by an increase in cGMP because applications of the cell-permeant form of this compound had a similar effect. Interestingly, and related to intercellular coupling, de Mello (1998) found that delivery of ANP (10$^{-8}$ M) to myocytes of cardiomyopathic hamsters decreased g$_j$ by ~48% and applications of dB-cGMP (10$^{-4}$ M) reduced g$_j$ by ~80%. Similar experiments dealing with coupling between glomus cells have not been done. 5) 5-HT has no effects on the carotid body chemosensory discharge in vitro, suggesting that the effects observed in vivo are of vascular origin. In the somatosensory cortex, 5-HT reduces dye coupling (Rorig and Sutor 1996), but in vascular smooth muscle, g$_j$ increases (Moore and Burt 1995). There are no studies on coupling and 5-HT in glomus cells.

The information presented here clearly shows that to understand the mechanisms of hypoxic uncoupling (or increased coupling) between glomus cells, much more work has to be done. It is almost certain that hypoxia starts a cascade of events in glomus cells that would provoke uncoupling in some cases.
and increased coupling in others. Most of these steps are unknown. However, this complex series of events could be in place to ensure sustained activity of the receptor by releasing transmitters from some glomus cells and recharging others to replace the transmitter load (Eyzaguirre and Abudara 1999). This mechanism is needed to provide proper ventilation during prolonged hypoxia, which happens at high altitudes, because the carotid body is the main or only O2 sensor in the body.

We thank Dr. H. M. Brown for reading this manuscript and for his advice and help during the course of this work. John Fisher and B. Evans provided expert technical assistance.

This work was supported by National Institutes of Health Project Grant 07938.

REFERENCES


PIETRUSCHKA F. Calcium influx in cultured carotid body cells is stimulated by acetylcholine and hypoxia. *Brain Res* 347: 140–143, 1985.


