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Effect of hypoxia on the intracellular chloride activity of cultured glomus cells of the rat carotid body

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Clusters of rat carotid body glomus cells were cultured for 1 to 12 days. Preparations were exposed to a control PO_2 of 134.9 ± 1.1 torr (mean ± SE), and an external chloride activity, $a_o(Cl)$, of 105.6 ± 2 mM. Thirty-six cells had a resting potential (E_m) of −25.2 ± 0.9 mV. The intracellular chloride activity, $a_i(Cl)$, was 32.8 ± 1.1 mM, and the calculated chloride equilibrium potential (E_{Cl}) was −30.9 ± 0.9 mV. E_{Cl} was more negative than E_m, indicating that Cl^- ions are not passively distributed. Hypoxia (5.4 ± 0.8 torr), induced by Na-dithionite ($Na_2S_2O_4$) 1.25 mM, elicited cell depolarization, increased $a_i(Cl)$ and a less negative E_{Cl} in about 80% of the cells. Fourteen per cent of the cells showed opposite effects. It is hypothesized that the increased $a_i(Cl)$ occurs because an outward-directed chloride pump is blocked by hypoxia. This effect is aided by depolarization of the cell. Decreased $a_i(Cl)$ may be due to cell hyperpolarization.

INTRODUCTION

In a previous publication we reported that the membrane potential (E_m) and intracellular chloride activity, $a_i(Cl)$, of rabbit glomus cells were influenced by the chloride activity, $a_o(Cl)$, of the bathing medium. When this ion was reduced from mean control values of 119 mM to 59 and then 8 mM, $a_i(Cl)$ fell from a mean of 21 mM to 17 and then 6 mM. These changes were accompanied by cell depolarizations of 6-8 mV (Oyama *et al.*, 1986). The effects of extracellular chloride on the resting potential in rabbits were similar to effects previously measured in cat glomus cells (Baron and Eyzaguirre, 1977). In those studies in cats and rabbits we concluded that the Em of the cells was influenced by chloride and that Cl^- ions were not passively distributed. In fact, the calculated chloride equilibrium potential (E_{Cl}) was more negative than the observed E_m under $a_o(Cl)$ of 119 and 59 mM (Oyama *et al.*, 1986).

It remained unknown whether carotid body stimulants such as hypoxia, hypercapnia or acidity would affect the normal chloride distribution across the cell membrane. This issue appeared important because of the influence of chloride on the glomus cell resting potential. As a beginning we decided to explore the effects of hypoxia on the $a_i(Cl)$ of glomus cells using rats. Some preliminary observations have been reported (Eyzaguirre, 1993).

METHODS

The methods for cell preparation and recording have been published (He *et al.*, 1991a, b, 1993; Pang and Eyzaguirre, 1992). In summary, carotid bodies were removed from 100-150 g anesthetized rats (sodium pentobarbitone 50 mg/kg). The organs were cleaned of surrounding connective tissue in ice-cold, Ca^{2+} and Mg^{2+} -free, Hank's Balanced Salt Solution. Afterwards, some

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were mechanically dissociated after incubation for 30 min (at 36.6° C) in the same solution containing 0.25% trypsin and 1% penicillin. The enzyme was later inactivated with 20% fetal bovine serum. Other carotid bodies were mechanically dissociated without enzymes. After removing debris, the cell suspensions were transferred to poly-L-lysine coated glass coverslips and immersed in enriched Ham's F-12 (10% fetal bovine serum, 80 µl insulin, 0.001 mM sodium selenite, 1 mM $NaHCO_3$, 15.7 mM HEPES, 0.11 mM $CaCl_2$ and 1% penicillin), pH 7.4. After the cell clusters had "settled" on the glass, the dishes were transferred to an incubator and cultured for 1 to 12 days in humidified 95% O_2 + 5% CO_2.

For recording, the cultures were mounted on the stage of a phase-contrast inverted microscope and bathed with a control solution containing (in mM) NaCl, 125; KCl, 5.7; $CaCl_2$, 4.8; $MgCl_2$, 1.3; Na-glutamate, 13; and dextrose, 7.2. pH was adjusted with HEPES-NaOH to 7.32 ± 0.01 (SE). The medium was equilibrated with air (about 21% O_2) –mean barometric pressure, 640 torr– and it flowed at 5-8 ml/min at 30-34° C. A similar test (hypoxic) solution containing sodium dithionite ($Na_2S_2O_4$) 1.25 mM was applied for 2-5 min. This reducing agent lowers the PO_2 of solutions (Lawson *et al.*, 1965) but it does not readily penetrate cell membranes (Burns and Shepard, 1979).

Each glomus cell was impaled with two microelectrodes for simultaneous recordings of the membrane potential (E_m) and the intracellular Cl^- activity, $a_i(Cl)$. The E_m was measured with conventional microelectrodes filled with 3 M KCl, resistance 40-70 MΩ. The $a_i(Cl)$ was calculated from recordings made with glass micropipettes (tip diameters < 1 µm) filled with a chloride ion exchanger (IE 170, WPI Co) and their inner surface made hydrophobic by silanization (Walker, 1980). The electrodes were back-filled with KCl 100 mM. The slope of the Cl^- electrodes (b) was 50-60 mV, measured in KCl solutions ranging from 4 to 200 mM. After the experiments, the slopes were measured again and those values were used for determining $a_i(Cl)$. The ion exchanger was 20x more selective for Cl^- than for HCO_3^-.

The E_m-detecting electrode was connected to an electrometer. The Cl^- electrode, connected to a high input impedance (10^{15} Ω) amplifier, also registered a potential difference across the cell membrane (ΔE). A subtracting amplifier read ($\Delta E - E_m$), the value used to calculate $a_i(Cl)$, as reported by Oyama *et al.* (1986). A common reference electrode (2 MΩ, 3 M KCl–[Ag-AgCl]), was placed downstream and used in conjunction with a virtual ground to compensate for possible solution-induced artifacts. Two small electrodes monitored the bath pH and oxygen tension, and a thermistor probe registered temperature. Data were recorded on FM tape (dc-1,350 Hz) for later analyses.

The activity coefficient of Cl^- was calculated as 74% of the concentration (Robinson and Stokes, 1970) and no effort was made to correct for possible interference of HCO_3^-.

$a_i(Cl)$ was calculated as:

$$a_i(Cl) = a_o(Cl) / 10\ [(\Delta E - E_m) / b]$$

Each cell was penetrated with the E_m and the Cl^- sensitive microelectrodes, operated by independent manipulators. Each one of these instruments had a Huxley-type base for a three-dimensional approach and a Narishige oil drive for final advance toward the cells. The first penetration was made with the E_m electrode immediately followed by impalement with the ion-sensitive probe. Good penetrations by the first electrode resulted in abrupt and steady negative deflections. Proper impalement by the second electrode did not disturb that recording (second trace in Fig 2; also Fig 3 in He *et al.*, 1991a). We proceeded with the experiment only when these two conditions were met.

After the experiments, tissues were fixed in formaldehyde-glutaraldehyde and excited with 400 nm (from a mercury vapor lamp). Fluorescence emission was viewed and photographed after a low band-pass filter set at 536 nm. More than 80% of the cells in the cultures were identified as glomus cells by their greenish fluorescence (He *et al.*, 1991a). Results obtained from fluorescing cells only are described below.

RESULTS

Control conditions

The preparations were exposed to a control PO_2 of 130-138 torr (mean 134.9 ± 1.1 SE). Variations in PO_2 were due to differences in barometric pressure throughout the experiments. The external chloride activity, $a_o(Cl)$ was 105.6 ± 2 mM (range 90-130 mM). This range was the result of preparing the solutions at different times.

Thirty-six cells were examined with the following results: The mean E_m was –25.2 ± 0.9 mV (range –20 to –44 mV), the mean $a_i(Cl)$ was 32.8 ± 1.1 mM (range 14.5-45 mM) and the mean E_{Cl} was –30.9 ± 0.9 mV (range –18.4 to –52.8 mV). These results indicated that the resting potential of rat glomus cells was almost identical to that in the rabbit (–25.9 ± 1.4 mV), whereas the $a_i(Cl)$ in rat glomus cells was significantly higher than in rabbit cells (20.9 ± 1.9 mM). Also, as in the rabbit, the calculated E_{Cl} was significantly more negative than E_m (Oyama *et al.*, 1986) indicating non-passive distribution of chloride.

Figure 1A shows a significant ($p < 0.01$) and positive correlation between $a_i(Cl)$ and $a_o(Cl)$, even within the relatively narrow range of external chloride used in this study. Figure 1B indicates that the chloride equilibrium potential at 32° C,

$$E_{Cl} = 60 \times \log_{10} [a_i(Cl)/a_o(Cl)],$$

was mainly determined by intracellular chloride. There was a significant ($p < 0.001$) and positive correlation between E_{Cl} and $a_i(Cl)$ (half-filled circles on the left), but none between E_{Cl} and $a_o(Cl)$ (open circles on the right). This lack of correlation is probably due to a weaker dependence of E_{Cl} on $a_o(Cl)$ than on $a_i(Cl)$. However, when external chloride is markedly changed, there is a clear effect on E_{Cl} (Oyama *et al.*, 1986). Another interesting feature was that the E_m was directly and significantly ($p < 0.001$) correlated with $a_i(Cl)$, as depicted in Figure 1C. There were no differences between $E_m/a_i(Cl)$ and E_m/E_{Cl}, which is not surprising since E_{Cl} was mainly determined by $a_i(Cl)$.

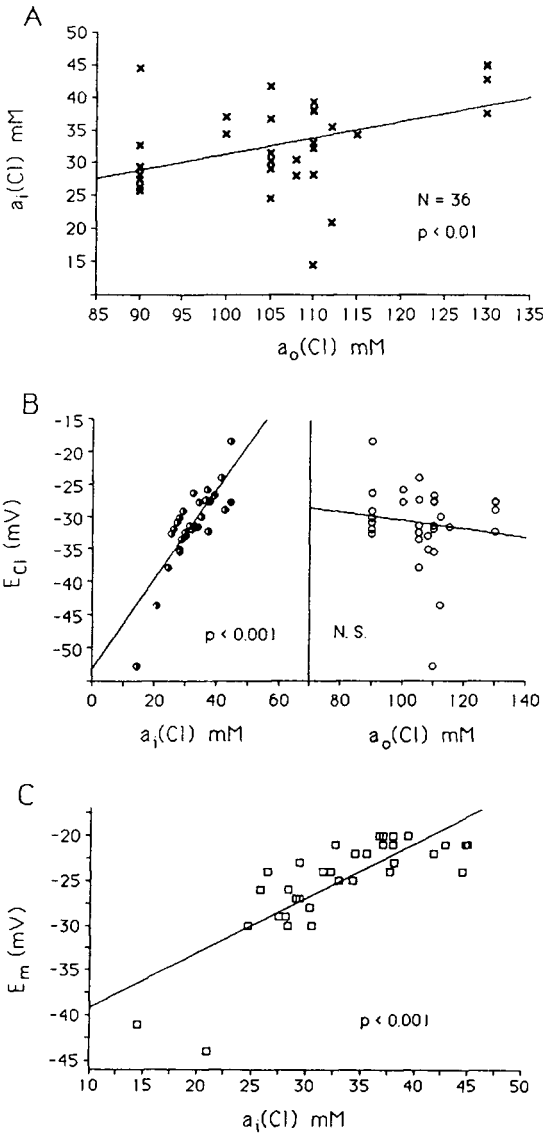

Fig. 1: Correlations of different resting parameters in glomus cells. A, between intracellular, $a_i(Cl)$, and extracellular, $a_o(Cl)$, chloride activities. p, level of significance of regression (F-test). N, number of cells. B, between the Cl^- equilibrium potential (E_{Cl}) and $a_i(Cl)$ (left side) and $a_o(Cl)$ (right side). Note significant regression for $a_i(Cl)$ but not for $a_o(Cl)$. C, between the resting potential (E_m) and $a_i(Cl)$. This regression is highly significant ($p < 0.001$).

Effects of hypoxia

Sodium dithionite ($Na_2S_2O_4$) lowered PO_2 of the bathing solutions to 5.4 ± 0.8 torr (range 1-24 torr). Although this agent does not readily penetrate cell membranes (Burns and Shepard, 1979), we were nevertheless con-

cerned that some of its effects could have been pharmacological. This point was tested in two ways. In one set of experiments, Na-dithionite 1.25 mM was superfused in a solution equilibrated with 100% O_2, but the drug was ineffective on the $a_i(Cl)$ and E_m of glomus cells. In a second group of experiments, dithionite 1.25 mM was applied as usual, in a solution equilibrated with air. During the maximal effect of the drug on $a_i(Cl)$ and E_m, we switched to another solution containing the same amount of dithionite but equilibrated with 100% O_2. The effect of $Na_2S_2O_4$ practically disappeared. Consequently, it is logical to assume that Na-dithionite acted principally by lowering the PO_2 of the medium (also, Pang and Eyzaguirre, 1992). However, the possibility of some pharmacological effects of $Na_2S_2O_4$ cannot be entirely excluded.

Figure 2 shows recordings from one experiment. PO_2 fell from a control level of 137 to 1 torr during the application of dithionite (lower trace). This was accompanied by cell depolarization of 10 mV (middle trace) and by an increased negativity (5 mV) of ΔE (upper trace). Calculations showed that the control $a_i(Cl)$ was 30.9 mM, which increased to 54.4 mM during hypoxia.

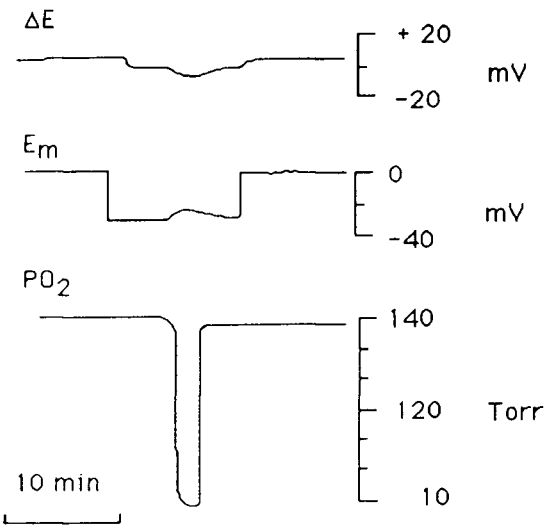

Fig. 2: Effect of $Na_2S_2O_4$ on the potential recorded by the Cl^- electrode (ΔE, upper trace), on the membrane potential (E_m, middle trace) and on PO_2 (lower trace). Time scale, 10 min. $a_i(Cl)$ was calculated after subtracting ($\Delta E - E_m$) and applying the formula described in the text.

The effects of hypoxia on E_m, $a_i(Cl)$ and E_{Cl} for all cells are illustrated in Figure 3A. The lower left columns (E_m) show that glomus cells significantly (asterisk) depolarized during hypoxia. $a_i(Cl)$ (middle upper column) significantly increased together with a decreased negativity in E_{Cl} (lower right column). These changes are more comprehensively seen in Figure 3B. The raw data have been sorted from most negative to most positive and plotted as

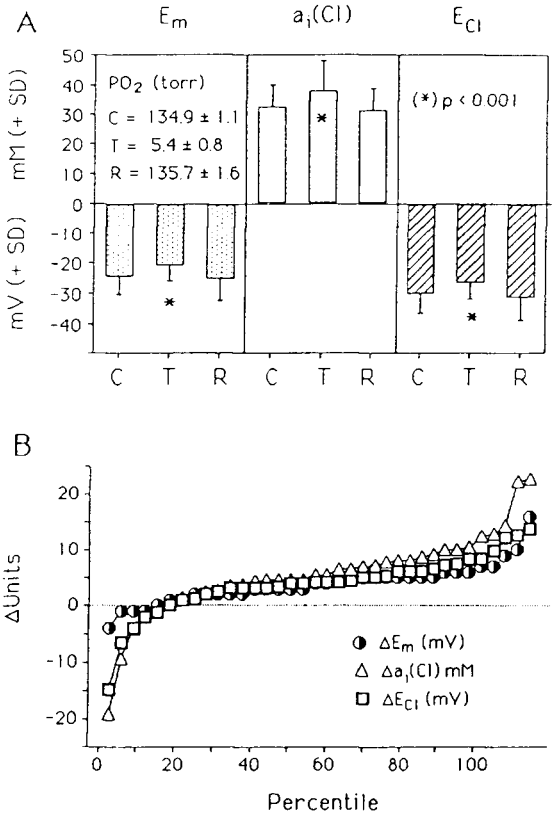

Fig. 3: A, effects of hypoxia on all glomus cells. The upper left block shows PO_2 values in the controls (C), during $Na_2S_2O_4$ (T) and during recovery (R). Lower left block, E_m changes (stippled bars). Upper middle block, $a_i(Cl)$ changes (open bars), and lower right block, effects on E_{Cl} (slanted bars). Ordinates, mean values + SD. Upper ordinate, $a_i(Cl)$ in mM. Lower ordinate, E_m and E_{Cl} in mV. Abscissae, C = controls; T = tests; R = recovery. Asterisks, levels of significance (non-parametric Wilcoxon-signed rank test). B, percentile distribution (from lowest to highest) of changes in resting potential (ΔE_m, half-filled circles), intracellular chloride activity ($\Delta a_i(Cl)$, open triangles) and Cl^- equilibrium potential (ΔE_{Cl}, heavily lined open squares). Note the similarity of distributions, since about 80% of cells depolarized, gained chloride and their E_{Cl} became less negative. About 14% of cells showed opposite effects. Ordinate, units describing ΔE_m, ΔE_{Cl} and $\Delta a_i(Cl)$. Abscissa, percentile distribution.

percentile distributions in line diagrams. About 80% of the cells underwent depolarization, their $a_i(Cl)$ increased and E_{Cl} became less negative. Fourteen per cent showed the opposite: cell hyperpolarization, increased E_{Cl} negativity and a decrease in $a_i(Cl)$.

Figure 4 illustrates the correlation between changes in E_m and in $a_i(Cl)$. There was a significant ($p < 0.001$) and positive correlation between ΔE_m and $\Delta a_i(Cl)$. A very similar correlation between ΔE_m and ΔE_{Cl} was found, since $a_i(Cl)$ and E_{Cl} basically express the same concept (not illustrated).

It is important to notice in Figures 3B and 4, as discussed below, that in five cells $a_i(Cl)$ increased and E_m became more negative during hypoxia.

DISCUSSION

The experiments show that, as in the cat and rabbit, the resting potential of rat glomus cells is associated with chloride ions, which are not passively distributed (Baron and Eyzaguirre, 1977; Oyama *et al.*, 1986). Lack of passive distribution of a given anion across the cell membrane may be a consequence of the activities of pumps or anion exchangers. Present results are compatible with the operation of a pump keeping chloride out of the cells, which would work against a concentration gradient. A hypoxic challenge seems to shut down this mechanism, resulting either in inward rush of Cl^- from the medium or weaker extrusion from the cell. As a consequence of increased $a_i(Cl)$, the Cl^- equilibrium potential shifts in a positive direction. It is possible that chloride movements across the glomus cell membrane occur through large conductance, voltage- and pH-independent Cl^- channels (Stea and Nurse, 1989). However, we have no information about the effects of hypoxia on these channels. They may or may not be affected.

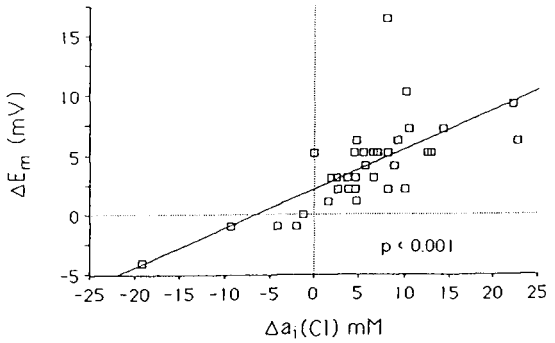

Fig. 4: Same data of Fig 3B. Correlation between changes in membrane potential (ΔE_m) and intracellular chloride activity ($\Delta a_i(Cl)$). The regression is highly significant ($p < 0.001$). Ordinate, ΔE_m (mV). Abscissa, $\Delta a_i(Cl)$ mM. Note that the vast majority of cells depolarized when $\Delta a_i(Cl)$ increased.

In most instances, hypoxia depolarized the cells (interior less negative) and this effect was directly correlated with an increase in $a_i(Cl)$. However, five cells (14%) showed a decrease in $a_i(Cl)$ during hypoxia, which was directly and significantly correlated with cell hyperpolarization (Fig 4). A more positive cell interior would tend to maintain Cl^- inside, or attract it from the outside. An increased internal negativity, on the other hand, could help in removing Cl^- ions against an adverse concentration gradient. Thus, these electric disturbances may work in conjunction with the chloride pump just postulated.

Glomus cells have a Cl^-/HCO_3^- exchange mechanism (Buckler *et al.*, 1991). Consequently, one could expect that an increase in intracellular Cl^- would displace bicarbonate from the cell, although simultaneous measurements of $a_i(Cl)$ and $a_i(HCO_3)$ have not been made. What we know is that the pH_i of many glomus cells decreases during severe hypoxia (Pang and Eyzaguirre, 1993). Part of this effect could be due to the increase in $a_i(Cl)$ possibly displacing bicarbonate. This idea, involving the large conductance Cl^- channels has been proposed by Stea and Nurse (1989). If this is the case, the minority of cells that lose chloride during hypoxia should become more alkaline. Buckler *et al.* (1991) have shown that removal of $[Cl^-]_o$ does induce alkalinity in glomus cells, probably by this mechanism. We have found (He *et al.*, 1991b; Pang and Eyzaguirre, 1993) that hypoxia can increase the pH_i of some clustered cells and we have proposed this mechanism as a possible explanation.

In conclusion, the resting potential of clustered rat glomus cells is closely asso-

ciated with Cl^- ions. The main effect of severe hypoxia was to increase the intracellular chloride activity. It is hypothesized that this phenomenon occurs because an outward oriented chloride pump is shut off by hypoxia. In addition, the concomitant cell depolarization could have contributed to the increased $a_i(Cl)$. In a minority of cells the opposite happened, and in them, loss of chloride, against an adverse concentration gradient, could have been induced by cell hyperpolarization. Gains and losses of intracellular chloride may have been accompanied by opposite changes in intracellular bicarbonate. We do not know, however, if similar findings would be obtained from entirely isolated cells. In isolation, rat glomus cells respond differently to hypoxia. They tend then to hyperpolarize and their pH_i decreases much more consistently that when they are in clusters (Pang and Eyzaguirre, 1992, 1993).

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