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# Hypoxia induces voltage-dependent $Ca^{2+}$ entry and quantal dopamine secretion in carotid body glomus cells

(glomus cells/$O_2$ sensing/cytosolic $Ca^{2+}$)

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ABSTRACT **We have investigated the changes of cytosolic $[Ca^{2+}]$ and the secretory activity in single glomus cells dispersed from rabbit carotid bodies during exposure to solutions with variable $O_2$ tension ($Po_2$). In normoxic conditions ($Po_2$ = 145 mmHg; 1 mmHg = 133 Pa), intracellular $[Ca^{2+}]$ was 58 ± 29 nM, and switching to low $Po_2$ (between 10 and 60 mmHg) led to a reversible increase of $[Ca^{2+}]$ up to 800 nM. The response to hypoxia completely disappeared after removal of external $Ca^{2+}$ or with the addition of 0.2 mM $Cd^{2+}$ to the external solution. These same solutions also abolished both the $Ca^{2+}$ current of the cells and the increase of internal $[Ca^{2+}]$ elicited by high external $K^+$. Elevations of cytosolic $[Ca^{2+}]$ in response to hypoxia or to direct membrane depolarization elicited the release of dopamine, which was detected by amperometric techniques. Dopamine secretion occurred in episodes of spike-like activity that appear to represent the release from single secretory vesicles. From the mean charge of well-resolved secretory events, we estimated the average number of dopamine molecules per vesicle to be ≈140,000, a value about 15 times smaller than a previous estimate in chromaffin granules of adrenomedullary cells. These results directly demonstrate in a single-cell preparation the secretory response of glomus cells to hypoxia. The data indicate that the enhancement of cellular excitability upon exposure to low $Po_2$ results in $Ca^{2+}$ entry through voltage-gated channels, which leads to an increase in intracellular $[Ca^{2+}]$ and exocytotic transmitter release.**

Glomus cells are the primary oxygen-sensitive chemoreceptors of the carotid body. Recent work has shown that these cells, which are of neuroectodermal origin, are electrically excitable (1–3) and that their special chemoreceptive properties are based upon the presence of $O_2$-sensitive $K^+$ channels (2, 4–8). Since reductions in blood $O_2$ tension ($Po_2$) lead to an increase in the excitability of glomus cells, it has been postulated that, as presynaptic-like elements, they release transmitters in response to hypoxia that activate afferent nerve fibers conveying to the brain the necessary information for an adequate hyperventilatory response (5, 9, 10). However, the participation of ion channels in chemotransduction has been questioned by the work of some investigators that either failed to show elevations of intracellular $[Ca^{2+}]$ in response to hypoxia (11) or attributed the increase of cytosolic $Ca^{2+}$ upon exposure to anoxic solutions to release of the cation from mitochondria (12). Here, we directly demonstrate that, as in nerve synapses, stimulus–secretion coupling in isolated glomus cells is mediated by $Ca^{2+}$ entry and the subsequent release of transmitters. We show that in isolated cells low $Po_2$ induces an increase of cytosolic $[Ca^{2+}]$ due to $Ca^{2+}$ influx through voltage-gated channels and that the elevation of intracellular $[Ca^{2+}]$ triggers the exocytotic secretion of dopamine, which can be resolved at the level of release from single vesicles. These experimental findings provide an explanation for the basic cellular mechanisms underlying sensory transduction in the carotid body, a physiological process that has remained obscure for decades.

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## METHODS

Experiments were performed on enzymatically dispersed glomus cells obtained from rabbit carotid bodies (2, 3). Cells were plated on slivers of glass coverslips treated with poly(L-lysine), and during the experiments, a coverslip was placed in a chamber of approximately 0.2 ml with continuous flow of solution. The external solution was equilibrated with air ($Po_2$ ≈ 145 mmHg; 1 mmHg = 133 Pa) or mixtures of $N_2$ and air to obtain the desired $O_2$ concentrations. $Po_2$ in the chamber was monitored with an $O_2$-sensing electrode (9). Macroscopic calcium currents were studied using the whole-cell configuration of the patch-clamp technique (3, 13) and recorded in isolation after blockade of the voltage-dependent $Na^+$ and $K^+$ channels. Cytosolic $[Ca^{2+}]$ was estimated in unclamped cells loaded with fura-2 by incubation for 10 min at 37°C with saline containing 1 μM fura-2 acetoxymethyl ester. Experiments were performed on an inverted microscope with standard optical components and equipped for epifluorescence and photometry (14). For the two excitation wavelengths, we used the filters shortwave-pass SWP 357 (excitation at ≈360 nm) and band-pass BP 380 (excitation at 380 nm; bandwidth, 10 nm). Fluorescence from the cells was measured by a dual wavelength photometer. The two output voltage signals from the photometer were digitized and displayed on-line on the screen of a computer in parallel with the estimated $[Ca^{2+}]$ concentration (15). Calibration of the fluorescence signals in terms of $[Ca^{2+}]$ was performed *in vitro* as described (16). Secretion was monitored in amperometric mode with a glass-sealed 8-μm diameter carbon electrode fabricated as described (17–20). In most experiments, we used an amplifier built in our laboratory that has in the headstage a Burr–Brown OPA 111 wired as a current-to-voltage converter with a feedback resistor of 500 MΩ. The high-resolution recordings (see below) were obtained with a standard List EPC-7 patch-clamp amplifier. The single events of high resolution, similar to those shown in Fig. 2*C*, were recorded with a carbon electrode covered with polyethylene, which, as shown before (19), decreases noise and permits the acquisition of signals at a broader bandwidth. We held the carbon fiber at a constant voltage of +950 mV, a potential more positive than the oxidation potential of dopamine, thus assuring the oxidation of dopamine released by the cells (see below). Cyclic voltammograms were obtained by applying voltage ramps from −600 to +1000 mV (at a rate of 170 V/s) to the carbon-fiber electrode. The signals are characterized by typical reduction (at approximately −400 mV) and oxi-

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dation (at approximately +800 mV) peaks. Amperometric signals were low-pass filtered at 20 Hz and stored on tape. The single events recorded with high resolution were low-pass filtered at 2000 Hz. The composition of solutions and other experimental variables are given in the figure legends. Most of our experiments were performed at 22–25°C. The effect of hypoxia on cytosolic $[Ca^{2+}]$ was tested in two cells at 31 and 36°C, respectively, with the same results.

## RESULTS

Modifications of cytosolic $[Ca^{2+}]$ in response to alterations of $Po_2$ were studied by double-wavelength microfluorimetry in isolated glomus cells loaded with fura-2 acetoxymethyl ester. Under normoxic conditions ($Po_2 \approx 145$ mmHg), resting cytosolic $[Ca^{2+}]$ was 58 ± 29 nM (mean ± SD, $n = 38$) and acute exposure to low $Po_2$ (between 50 and 10 mmHg) elicited a reversible elevation of $[Ca^{2+}]$ of 100–800 nM above the basal level ($n = 19$). The parallel time courses of the two variables ($Po_2$ and intracellular $[Ca^{2+}]$) are shown in Fig. 1*A*, which includes the recording of the $O_2$-measuring electrode placed in the vicinity of the cell. As with the electrophysiological responses of glomus cells to the repeated exposure to hypoxia (2, 9), the changes of $[Ca^{2+}]$ were completely reversible and, in a given cell, highly reproducible (Fig. 1*B*).

Since hypoxia favors action potential firing in rabbit glomus cells (2, 5) and these cells contain a large population of high-voltage-activated $Ca^{2+}$ channels (2–5), the elevation of cytosolic $[Ca^{2+}]$ in response to either membrane depolarization or the hypoxic stimulus should have a similar dependence on extracellular $Ca^{2+}$. Brief exposure to a high $K^+$ (30 30 mM $K^+$) solution (presumably causing a transient membrane depolarization) produced in all cells tested an almost 10-fold reversible increase in cytosolic $[Ca^{2+}]$ (458 ± 274 nM, $n = 14$). The increase of $[Ca^{2+}]$ was abolished when $Ca^{2+}$ was removed from the external solution (Fig. 1*C Left*) or when 0.2 mM $Cd^{2+}$, a well-known powerful $Ca^{2+}$ channel blocker was added (Fig. 1*C Right*). In fair agreement with the fluorescence data, these same solutions also abolished the macroscopic voltage-dependent calcium currents recorded from cells subjected to whole-cell voltage clamp (Fig. 1*D*). We have examined the effect of hypoxia in cells before, during, and after exposure to the 0 $Ca^{2+}$ or 0.2 mM $Cd^{2+}$ external solutions. Without exception, this treatment completely abolished the increase of $Ca^{2+}$ induced by low $Po_2$ (Fig. 1*E*). Brief removal of $Ca^{2+}$ (Fig. 1*E Left*) or addition of 0.2 mM $Cd^{2+}$ to the external solution (Fig. 1*E Right*) did not cause any deleterious action on the cells since the response to hypoxia appeared normal when the standard external solution was reintroduced in the chamber. Similar qualitative results were obtained with nifedipine (0.1 μM), which partially abolished the hypoxia-induced elevations of cytosolic $Ca^{2+}$ (data not shown). These observations strongly suggest that the increase of cytosolic $[Ca^{2+}]$ resulting from the exposure to low $Po_2$ or in response to high $K^+$-induced depolarization is due to $Ca^{2+}$ entry through voltage-gated channels of the membrane. Rabbit glomus cells mostly express high-voltage-activated $Ca^{2+}$ channels (3) but saturating concentrations of dihydropyridine antagonists only block ≈60% of the current (21). Thus dihydropyridine-insensitive channels are also present in these cells. However, the participation of the different $Ca^{2+}$ channel types in the secretory activity of glomus cells is not well studied and must be addressed in future experimental work.

The secretory response of isolated glomus cells to membrane depolarization was demonstrated with a carbon electrode placed adjacent to cells subjected to whole-cell patch-clamp recording. When the carbon fiber was polarized to +950 mV, we were able to detect, in amperometric mode (18–20), minute amounts of dopamine, which is the major

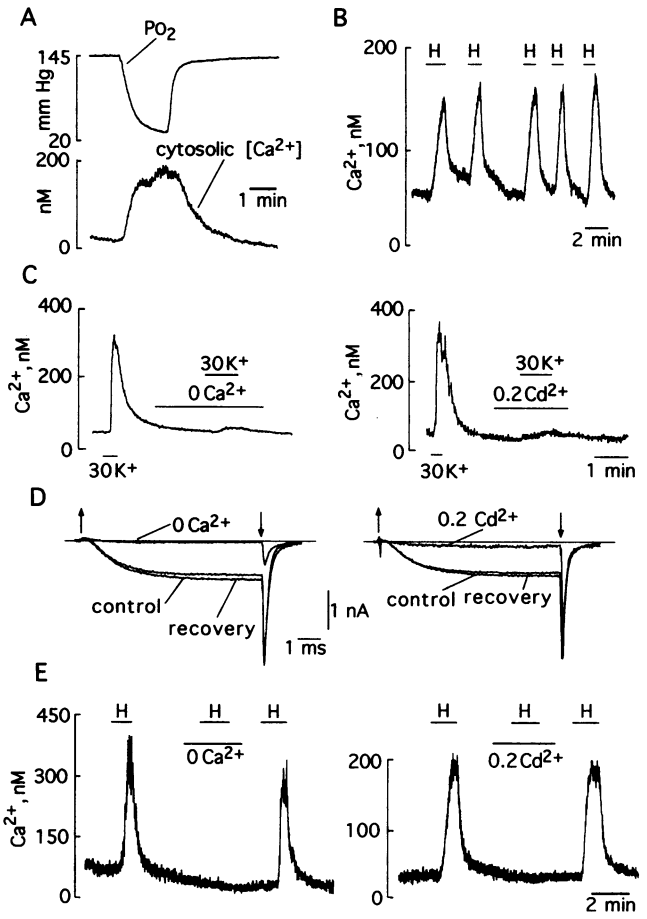


FIG. 1. Hypoxia promotes voltage-dependent $Ca^{2+}$ influx and elevation of cytosolic $[Ca^{2+}]$. (*A*) Parallel changes of $O_2$ tension in the chamber and the modifications of cytosolic $Ca^{2+}$ in a fura-2-loaded glomus cell. $[Ca^{2+}]$ increases in parallel with the depression in $O_2$ tension, but after reestablishing a normal $Po_2$ value (which was achieved in less than 20 s), return of the $Ca^{2+}$ signal to basal levels is somewhat slower, with an average time constant of 38 ± 19 s ($n = 9$). This delay probably reflects the time required for complete reequilibration of the $Ca^{2+}$-sequestering and extruding mechanisms of the cell. (*B*) Changes of cytosolic $[Ca^{2+}]$ during repeated exposure of a glomus cell to hypoxia (H; $Po_2 = 30$ mmHg). (*C*) Elevation of cytosolic $[Ca^{2+}]$ in two unclamped cells during exposure to an external solution with 30 mM $K^+$. The increase of $Ca^{2+}$ induced by high $K^+$ was abolished by removal of external $Ca^{2+}$ (*Left*) or the addition of 0.2 mM $Cd^{2+}$ to the external solution (*Right*). The application of the various test solutions is indicated by the horizontal bars. In *A–C*, the standard external solution contained 140 mM NaCl, 2.7 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 10 mM Hepes (pH 7.3). NaCl was replaced equimolarly for KCl, and $CdCl_2$ was added. When $Ca^{2+}$ was removed, the concentration of $MgCl_2$ was increased to 4 mM. (*D*) Calcium currents recorded in two patch-clamped glomus cells during 8-ms step depolarizations to +20 mV from a holding potential of −80 mV. The 0 $Ca^{2+}$ and 0.2 mM $Cd^{2+}$ external solutions reversibly abolished the current. External solutions were as in *C* but without glucose and with 1 mM tetrodotoxin added. The pipette solution composition was 110 mM CsCl, 20 mM CsF, 5 mM EGTA, 10 mM Hepes, and 2 mM MgATP (pH 7.2). (*E*) Changes of cytosolic $[Ca^{2+}]$ in response to hypoxia (H; $Po_2 = 30$ mmHg) in two unclamped cells bathed in a solution of standard ionic composition or after introduction of the 0 $Ca^{2+}$ (*Left*) or 0.2 mM $Cd^{2+}$ (*Right*) solutions to block the voltage-gated $Ca^{2+}$ channels. Solutions were as in *A*.

oxidizable substance synthesized and released by carotid body cells (see below). Opening of $Ca^{2+}$ channels by depolarizing voltage steps was followed by a burst of spike-like current transients, each spike representing a package of dopamine released from single secretory vesicles (18, 19).

With large stimuli, the spikes partially fused into a broad concentration envelope (Fig. 2*A*, top trace). The spiking activity was reversibly abolished when $Ca^{2+}$ channels were blocked by 0.2 mM $Cd^{2+}$ added to the external solution (Fig. 2*A*, middle and bottom traces). As in chromaffin cells (19), the spikes appeared with a variable first latency that with depolarizations between 0 and +20 mV (potentials at which we obtained the peak inward $Ca^{2+}$ current) averaged 103 ± 60 ms (mean ± SD, $n$ = 29 events). Fig. 2*B* illustrates similar results obtained in unclamped cells depolarized by the application of high extracellular $K^+$ before and after blockade of $Ca^{2+}$ channels with $Cd^{2+}$. When cytosolic [$Ca^{2+}$] and dopamine secretion were monitored simultaneously, the parallel time course of the two became apparent.

Representative amperometric spikes recorded at high resolution from a glomus cell are shown on an expanded time base in Fig. 2*C*. They have the fast rising phase and slower decay typical of the secretory events occurring in the cell region facing the carbon-fiber electrode (18, 19). In many of the spikes, the upstroke was preceded by a "foot" (arrow in Fig. 2*C*) that, as suggested (19, 20), probably originates from the leak of substance through the fusion pore formed by the secretory vesicle and the cell membrane before complete exocytosis occurs. The mean charge per secretory event was 0.044 ± 0.027 pC (mean ± SD, $n$ = 129 fast events from a single cell), which, assuming that two electronic charges are transferred in the oxidation of a dopamine molecule, yields an estimate of 138,000 ± 84,000 (mean ± SD) molecules per vesicle. Fig. 2*D Upper* is a cyclic voltammogram obtained by direct application of dopamine (≈10 μM) to the carbon-fiber electrode, and Fig. 2*D Lower* is the signal recorded during glomus cell secretion. The similarity of the two voltammograms, with identical oxidation and reduction peaks, indicates that the secretory product monitored in amperometric mode is indeed dopamine.

In glomus cells maintained under normoxic conditions, the secretory events were absent or appeared at a very low frequency (1.2 ± 1 spikes per min; mean ± SD; $n$ = 5). Exposure to low $Po_2$ was accompanied by an almost 15-fold reversible increase in the average frequency of dopamine spikes (14.6 ± 6 spikes per min, $n$ = 5), which occurred in parallel to the changes in cytosolic [$Ca^{2+}$]. The correspondence of the changes in $Po_2$, intracellular [$Ca^{2+}$], and dopamine release is illustrated in Fig. 3 with data from an experiment in which we monitored the three parameters during exposure of the cell to low $Po_2$ and in response to high external $K^+$. In this cell, which exhibited some spontaneous secretory activity, both the increase of [$Ca^{2+}$] and the secretory response elicited by hypoxia (Fig. 3*A*) were almost completely abolished when we prevented ion flux through calcium channels with nickel (Fig. 3*B*). After wash-out of the nickel, introduction of the high $K^+$ solution evoked a rapid increase of internal [$Ca^{2+}$] and a typical episode of quantal dopamine release (Fig. 3*C*).

## DISCUSSION

Our results indicate that low $Po_2$, which is the most powerful natural stimulus for the carotid body, elicits an increase of cytosolic [$Ca^{2+}$] due to transmembrane $Ca^{2+}$ influx through voltage-gated channels. This conclusion appears to us inescapable although previous investigators have either failed to demonstrate elevations of intracellular [$Ca^{2+}$] in response to hypoxia or have attributed the increase of $Ca^{2+}$ upon exposure to anoxic solutions to release of the cation from mitochondria (11, 12). These studies, performed on clusters of glomus cells that were not characterized electrophysiologi-

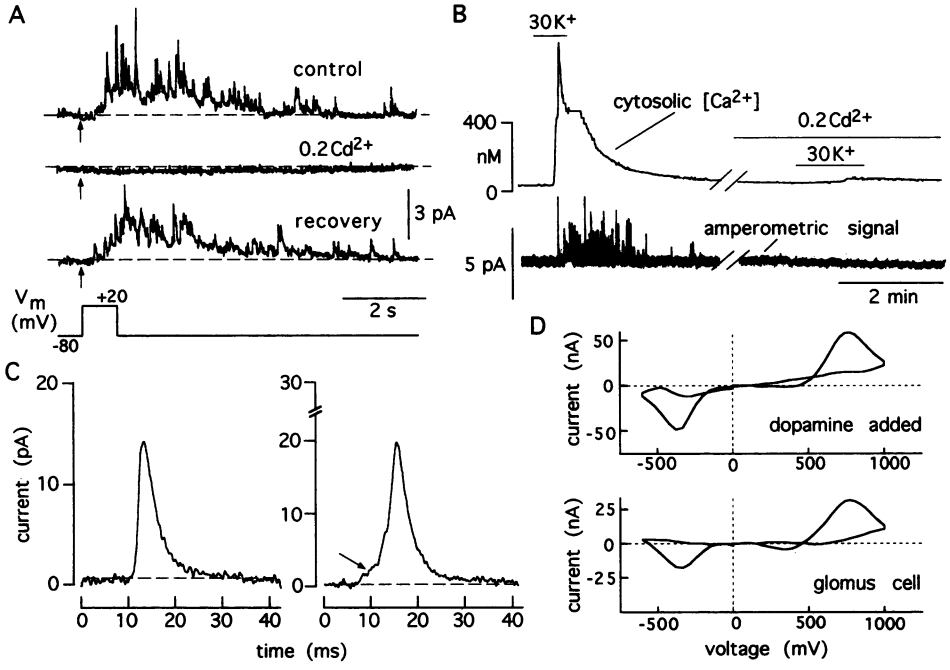


FIG. 2. Quantal dopamine secretion in response to membrane depolarization. (*A*) Dopamine released from a voltage-clamped glomus cell subjected to 800-ms depolarizing steps from −80 to +20 mV. Dopamine is released as discrete spike-like events representing the release from single secretory vesicles. Note that the secretory activity lasts longer than the depolarizing pulse. External solution composition was as in Fig. 1 *A–C*. The internal solution contained 20 mM KCl, 125 mM potassium glutamate, 7 mM $MgCl_2$, 10 mM Hepes, 0.2 mM MgATP, 0.05 mM GTP (Na salt), and 0.3 mM $K_2$EGTA (pH 7.3, adjusted with NaOH). (*B*) Simultaneous recording of cytosolic [$Ca^{2+}$] (upper trace) and dopamine secretion (lower trace) from a fura-2-loaded cell by the same experimental protocol of Fig. 1*C*. Note the parallel time course of the burst of dopamine spikes and the elevation of cytosolic [$Ca^{2+}$]. The break in the recording corresponds to approximately 60 s. (*C*) Representative single secretory events at an expanded time scale. In many cases, as indicated by the arrow, the upstroke is preceded by a "foot." (*D*) (*Upper*) Fast cyclic voltammogram obtained after the immersion of the electrode into a solution with dopamine. (*Lower*) Similar signal recorded from a glomus cell.

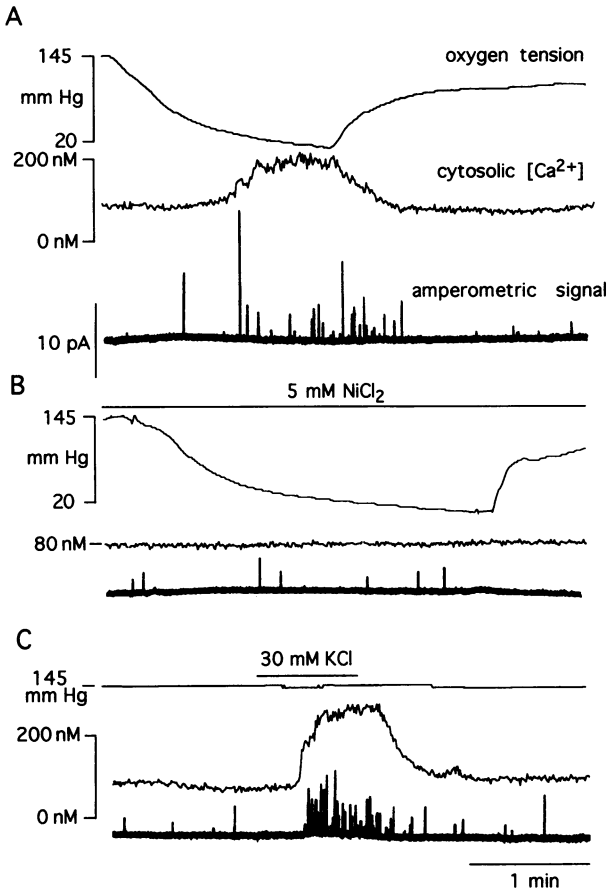


FIG. 3. Parallel changes of $O_2$ tension, cytosolic [$Ca^{2+}$], and dopamine secretion in a glomus cell in response to hypoxia (*A*) and to depolarization by high external $K^+$ (*C*). The secretory response to hypoxia was prevented by blockade of voltage-gated $Ca^{2+}$ channels with nickel added to the external solution (*B*). Solutions and other experimental variables were as in Figs. 1 and 2.

cally, ascribed a secondary role in chemotransduction to ion channels. In our preparation of glomus cells exhibiting the typical oxygen-sensitive $K^+$ current, we have never seen indications supporting the view that release of $Ca^{2+}$ from internal stores significantly contributes to the response of the cells to low $Po_2$. Our observations in rabbit glomus cells (see also refs. 22 and 23) are in excellent agreement with a recent report (24) showing that low $Po_2$ induces depolarization and the increase of cytosolic $Ca^{2+}$ in glomus cells from newborn rats.

In isolated glomus cells, elevations of cytosolic $Ca^{2+}$ elicited in response to hypoxia or direct membrane depolarization trigger exocytotic dopamine secretion that can be resolved at the level of release from single secretory vesicles. These findings fit quite well with biochemical studies in explanted carotid bodies (25, 26) or dispersed rat glomus cells (27), indicating that dopamine is a major substance released by the carotid body in response to hypoxia and that secretion requires extracellular $Ca^{2+}$. We have also shown, in excellent agreement with recent work in chromaffin cells (19), that in dialyzed glomus cells, $Ca^{2+}$ influx through voltage-gated channels can trigger quantal transmitter release. Upon membrane depolarization, the secretory events are observed with a variable latency with average values in the range of those of chromaffin cells (19). However, the estimated average amount of dopamine released during each well-resolved secretory event is about 15 times smaller than a previous estimate in adrenomedullary cells (19), which is in accord with the larger size of chromaffin granules compared to those of glomus cells (28, 29). In the context of our previous electrophysiological work (2, 8), the present results provide direct evidence for an explanation at the cellular level of the mechanisms of chemosensory transduction in the carotid body. The sequence of events is initiated by the conversion by $O_2$-sensitive $K^+$ channels of $Po_2$ changes into modifications in the glomus cell membrane voltage, which is the variable regulating $Ca^{2+}$ entry and secretion. These phenomena are similar to those observed in nerve synapses and, thus, make the glomus cell a well-defined model for study of the mechanisms of exocytotic transmitter release.

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