A new method to detect rapid oxygen changes around cells: How quickly do calcium channels sense oxygen in cardiomyocytes?

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Scaringi JA, Rosa AO, Morad M, Cleemann L. A new method to detect rapid oxygen changes around cells: How quickly do calcium channels sense oxygen in cardiomyocytes? J Appl Physiol 115: 1855–1861, 2013. First published October 24, 2013; doi:10.1152/japplphysiol.00770.2013.—Acute hypoxia is thought to trigger protective responses that, in tissues like heart and carotid body, include rapid (5–10 s) suppression of Ca2+ and K+ channels. To gain insight into the mechanism for the suppression of the cardiac L-type Ca2+ channel, we measured O2-dependent fluorescence in the immediate vicinity of voltage-clamped cardiac cells subjected to rapid exchange of solutions with different O2 tensions. This was accomplished with an experimental chamber with a glass bottom that was used as a light guide for excitation of a thin ruthenium-based O2-sensitive ORMOSIL coating. Fluorescence imaging showed that steady-state PO2 was well controlled within the entire stream from an electromagnetically controlled solution “puffer” but that changes were slower at the periphery of the stream (τ1/2 ~ 500 ms) than immediately around the voltage-clamped myocyte (τ1/2 ~ 225 ms) where, in turn, firmly attached cells produced an additional local delay of 50–100 ms. Performing simultaneous voltage clamp and O2 measurements, we found that acute hypoxia gradually and reversibly suppressed the Ca2+ channel (CaV1.2). Using Ba2+ as charge carrier, the suppression was significant after 1.5 s, reached ~10% after 2.5 s, and was nearly completely reversible in 5 s. The described fluorescence measurements provide the means to check and fine tune solution puffers and suggest that changes in PO2 can be accomplished within ~200 ms. The rapid and reversible suppression of barium current under hypoxia is consistent with the notion that the cardiac Ca2+ channel is directly modulated by O2.

omosil; dissolved oxygen sensing; hypoxic suppression of Ca2+ current

While it has long been known that cardiac myocytes respond rapidly to hypoxia by suppressing L-type Ca2+ channel current (carried by Ca2+ or Ba2+), this response is not yet fully understood (8). We have previously suggested that the Ca2+ channel functions as an acute oxygen sensor for the heart, in which the decrease in calcium current (I_Ca) triggered by hypoxia involves the Ca2+/CaM binding domain of the channel’s C-terminus (18, 23). Others have suggested that the suppressive effects of hypoxia on I_Ca is mediated by changes in production of mitochondrial ROS, which in turn interact with thiol groups on the C-terminus of the Ca2+ channel (12–14). In part, these alternative possibilities may be distinguished through the kinetics of the response of L-type Ca2+ channels to hypoxia.

To address this issue, we developed a novel method for sensing dissolved O2 in the immediate vicinity of patch-clamped myocytes. Using a ruthenium-doped ORMOSIL coating as an O2 sensor (5, 20), we prepared coverslips, the fluorescence of which was rapidly and reproducibly quenched by the binding of dissolved O2. Plating cardiac cells onto these coverslips, we verified that hypoxia rapidly suppressed current carried by L-type Ca2+ channels and established that the suppressive effect of acute hypoxia results from hypoxic conditions lasting as little as 1.5 s in duration.

MATERIALS AND METHODS

Isolation of rat cardiac myocytes. Cardiac myocytes from the right ventricle of male Sprague Dawley rats were isolated as previously described (17). Protocol for animal handling and cell isolation (AR 2791) was approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina according to national legislation and guidelines.

Hearts were surgically extracted from animals anesthetized by isoflurane (3%, vaporized), retrogradely cannulated through the aorta, and washed for 5 min by perfusion of calcium-free Tyrode’s solution (in millimoles: 136 NaCl, 30 taurine, 10 HEPES, 10D-glucose, 5.4 potassium l-glutamate, and 1 MgCl2 at pH 7.2 using NaOH) using a peristaltic pump at 7 mL/min. After hearts stopped completely, hearts were enzymatically digested by a mixture of collagenase type 2 (1.32 mg/mL, 270 units/mg, Worthington) and protease type XIV (0.26 mg/mL, 3.5 units/mg, Sigma) for 15–20 min. Postdigestion, hearts were washed with 0.2 mM Ca2+ Tyrode’s solution for 8 min. All solutions were maintained at 37°C using a heating circulator bath, and perfusion steps were conducted under a heating lamp to maintain heart temperature. The right ventricle was dissected from the digested heart and cells were released from the tissue by gentle manual dissociation using forceps. Cells were plated onto oxygen sensor-doped ORMOSIL-coated coverslips in 0.2 mM Ca2+ Tyrode’s solution and were allowed to settle for a minimum of 45 min prior to patch clamp experiments.

Coverslip coating materials and procedure. Tetramethylorthosilicate (TMOS, 98%), trimethoxy(propyl)silane (TMPS, 97%), ACS grade hydrochloric acid (HCl, 37%), nitric acid purified by redistil...
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Solution (HNO₃, 70%), and absolute ethanol (EtOH, 200 proof) were purchased from Aldrich. Tris(4,7-diphenyl-1,10-phenanthroline) ruthenium(II) dichloride complex [Ru(dpp)₃]Cl₂, ≥95% (HPCE) was obtained from Fluka.

Method for the synthesis of [Ru(dpp)₃]Cl₂-doped ORMOSIL coating was adapted from those previously described (5, 20). Twenty four hours prior to ORMOSIL preparation, glass coverslips (22 × 40 mm, Corning, 2935–224) were placed into a bath of HNO₃ to prepare the surface for coating. Immediately prior to coating preparation, coverslips were removed from HNO₃ bath, washed by successive rinses with EtOH and distilled water, and air dried. ORMOSIL matrix was prepared by mixing TMOS (100 µL, 0.672 mmol) and TMPS (160 µL, 0.908 mmol) together with a magnetic stir bar in a 25 mL round bottom flask. Upon pipetting both solutions into the reaction vessel, HCl (200 µL, 0.01 M) was immediately added in a dropwise manner. The solution was stirred for 1 h in a closed flask. Meanwhile, a 5 mM stock solution of [Ru(dpp)₃]Cl₂ was prepared by combining 1.00 mg (855 nmol) with 171 µL of EtOH, then vortexing until homogeneous. The 5 mM [Ru(dpp)₃]Cl₂ stock solution was combined with the completed ORMOSIL matrix in a microcentrifuge tube in a 1:1 ratio, then mixed by vortexing. Immediately, a portion of this mixture was pipetted (4 µL) onto a prepared glass coverslip and spread by drawing a second coverslip over its surface. Coverslips remained in ambient conditions for a minimum of 24 h before plating with cells.

Patch clamp recordings. Freshly isolated cardiac myocytes plated onto [Ru(dpp)₃]Cl₂-doped ORMOSIL-coated coverslips were voltage-clamped in the whole cell configuration on an inverted microscope using a Dagan 8900 Patch Clamp amplifier (Dagan, Minneapolis, MN), Digidata 1440A digitizer and pClamp 10.2 software (Axon Instruments, Union City, CA). Ba²⁺ currents (I Ba) through l-type Ca²⁺ channels were measured using patch pipettes with 2–5 MΩ resistance filled with an internal solution containing (in millimoles): 137 NaCl, 10 HEPES, 11 D-glucose, 2 BaCl₂, and 1 MgCl₂ at pH 7.2 using CsOH. Ba²⁺ was chosen as the charge carrier because, unlike ICa, the suppressive effect of hypoxia on IBa is not mitigated by phosphorylation (23). The extracellular Tyrode’s solution contained (in millimoles) 137 CsCl, 11 D-glucose, 11 HEPES, 11 TEACl, 5.5 MgATP, 5.5 NaCl, 2.2 EGTA, and 0.92 CaCl₂ at pH 7.2 using CsOH. D-alanine (855 nmol) with 171 µL of EtOH, then vortexing until homogeneous. The 5 mM [Ru(dpp)₃]Cl₂ stock solution was combined with the completed ORMOSIL matrix in a microcentrifuge tube in a 1:1 ratio, then mixed by vortexing. Immediately, a portion of this mixture was pipetted (4 µL) onto a prepared glass coverslip and spread by drawing a second coverslip over its surface. Coverslips remained in ambient conditions for a minimum of 24 h before plating with cells.

Oxygen measurement. The retention of [Ru(dpp)₃]Cl₂’s luminescent properties was verified by excitation (measured emission λ = 610 nm) and emission (excitation λ = 470 nm) spectra of sensor-coated coverslips at room temperature using BioTek Synergy™ H1 Hybrid Multi-Mode Microplate Reader (Fig. 1A). Oxygen-quenching fluorescent characteristics were confirmed by rapidly dispensing O₂ and N₂ saturated H₂O onto a sensor-doped ORMOSIL-coated six-well plate.

During voltage clamp experiments, the ruthenium-based sensor was excited by evanescent illumination at 473 nm from a 400 mW diode laser. In this configuration the coated coverslips (22 × 40 mm, Corning, 2935–224) were used as an exchangeable bottom and light guide in the experimental chamber and light was admitted along the short edge via optical fibers (Light guide-based TIRFM, TIRF Labs, Cary, NC). Emitted light (>515 nm) from the paracellular vicinity was detected by a photomultiplier tube behind an aperture that limited the detection area to the voltage-clamped cells and its immediate surroundings (~100 µm radius around cell) or wider area. Using this optical system, we verified that our perfusion system was capable of changing the O₂ concentration around the cell with a time to half maximum (τ ½) of 225 ms ± 14 ms (± SE), n = 6.

Fluorescence distribution was additionally verified in the epillumination mode with a Leica microscope (DMI6000 B, Buffalo Grove,
IL) equipped with an iXon3 897 camera (Andor Technology, Belfast, UK). Fluorescence images \( F(x,y,t) \) were typically recorded at 20 Hz and were displayed ratiometrically \( \frac{F(x,y,t)}{F_{\text{normoxic}}(x,y)} \) (2) after division with the normoxic steady-state fluorescence distribution \( F_{\text{normoxic}}(x,y) \). This distribution was either measured directly (Fig. 1C) or was calculated as a weighted average \( F_{\text{normoxic}} = (2 \times F_{N2} + F_{O2})/3 \), Fig. 2) from the steady-state fluorescence distributions measured with solutions bubbled with \( N_2 (F_{N2}) \) or \( O_2 (F_{O2}) \). The weights (2:1) were derived from the average spectrofluorometric and imaging responses \( (F_{N2} - F_{\text{normoxic}})/(F_{\text{normoxic}} - F_{O2}) = 2.4 \pm 0.5, n = 6 \).

Statistical analysis (or data analysis). Suppression of IBa was calculated by taking the difference between the peak current during hypoxia and the projected current with no hypoxia, accounting for rundown (Fig. 4). Student’s \( t \)-test was performed to check for significance between the control and experimental groups for each duration of hypoxia (0.5, 1, 1.5, 2, and 2.5 s). Less than 5% probability of equality between groups is denoted by * and less than 1% is indicated by **.

RESULTS

Oxygen measurement. After preparing the Ru-based \( O_2 \)-sensitive ORMOSIL coating, we examined the magnitude, uniformity, and time course of the responses of the coated coverslips to solutions with different \( O_2 \) tensions. As a first step, we coated the bottom of a polystyrene six-well plate and used a spectrofluorometer to measure the absorption and emission spectra, which displayed up to a threefold reduction in fluorescence intensity.

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Fig. 2. Local changes in \( O_2 \)-dependent fluorescence immediately following solution exchange. The insets show a bright field image (BFI) of the puffer outlet with several cells in front of it, regions of interest (ROI), and two sequences of numbered sample frames measured at 20 Hz when switching from fully oxygenated to hypoxic solution (#146–154) and back (#183–191). Notice that the measured \( O_2 \) changes occur earlier at the center of the stream hitting the coverslip (red and green ROIs) than at the periphery and that the coating under the cells (red ROI, white arrows) responds with a delay of ~50–100 ms compared with the immediately surrounding regions of the coverslip (green ROI). The sample frames were measured ratiometrically relative to the normoxic steady-state fluorescence distribution as described in the Methods section. The color code is identical to Fig. 1 (blue: 100% \( O_2 \); green: normoxia; yellow/orange: hypoxia). The lower row of images show the delayed spread of hypoxia (consecutive frames 1, 2, and 3) under an intact cardiomyocyte (BFI-2).
when the superfusing solution was changed from one that was bubbled with 100% N₂ to another bubbled with 100% O₂ (Fig. 1A). Solution equilibrated with ambient air produced intermediate responses. Next we reproduced the geometry of biological experiments where a small area of the coated coverslips was exposed directly to rapid flow of solutions containing different concentrations of O₂ (Fig. 1B). Figs. 1C and 1D show the steady-state images and the time course of the fluorescence signals measured when PO₂ of the superfusing solutions was changed between 100%, 0% (100% N₂), and 20% (atmospheric) oxygen. The O₂-dependent fluorescence signals changed most rapidly directly in front of the outflow tube (green trace in Fig. 1D and region of interest (ROI) in Fig. 1C), spread with a slight delay in concentric circles (red and blue), and had a relatively well defined outer limit that in the shown sample frames (b and c in Fig. 1D) is seen only in lower left-hand corner (orange). Rapidly exchanged solutions, shown in Fig. 1D, quickly reach a steady-state level but consistently showed slight delays in response time in areas farther away from the central puffing region.

Figure 2 shows images of O₂-dependent fluorescence recordings at 20 Hz from an oxygen-sensing coverslip plated with cells as seen in the bright field image (BFI). Selected frames recorded at the onset (#146–154) and termination (#183–191) of a brief hypoxic interval confirmed that the solution directly in front of the puffer outlet changes first (red and green ROIs and curves). This change in fluorescence expanded radially (blue ROI) but terminated at the interface of superfusing stream and the bath solution (orange ROI). Analysis of the frames recorded immediately after the solution changes (frames #147, 148, 184, and 185) showed that there was a delay of ~100 ms in O₂ signals measured underneath the cells (red ROI, white arrows) compared with their immediate surroundings (green ROI), suggesting that the plated myocytes contribute to the formation of an unstirred layer with a significant diffusion delays. Such delays were seen not only in association with the rounded, irreversibly contracted cells that dominated the scene after several hours of experimentation but also under the intact cells (lower row of images in Fig. 2) that were used in voltage clamp experiments.

**Suppression of the cardiac Ca²⁺ channel by acute hypoxia.** We have previously reported that the current through cardiac Ca²⁺ channels is suppressed by ~10% within 5 s and by ~20–30%, depending on the charge carrier, within 45–60 s of exposure to hypoxic solutions (23). To gain further mechanistic insight, we carried out simultaneous measurements of O₂ withdrawal-induced rise in fluorescence signal and IBa in patch-clamped myocytes. IBa was activated three times, at 5 s intervals by voltage clamp depolarizations from Vm = 50 to 0 mV (Fig. 3A), with the middle pulse occurring while the cell was experiencing hypoxia as evidenced by the O₂ signal. The switch from fully oxygenated to hypoxic solution was digitally triggered and allowed to continue 100 ms after the depolarization had ended, assuring that changes in flow pressure did not affect IBa (6, 17). Fig. 3B shows sample IBa traces where a, b, and c correspond to identically labeled peak

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**Fig. 3.** Simultaneous measurements of O₂ signal and Ba²⁺ current through the Ca²⁺ channel. A: standard voltage clamp protocol with 5 s pulse intervals and 30 ms depolarizations from −50 to 0 mV. All traces in figure are low pass filtered at 500 Hz. B: overlay of IBa from single sweep of protocol with 2.5 s exposure to N₂-bubbled solution. Letters a, b, and c correspond to labeled depolarizations from A. C: example of rapid changes in O₂ tension measured in the paracellular vicinity of a voltage clamped cell. Multiple overlaid sweeps are shown with exposure to hypoxia ranging from 0.5 to 2.5 s. D: example of slower and less reproducible changes in PO₂ measured in the paracellular vicinity of a voltage-clamped cell under less optimal conditions. E: timing of the command potentials to the puffer (top) and voltage-clamp apparatus (Vm, bottom).
values of \( I_{\text{Ba}} \) in Fig. 3A at times before, during, and after the exposure to hypoxia. It is apparent that the peak \( I_{\text{Ba}} \) was suppressed by \( \sim 20\% \) within 2.5 s of hypoxia and that the suppression of the current at the end of the 100 ms depolarization pulse was proportionally larger. The recovery of current 5 s after reintroduction of \( O_2 \) was nearly complete. By simultaneously measuring dissolved \( O_2 \) tension and \( I_{\text{Ba}} \), we found that a rapid (\( \tau_{1/2} = 225 \) ms), consistent, and accurately timed change of solutions could be achieved (Fig. 3C). However, this result was only secured by placing the puffer close to the voltage-clamped cell and by consistently flushing the tubing which carried the \( N_2 \) and \( O_2 \) perfused solutions so that a homogeneous oxygen tension was maintained. Without such precautions or with imprecise placement of the solution puffer, the measured paracellular \( O_2 \) concentration was found to change more slowly and the steady-state signals were more variable (Fig. 3D). In the absence of consistent, rapidly changing \( O_2 \) signals, it would be impossible to assess very short durations of hypoxia (\(<1\) s) as a steady state is never reached. The \( O_2 \) signals in Fig. 3, C and D, were generated by a sequence of hypoxic solution pulses that increased in duration in increments of 500 ms, shown schematically in Fig. 3E (cf. T in Fig. 3A).

While monitoring \( O_2 \) tension to assure that solutions were being rapidly exchanged, we found that short durations of acute hypoxia transiently diminished \( L \)-type \( Ca^{2+} \) current carried by \( I_{\text{Ba}} \). Fig. 4A shows sample experimental and control traces from myocytes that were exposed to puffs of 2.5 s duration prior to the second depolarization pulse with solutions equilibrated either with \( N_2 \) or \( O_2 \), respectively. When myocytes were subjected to the puffs of \( N_2 \) equilibrated solutions, the cell experienced a 6% decrease in \( I_{\text{Ba}} \) that recovered partially after 5 s, as compared with no change in current amplitude or kinetics when the cell was perfused with only oxygen-containing solutions. Fig. 4B shows the peak current density from an experiment (black and white filled circles) plotted against time, with durations of hypoxia ranging from 2.5 to 0.5 s. The hollow points, representing depolarizations during which time the cell experienced \( N_2 \), show that short durations of hypoxia suppress \( I_{\text{Ba}} \), but the effect is smaller with shorter durations of \( N_2 \) equilibrated solutions. We found that durations of hypoxia equal to or longer than 1.5 s consistently and transiently suppressed \( I_{\text{Ba}} \).

There was always significant rundown of the current (black curve) even in presence of \( Ba^{2+} \) as the charge carrier. Rundown is the slow decrease of measured ionic current as a function of time, likely through the loss of molecules from within the cytoplasm that modulate channel function (11). These findings also suggest that the recovery of \( I_{\text{Ba}} \) after reintroduction of fully oxygenated solution was \( \sim 80-90\% \) complete after 5 s.

Fig. 5 shows the average suppression of \( I_{\text{Ba}} \) activated with 30 ms depolarizations as cardiomyocytes were exposed to hypoxia for 0.5, 1, 1.5, 2, and 2.5 s. The suppression of \( I_{\text{Ba}} \) increases in a linear fashion with increasing durations of hypoxia, with the strongest suppression of 10% occurring with 2.5 s of hypoxia exposures. In sharp contrast, when control myocytes were subjected to the switch of identical fully oxygenated solutions there were no significant changes in \( I_{\text{Ba}} \).

**DISCUSSION**

Here we have demonstrated a method to measure rapid changes in \( O_2 \) tension in the immediate vicinity of individual cells (\( \tau_{1/2} = 225 \) ms) and have used this approach to show that...
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Fig. 5. Rapid suppression of I_{Ba} at the onset of acute hypoxia. Mean suppression of I_{Ba} by durations of hypoxia ranging from 0.5 to 2.5 s (black). Mean control suppression (diagonal lines) represent experiments in which the cells were alternatively puffed with two O_{2} perfused solutions.

the hypoxic suppression of the current through the cardiac Ca^{2+} channel takes effect within 1.5 s of hypoxic insult and is rapidly reversible. The findings are relevant to identifying the mechanisms for rapid O_{2} sensing in biological tissues and for evaluating the efficiency of rapid perfusion techniques and the speed with which the concentration of O_{2} and other compounds can be altered at the surface of single isolated cells.

Hypoxic suppression of the cardiac Ca^{2+} channel. We have previously reported that I_{Ca} in cardiac cells is suppressed by ~20% within 50 s of hypoxia and have argued that the speed of this response was too fast to reflect a significant buildup of reactive oxygen species or change in the ATP/ADP balance (23). Like other investigators of acute hypoxia (9, 21, 25, 28), we previously puffed deoxygenated solution from reservoirs bubbled with 100% N_{2} onto voltage-clamped cells and collected electrophysiological data without directly measuring the local paracellular PO_{2}. The work reported here was undertaken to determine directly how rapidly the suppression of the cardiac Ca^{2+} channel follows the reduction in PO_{2}. As shown in Figs. 4 and 5, the suppression of the peak I_{Ba} through the Ca^{2+} channel developed linearly with time, reaching a 10% suppressive effect after 2.5 s exposure to N_{2}-bubbled solution. In some cells the suppression was considerably larger, especially with regard to the maintained component of I_{Ba} (Fig. 3B). We have observed that the suppression depends on the permeating ion, the channel phosphorylation state, and isoform of the channel (23), although there may yet be other factors that accentuate or modulate hypoxic regulation. Control experiments, where we switched back and forth between different fully oxygenated solutions (Figs. 4 and 5), suggest that the observed suppression was due to hypoxia and not to artifacts such as mechanical shock waves generated by the valves of the electromagnetically controlled solution puffing system (6, 24).

Considering the gradual rundown of I_{Ba} (Fig. 4B), present also in the control experiments and those where no solution exchange took place, the full recovery of hypoxic suppression on reoxygenation suggests specificity to I_{Ca} suppressive effect (Figs. 3B and 4B).

The speed and reversibility of the measured hypoxic suppression of I_{Ba} suggest that the effect of O_{2} on the Ca^{2+} channel is fairly direct and is not mediated by a slowly activating signaling process such as phosphorylation or alterations in concentrations of cellular constituents such as ROS, ADP, and ATP. This begs the question whether the heme-oxygenases that have been implicated in O_{2} sensing in the carotid body (19, 27) and ganglionic nociceptive neurons (7) might also govern the hypoxic regulation of the cardiac Ca^{2+} channel. Published experiments with inhibitors of heme-oxygenases protoporphyrins are consistent with this possibility (23), but it remains to be determined to what extent heme-oxygenases may serve as a general extramitochondrial O_{2} sensor. Alternatively, hypoxia may induce ROS signals that locally occur faster than with the delay of ~3 min found with global cellular ROS measurements (13). This possibility is supported by reports of stretch-activated ROS production by sarcolemmal NADPH oxidase (22) and spontaneous mitochondrial superoxide flashes (26), both of which take place on a timescale of seconds or faster, but are not known to do so in response to hypoxia. The methods described here may be used in future experiments to distinguish between these possibilities by comparing the time course of the changes in PO_{2} and I_{Ca} to local ROS signals or responses mediated by heme-oxygenase.

Verification of rapid changes in paracellular PO_{2}. The use of O_{2}-sensitive coverslips allowed for the measurement of PO_{2} directly under and around cardiomyocytes. Cells were adhered to a thin, inert coating consisting of a fluorescent ruthenium-based sensor imbedded in a matrix of organically modified silicates. In our implementation we used coverslips as a light guide for continuous evanescent wave excitation and measured fluorescence intensity. Epi-illumination, as used in the imaging studies, produced some autofluorescence from the polyethylene outflow tube of the solution puffer, but the responses were not significantly degraded. Similarly, instead of fluorescence intensity, it may do as well to measure fluorescence lifetimes (15). In either case the O_{2}-dependent signal decreases nearly linearly with PO_{2}, which in turn implies that it may be rather difficult to measure very small values of PO_{2} (~5 mmHg) without using a more sensitive sensor (with a larger Stern-Volmer constant). On the other hand, for our present purpose the kinetics of the measurements were of greater importance. By ratiometric imaging of the PO_{2}-dependent fluorescence we found that transitions between hypoxic and fully oxygenated solution occurred rapidly (~1/2 = 225 ms) only right in front of, and close to, the outflow tube of the solution changer. It appeared that the stream of solution, which hit the coverslip at an incident angle of ~45°, rapidly fanned out to cover a larger area and thereby slowed down considerably at the sides and with increasing distance from the exit port. It is known that much faster transition times (~<5 ms) can be achieved in electrophysiological measurements where a small voltage-clamped HEK-295 cell is lifted off the coverslip and placed in one of the streams from a piezo-electric solution changer (1), but such procedures are hardly practical with the larger ventricular cardiomyocytes (~100 μm × 20 μm × 10 μm). Furthermore Fig. 2 shows that the PO_{2} signal under the attached cells were delayed by ~50 ms, suggesting a diffusion (D = 2μm^2/ms) delay through an unstimulated layer comparable to the thickness of the cell. Therefore the value of ~50 ms may...
represent a practical limit for the speed of application of O2 and other compounds that must penetrate the intact cells before they take full effect. The measurements of PO2 suggest that it is important to verify the speed and effectiveness of solution puffers and that the electromagnetically controlled puffer used in this study was adequate to implement changes in PO2 well before the gradual hypoxic suppression of the Ca2+ channel reached a significant level after about 1.5 s. The technique described here may prove useful in testing dose-dependence and kinetics of various hypoxic responses in different cell types.

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