Capillaries demonstrate changes in membrane potential in response to pharmacological stimuli

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MCGAHERN, Eugene D., JAMES M. BEACH, AND BRIAN R. DULING. Capillaries demonstrate changes in membrane potential in response to pharmacological stimuli. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H60–H65, 1998.—It has been proposed that capillaries can detect changes in tissue metabolites and generate signals that are communicated upstream to resistance vessels. The mechanism for this communication may involve changes in capillary endothelial cell membrane potentials which are then conducted to upstream arteries. We have tested the capacity of capillary endothelial cells in vivo to respond to pharmacological stimuli. In a hamster cheek pouch preparation, capillary endothelial cells were labeled with the voltage-sensitive dye di-8-ANEPPS. Fluorescence from capillary segments (75–150 µm long) was excited at 475 nm and recorded at 560 and 620 nm with a dual-wavelength photomultiplier system. KCl was applied using pressure injection, and acetylcholine (ACH) and phenylephrine (PE) were applied iontophoretically to these capillaries. Changes in the ratio of the fluorescence emission at two emission wavelengths were used to estimate changes in the capillary endothelial membrane potential. Application of KCl resulted in depolarization, whereas application of the vehicle did not. Application of ACh and PE resulted in hyperpolarization and depolarization, respectively. The capillary responses could be blocked by including a receptor antagonist (atropine or prazosin, respectively) in the superfusate. We conclude that the capillary membrane potential is capable of responding to pharmacological stimuli. We hypothesize that capillaries can respond to changes in the milieu of surrounding tissue via changes in endothelial membrane potential.

MATERIALS AND METHODS

Animal preparation. Male Golden hamsters (90–150 g body wt) were prepared for in vivo examination of the cheek pouch (8). Each animal was anesthetized with an intraperitoneal injection of pentobarbital sodium (80–100 mg/kg). Body temperature was maintained at 36.5–37.5°C by radiative and conductive heating. A tracheostomy tube was placed, and the animal was allowed to breathe room air spontaneously during the course of the experiment. The left femoral vein was cannulated for administration of fluid and anesthesia. Intravenous anesthesia (pentobarbital in normal saline, 4 mg/ml) was infused continuously with a syringe pump (Sage Instruments) at a rate of ~0.45 ml/h during the experiment. Supplemental intraperitoneal injections of 0.1 ml of 70% pentobarbital were administered as needed during dissections. The left cheek pouch was exteriorized, opened, cleared of connective tissue, mounted on the stage of a microscope (Zeiss), and observed with a ×20 (NA = 0.33) air (Zeiss) or ×20 (NA = 0.55) water immersion (Olympus) objective.

The cheek pouch was superfused at 5–6 ml/min with a modified Ringer bicarbonate solution (pH 7.36–7.44) containing (in mM) 132 NaCl, 4.7 KCl, 2.0 CaCl2, 1.2 MgSO4, and 20 NaHCO3. The superfusate was warmed (36.5–37.5°C) and equilibrated by bubbling with 5% CO2-95% N2O2 (5%) was added to the gas mixture to enhance arteriolar tone when necessary. Video images of the transilluminated preparation (Dage-MTI SIT 66) were visualized on a monitor (Dage-MTI), and diameter and distance measurements were made using a modified video analyzer (Colorado Video) and Image-1 software (Universal Imaging). Resting arteriolar tone was assessed at the beginning of each experiment and at various points during each experiment by applying topical ACH (10–4 M) and measuring the diameter of the maximally dilated vessel. Preparations demonstrated a resting arteriolar diameter ranging from 55 to 85% of the diameter attained after application of ACH. Recording of capillary signals was performed only as long as arteriolar tone was present in the preparation, usually between 3 and 5 h.

Labeling of capillaries. An 8 mM stock solution of di-8-ANEPPS (Molecular Probes) was prepared in dimethyl sulfoxide. Labeling solution was prepared by diluting stock solution in 3-(N-morpholino)propanesulfonic acid (MOPS)-buffered saline containing (in mM) 145 NaCl, 4.7 KCl, 2.0 CaCl2, 1.2 MgSO4, 1.2 NaH2PO4, 5.0 glucose, 2.0 pyruvate, 0.02 EDTA, 2.8 NaOH, and 2.0 MOPS to a final concentration of 12 µM. Pluronic F-127 detergent was added (0.1%) to increase the solubility of the dye. The final concentration (%vol) of dimethyl sulfoxide in the labeling solution was 0.12%. The dye solution was vortexed and stored in the dark until use. Micropipettes for dye perfusion were prepared from stock glass capillary tubes (1.2 mm in diameter; World Precision Instruments) pulled with a pipette puller (David Kopf Instruments).
ments) and tripled-beveled to ~5-µm tips. Cheek pouch arterioles and capillaries were labeled by perfusing dye into an arteriole with a micropipette using a picospritzer (PV-820, World Precision Instruments). During perfusion, the proximal segment of the arteriole was occluded with a micropipette with a rounded tip (~30 µm in diameter) and perfusion pressure was kept at the lowest level, usually 5–25 lb/in.2, that would displace blood and label associated capillary networks. Time of infusion was ~15–20 min, and the amount of dye solution infused varied between 0.04 and 0.1 ml. Adequacy of dye staining was determined by periodic visual inspection of the preparation under epi-illumination. Time of dye infusion and the amount of vessel area dyed had greater impacts on the adequacy of capillary labeling than the amount of dye infused. When labeling was found to be adequate, the perfusion pipette and occluder were removed and blood flow resumed through arterioles and capillaries. After a 10-min equilibration period, studies were begun.

Drug application. Drugs were applied topically to short capillary segments by pressure ejection using a picospritzer (KCl, 140 mM in Ringer bicarbonate superfusate solution) or iontophoresis (1 M ACh or PE in distilled water). For KCl applications, a pipette containing drug and a second pipette containing vehicle solution were positioned next to a capillary segment by micromanipulators (Leitz), thus allowing easy selection of either vehicle or drug stimulus. Pressure ejection was accomplished without disturbing the capillary or the blood flow within it. For iontophoresis of ACh and PE, pipette tips were left unbeveled (size of tip < 1 µm). Ejection was driven by an iontophoresis programmer (3 s, 600 nA; World Precision Instruments). Antagonist studies were accomplished by adding atropine 10^{-6} M for ACh or prazosin 10^{-7} M for PE to the superfusate and allowing 5–10 min for stabilization before iontophoresis of drug.

Recording of membrane potential. Epi-illumination was restricted to capillary segments 75–150 µm long (Fig. 1) by adjusting the iris of a diaphragm in the light path. There was no fluorescence noted from nonvascular cells in these recording windows. Dye was excited using a 75-W xenon arc lamp filtered for excitation at a 475-nm center with 15-nm band pass. Emission wavelengths of 560 and 620 nm were selected using a 500-nm long-pass filter (Zeiss) and recorded using a dual-wavelength photomultiplier system (Nikon CCTV dual-image adapter and Hamamatsu PMT model 1104). Capillary segments demonstrating a steady fluorescence ratio (F_{620}/F_{560}) were selected for study. The fluorescence ratio was recorded for 10 s before drug was applied and for 30 s after application. In the case of KCl, results of some rapid sequential applications were recorded. In the cases of ACh and PE, 2–3 min were allowed to pass between each measurement. Changes in capillary endothelial membrane potential were assessed based on a prior calibration in which a change in F_{620}/F_{560} (%ΔR) of 1% represented an ~10-mV change (2). A negative value represented depolarization, and a positive value represented hyperpolarization. Raw data were recorded at 25 Hz with a 12-bit data acquisition board (Workbench, Strawberry Tree) and analyzed off-line.

Data analysis. Statistical analysis was performed for paired sequential applications of KCl (same duration) on the same capillary. Mean (± SE) differences in %ΔR, time of initiation of KCl application to time of initial response, and slope of depolarization over the first 3 s were determined for the first and second applications and compared using a paired two-tailed t-test, with significance denoted by P < 0.05.
For each individual response generated by ACh and PE applications, %ΔR was recorded at 25 Hz. A data point was generated every 2.5 Hz by averaging blocks of 10 recorded %ΔR values. The variation over this time (0.4 s) was minuscule. Data were referenced to a common time 0 at the instant of drug application. Combining data from the various individual curves, a mean (± SE) %ΔR was thus generated every 0.4 s. Comparisons between results with and without a receptor antagonist were performed in an unpaired fashion using a Kruskal-Wallis test for area under a curve and by a random coefficient model as described by Crowder and Hand (5). Significance for difference between curves using each test was denoted by P < 0.05.

RESULTS

KCl application. Pulsed applications of KCl (range: 0.5–11 s in duration) onto labeled capillary segments resulted in depolarization in 48 of 49 instances (17 capillaries, 5 animals) (Fig. 2). Among different capillaries, the maximum %ΔR was quite variable and did not correlate well with the duration of the KCl application. Representative tracings of responses are shown in Fig. 3. In those instances in which application of the vehicle was paired with application of KCl, the vehicle had no effect on membrane potential (18 applications, 12 capillaries, 4 animals). In individual capillaries, the fluorescence change correlated well with pulse duration, and a steady state could be achieved after 8–11 s (Fig. 4). The reproducibility of individual responses was good. In paired sequential applications of KCl (same duration) on the same capillary, there were no mean differences between the first and second applications for %ΔR (3.06 ± 0.77 vs. 3.19 ± 0.90%; n = 10), latency of response (0.65 ± 0.14 vs. 0.61 ± 0.13 s; n = 10), or slope of depolarization over the first 3 s (0.90 ± 0.27 vs. 0.92 ± 0.29 %/s; n = 8).

Acetylcholine and phenylephrine application. Application of ACh (57 applications, 22 capillaries, 6 animals) caused a hyperpolarization (Fig. 5A) that began immediately after application. The change in fluorescence corresponds to a maximal change in membrane potential of ~3.5 mV (0.35 %ΔR) ~6 s after drug application. Membrane potential returned to baseline ~20 s after drug application. The fluorescence signal showed a slight, though not statistically significant, overshoot.

Addition of atropine (10−6 M) to the superfuse solution (18 applications, 9 capillaries, 4 animals) reduced the response to ACh to a brief transient (Fig. 5B). The response during atropine superfusion was reduced to ~1.5 mV (0.15 %ΔR). Again, a very slight depolarization is suggested after the signal returned to baseline.

Application of PE (36 applications, 12 capillaries, 5 animals) caused a depolarization (Fig. 6A) that began without detectable latency and reached a maximum of approximately −3.5 mV (~0.35 %ΔR) 4–7 s after application. There was no recovery from the PE application over the time of the observation.

Prazosin (10−7 M) in the superfuse (22 applications, 12 capillaries, 4 animals) completely blocked the PE response (Fig. 6B).

Fig. 2. Distribution of change in fluorescence ratio F620/F560 (%ΔR) obtained from KCl applications of varying time duration to capillaries.

Fig. 3. %ΔR resulting from KCl application (bar denotes 5-s duration) to 6 different capillaries.

Fig. 4. %ΔR from sequential applications of KCl (bars denote times of 2, 5, and 15 s) to same capillary. A steady-state change in F620/F560 is achieved at ~8 s during last application.
For each agonist, the membrane potential response obtained from agonist alone was significantly different from that obtained in the presence of antagonist in the superfusate.

DISCUSSION

KCl, ACh, and PE were chosen for this study because each is known to induce conducted arteriolar responses (6, 9, 22, 23, 27, 28). These responses are thought to be due to changes in membrane potential originating in and/or conducted through endothelial or smooth muscle cells. KCl, ACh, and norepinephrine have also been reported to induce changes in arteriolar blood flow when applied to capillaries (7, 26), presumably from signals generated in the capillaries and then conducted upstream. Also, each of the drugs chosen has a potentially different mechanism of action for inducing changes in membrane potential in capillary endothelial cells.

KCl causes depolarization of endothelial cells by altering K⁺ distribution across the cell membrane and thus would be expected to elicit a change in membrane potential in capillaries. In arteriolar endothelial cells, depolarization and repolarization in response to KCl are characteristically rapid (2, 27, 28). In our experiments, responses in capillaries were slightly slower than those in arteriolar cells. The degree of depolarization was on the same order as that found in experiments by Miao and Joyner (18). They recorded changes in membrane potential in capillaries and venules in hamster mesentery using the fluorescent probe bisoxonol. In their experiments, suffusion of capillaries and venules with a 100 mM K⁺ solution resulted in a 50% change in fluorescence (~8–9 mV depolarization), whereas suffusion and perfusion together of those vessels resulted in a 225% fluorescence change (~36 mV depolarization).

There were substantial differences in the rate and magnitude of KCl responses between capillaries or within the same capillary during different viewing periods in our experiments. This might be explained by a number of potential factors. First, attempts were made to avoid any significant disturbance of connective tissue or superfusate during application of drug to avoid altering the capillary image. Thus the injection pressure (and therefore the amount of drug applied) was necessarily adjusted from capillary to capillary to...
achieve this goal. Second, capillaries vary in their depths within tissue. Thus the amount of, and the rapidity with which, drug actually reached each capillary likely varied. Minor inconsistencies in washout of drug from superfusate flow may also have been present. Third, with the use of a voltage-dependent dye to measure changes in membrane potential, only a relative change in membrane potential from baseline can be determined, not the actual membrane potential. Thus variability in resting membrane potential between capillary segments, or variations in a particular capillary segment between viewing periods, could result in variations in the signal generated by drug application.

ACh binds to muscarinic receptors and activates a potassium current, which causes endothelial cell hyperpolarization and possibly increased intracellular Ca\(^{2+}\) (3, 20). This hyperpolarization of membrane potential in turn is conducted to surrounding smooth muscle cells through gap junctions (3, 21). Previous investigators have determined certain characteristics of endothelial response to ACh stimulation in vitro studies. The magnitude of change in membrane potential in response to ACh stimulation may depend on the baseline endothelial cell resting potential (3, 16). In addition, depolarization of membrane potential after an initial hyperpolarization has been noted (16, 17). Finally, changes in membrane potential induced by ACh in endothelial cells recover slowly, and rapidly repeated stimulations result in responses that are significantly attenuated, if present at all, compared with the initial response (3, 4).

Our results are consistent with these previous findings in that the initial signals obtained were of a hyperpolarizing nature, with a subsequent slight depolarization. Recovery of the membrane potential to baseline was gradual. In addition, when attempted in preliminary studies, we could not reliably obtain repetitive responses to rapid, sequential applications of ACh as we could with KCl.

Phenylephrine acts primarily through adrenergic receptors, and there is evidence to suggest that some endothelial cells may possess \(\alpha_2\)-receptors (13, 24). Some investigators have suggested that PE may induce production of endothelium-derived relaxing factor (1, 13, 21), although this could not be shown in isolated arterioles (28). Given the limited evidence for \(\alpha_2\)-activity in endothelial cells, the possibility should also be considered that the capillary responses to PE may be secondary to an action on surrounding pericytes or perhaps nerves. Pericytes grown in culture have demonstrated depolarization in response to stimulation of \(\alpha_2\)-receptors (12), and there is evidence that pericytes connect to capillary endothelial cells via gap junctions (11, 14, 25). In addition, in arterioles, changes in membrane potential generated in smooth muscle cells by PE are conducted to endothelial cells (28). From these collective findings, one could hypothesize that, in our experiments, PE may cause depolarization in pericytes which is conducted to capillary endothelial cells.

We found that typically membrane potential did not fully recover in the time that a capillary was observed after application of PE. Thus we did not attempt rapid, repeated stimulations of capillary segments with PE. A relatively long period of depolarization compared with duration of drug application may be characteristic of adrenergic binding sites on capillary endothelial cells or surrounding pericytes.

Because we were attempting to detect direct capillary responses, care was taken to minimize the possibilities that the changes in membrane potential observed resulted from stimulation of upstream arterioles with conduction back into capillaries. Capillary segments studied were a minimum of 125–200 \(\mu\)m from the nearest arteriole. We determined, by periodically applying drugs at various distances from arterioles under transillumination, that an arteriolar response due to diffusion over this distance was not present or was delayed on the order of 5–10 s. Multiple random observations under transillumination also revealed that arterioles may have an extremely delayed and minor change in the diameter of upstream arterioles that occurred during application of drug. Multiple observations were also performed during the course of our experiments to determine that local application of drug had either no effect on blood flow within the capillary being visualized or that any changes in flow were delayed well beyond the time course of membrane potential signals being obtained. Time courses showed that changes in capillary membrane potential occurred immediately after application of drug (Figs. 5 and 6). These changes were faster than what might be expected from diffusion effects and suggest that changes in membrane potential were being generated directly from the capillary or pericapillary cells.

The recording windows for viewing capillary segments encompassed connective tissue and other cells in the extravascular space. Membrane potential signals could therefore have been recorded from nonvascular cells if significant amounts of dye had entered the cell membranes after leakage through the vascular wall during loading or from dye that had dissociated from capillaries over time. Because the fluorescence yield from the dye is greatly enhanced in the cell membrane (10), any labeled extravascular cells present in the recording window would be visible. Because we did not observe fluorescence from nonvascular cells adjacent to capillaries or in the connective tissue within recording windows, we conclude that membrane potential responses originated solely from labeled capillary endothelial cells. The dye can be present in aqueous and nonmembrane compartments; however, this will contribute only a background intensity that is not voltage dependent. The effect of background fluorescence on arteriolar recordings has been addressed by Beach et. al. (2). In addition, nonspecific effects of KCl on the dye signal have been addressed by Loew and co-workers (10, 15, 19). The voltage response obtained was shown to follow a Nernst relation in lipid vesicles and cell cultures in response to changes in K\(^+\) concentration, and hence it is unlikely that KCl produced a nonspecific effect on the dye signals that we obtained in our experiments.
In summary, these experiments demonstrate the ability to detect changes in capillary membrane potential in vivo. These changes may be in either a depolarizing or hyperpolarizing direction, and they may be induced by stimuli that act either directly on capillaries or possibly on pericytes or nerves. Changes in membrane potential may be the means by which capillaries communicate with upstream vessels to optimize delivery of blood flow and oxygen to downstream tissue.

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