Membrane Potentials in Retinal Capillary Pericytes: Excitability and Effect of Vasoactive Substances

Horst Helbig,* Sylvia Kornacker,† Susanne Berweck,‡ Frank Stahl,†
Albrecht Lepple-Wienhues,† and Michael Wiederhold†

Retinal capillary pericytes are believed to have a contractile function and to regulate retinal blood flow at the microvascular level. Membrane potential is an important control element for contractility in smooth muscle cells. In the present study, bovine retinal capillary pericytes have been grown in tissue culture and membrane potentials have been measured using glass microelectrodes. Resting potentials averaged $-31 \pm 7$ mV ($n = 203$). Relative K$^+$ conductance was low, with a transference number for K$^+$ of 0.16. Readdition of K$^+$ to K$^+$-depleted cells transiently hyperpolarized the membrane potential, probably by stimulating the electrogenic Na$^+$/K$^+$ transport. Repetitive spike-like depolarizations (action potentials) were induced by stimulating the Na$^+$/K$^+$-ATPase, by applying norepinephrine ($10^{-5}$ mol/l), and by adding 10 mmol/l Ba$^{2+}$. These action potentials depended on the presence of extracellular Ca$^{2+}$ and were inhibited by the Ca$^{2+}$ antagonist nifedipine ($10^{-6}$ mol/l). Norepinephrine ($10^{-5}$ mol/l) depolarized the membrane by $7.4 \pm 3.5$ mV (mean ± SD, $n = 49$). This response was blocked by the alpha-antagonist prazosin ($10^{-5}$ mol/l). Histamine also led to a membrane depolarization of $8.6 \pm 2.8$ mV ($n = 49$), which could be inhibited by the H$_1$-antagonist diphenhydramine. Endothelin ($10^{-7}$ mol/l), vasopressin ($10^{-6}$ mol/l), and acetylcholine ($10^{-4}$ mol/l) had no major effects on membrane potential.

The conclusion is that retinal capillary pericytes are excitable cells and react to several vasoactive substances. Invest Ophthalmol Vis Sci 33:2105-2112, 1992

The capillary wall is composed of two different cell types—endothelial cells and pericytes. Endothelial cells form the capillary tube. Pericytes envelop the endothelial tube from the arteriole to the postcapillary venule. Pericytes envelop pericytes imperceptibly merge into the vascular smooth muscle cells of the precapillary arterioles, forming “intermediate cells” at the transition zone between smooth muscle cells and pericytes.1,2 Very early on, this relation between pericytes and smooth muscle cells led investigators to postulate a contractile function for pericytes.1,2 This assumption is supported by more recent data. Pericytes are equipped with a contractile apparatus, possessing smooth muscle actin3 and contract in vitro in response to vasoactive hormones.4 Tilton2 described morphologic changes of pericytes in situ in response to vasoactive hormones and concluded that these morphologic alterations would reflect pericyte contraction. With this contractile function, pericytes could significantly contribute to the regulation of blood flow at the microvascular level.

A variety of additional functions have been proposed for pericytes. They may serve as a mechanical component in the structural maintenance of the capillary wall, may contribute to the control of blood-tissue barrier integrity, and may play a role in phagocytosis. Pericytes also may be involved in the regulation of endothelial cell growth and function, synthesis of vascular basement membrane, and modulation of capillary growth.2

Pericytes throughout the capillary network are morphologically diverse. This likely reflects different functional requirements of various organs.2 In the retina, pericytes form a particularly tight coverage of the circumference of the capillary endothelial tube, suggesting an important function of pericytes in retinal microcirculation.6

The importance of pericytes in the physiology of retinal microcirculation is emphasized in several pathologic conditions. An early finding in the pathology of diabetic retinopathy is selective loss of pericytes from the capillary wall, which has been viewed as a trigger for ocular neovascularization.7 On the other hand, the greater integrity of retinal microcirculation

---

From the Departments of *Ophthalmology and †Clinical Physiology, Universitätsklinikum Steglitz, Freie Universität Berlin, Hindenburgdamm 30, D-1000 Berlin 45, FRG.
Supported by the Deutsche Forschungsgemeinschaft grant DFG Wi-328/11.
Submitted for publication: July 5, 1991; accepted January 6, 1992.
Reprint requests: Horst Helbig, Department of Ophthalmology, Universitätsklinikum Steglitz, Freie Universität Berlin, Hindenburgdamm 30, D-1000 Berlin 45, FRG.

2105
compared to cerebral microcirculation in acute hypertension might be a result of the tighter coverage of retinal capillaries with pericytes.\(^6\)\(^8\)

In contrast to the importance of pericytes for microcirculation in retina and other tissues, very little is known about the physiology and pathophysiology of these cells. In the present study, bovine retinal pericytes were grown in tissue culture and membrane potentials were measured using conventional glass microelectrodes. We report that pericytes are excitable cells and react to several vasoactive substances.

### Materials and Methods

#### Tissue Culture

Primary cultures of bovine retinal capillary pericytes were established essentially as described by Gitlin and D'Amore.\(^9\) Briefly, bovine eyes were obtained from the local abattoir and transported to the laboratory cooled on ice. After dissection in ethanol and rinsing in sterile phosphate buffered saline (PBS), eyes were cut circularly at the equator, and the anterior part and the vitreous were removed. The retina was peeled from the underlying tissue and transferred into PBS. Care was taken to avoid attachment of retinal pigment epithelium to the retinas. Retinas were minced using crossed scalpel blades, the minced tissue was washed in PBS and incubated for 60 min in a 0.4% collagenase solution (Sigma, St. Louis, MO) in PBS. After digestion in ethanol and rinsing in sterile phosphate buffered saline (PBS), eyes were cut circularly at the equator, and the anterior part and the vitreous were removed. The retina was peeled from the underlying tissue and transferred into PBS. Care was taken to avoid attachment of retinal pigment epithelium to the retinas. Retinas were minced using crossed scalpel blades, the minced tissue was washed in PBS and incubated for 60 min in a 0.4% collagenase solution (Sigma, St. Louis, MO) in PBS. The digested preparation was washed in PBS, filtered through sterile gauze to retain larger vessel fragments, and subsequently transferred into plastic tissue culture flasks (Nunc, Roskilde, Denmark) in Dulbecco's modified Eagle's medium supplemented with 2 mmol/L-glutamine, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, and the following ion concentrations (in mmol/L): 123 NaCl, 28 NaHCO\(_3\), 4 KCl, 1.7 CaCl\(_2\), 1 KH\(_2\)PO\(_4\), and 0.9 MgSO\(_4\). Solutions were gassed with a 5% CO\(_2\)/95% air mixture, resulting in a pH near 7.4. Control Ringer's solution contained 5 mmol/L glucose and the following ion concentrations (in mmol/L): 123 NaCl, 28 NaHCO\(_3\), 4 KCl, 1.7 CaCl\(_2\), 1 KH\(_2\)PO\(_4\), and 0.9 MgSO\(_4\). Solutions were gassed with a 5% CO\(_2\)/95% air mixture, resulting in a pH near 7.4. In solutions containing Ba\(^2+\), BaCl\(_2\) iso-osmotically replaced NaCl. Ba\(^2+\)-containing solutions were SO\(_4^{2-}\)-free, as were the respective control solutions. In solutions that contained higher K\(^+\) concentrations, K\(^+\) replaced Na\(^+\). In nominally K\(^+\)-free solutions, Na\(^+\) replaced K\(^+\).

Ouabain (g-strophanthin) was purchased from Merck (Darmstadt, FRG). Vasopressin, nifedipine, histamine, diphenhydramine, norepinephrine, and prazosin were obtained from Sigma Chemicals. Endothelin-1 (human, porcine) was purchased from Peninsula Laboratories Inc. (Belmont, CA).

#### Membrane Potentials

Successful impalement of a cell led to a steep initial negative deflection of the recorded voltage, which then was followed by a further increase over several minutes until a stable value was reached. The comparatively slow second phase increase in membrane potential was attributed to an improved sealing of the electrode in the membrane. Only voltage recordings

To positively identify our cell preparation as pericyte cultures, we performed indirect immunofluorescence staining for alpha-smooth muscle actin, which can be used to differentiate pericytes from other retinal cells.\(^3\) Cells were fixed in acetone, primary antibody was anti-alpha smooth muscle actin (1:100; Sigma), and secondary antibody was fluorescein-labelled goat antimouse IgG (1:50; Sigma). In the second subpassage, more than 95% of the cells stained for alpha-smooth muscle actin, indicating that our preparation consisted mainly of pericytes.

#### Measurements of Membrane Potentials

The experimental setup has been described and discussed elsewhere in detail.\(^11\)\(^12\) Briefly, a culture dish grown with confluent cells was placed in a temperature controlled container (37°C). A flow chamber was pressed on the culture dish, isolating a small channel that could be superfused with up to eight different test solutions with 90% fluid exchange within 3 sec. Cells in the flow channel were impaled with glass microelectrodes filled with 0.5 mol/L KCl (resistance in Ringer solution: 50–120 MΩ). Membrane voltage was recorded on a chart recorder.

### Results

#### Membrane Potentials

Successful impalement of a cell led to a steep initial negative deflection of the recorded voltage, which then was followed by a further increase over several minutes until a stable value was reached. The comparatively slow second phase increase in membrane potential was attributed to an improved sealing of the electrode in the membrane. Only voltage recordings
that remained within ±2 mV for at least 3 min were considered stable. Impalements that gave a potential difference of less than −20 mV were regarded as leaky and were excluded. In pericytes, the steady state membrane potentials in Ringer’s solution in 203 recordings averaged −31.2 ± 6.7 mV (mean ± standard deviation). The range was from −20 to −50 mV (Fig. 1), but within the same culture dish the potentials recorded from different cells varied by only a few millivolts.

**K⁺ Conductance**

Increasing the extracellular K⁺ concentration led to a rapid and reversible depolarization. The effect of step changes of extracellular K⁺ concentration from 5 mmol/1 in control Ringer’s solution to 10, 20, 40, and 80 mmol/1 is shown in Figure 2. Increasing K⁺ concentrations led to depolarizations with relatively small amplitudes. Increasing extracellular K⁺ concentration from 5 to 40 mmol/1 induced an average depolarization of 8.8 ± 3.2 mV (n = 5). From these data, a transference number for K⁺ in pericytes of 0.16 can be calculated.

**Na⁺/K⁺-ATPase**

Superfusing the monolayer with a nominally K⁺-free solution led to a depolarization of 5.2 ± 1.5 mV (n = 88, Fig. 3A). After 5 min of K⁺ depletion, the cells responded to readdition of K⁺ with a fast hyperpolarization of −19.8 ± 9.4 mV (n = 61) above the steady state potential. Subsequently, the membrane potential declined toward its initial value within 5–10 min (Fig. 3A). The addition of ouabain (10⁻⁴ mol/l) under steady state conditions depolarized the membrane potential by 4.2 ± 1.5 mV (n = 5). Removing extracellular K⁺ in the presence of ouabain (10⁻⁴ mol/l) resulted in a small hyperpolarization (2.8 ± 0.4 mV, n = 5). Readding K⁺ in the presence of ouabain caused a depolarization (3.0 ± 0.5 mV, n = 5).

![Figure 2](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933392/)

**Fig. 2.** Typical recording showing the effect of step changes of extracellular K⁺ concentration from 5 mmol/l in control Ringer’s solution to 10, 20, 40 and 80 mmol/l on the membrane potential. Similar results were obtained in three different recordings.

![Figure 3A](https://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933392/)

**Fig. 3A.** Original recording showing the effect of removing extracellular K⁺ and readdng K⁺ after 5 min of K⁺ depletion. In 50 of 61 similar experiments, we observed a voltage response as shown in (A). (B) Same experimental conditions as in (A). In 11 of 61 experiments, we observed repetitive depolarizations during the phase of slow depolarization toward the resting potential.
Excitability "Action Potentials"

In 203 experiments, recordings under steady state conditions were electrically quiescent. However, in 11 out of 61 experiments in which K⁺ was readded to K⁺-depleted cells, we observed repetitive depolarizations during the phase of slow depolarization toward the resting potential. A consistent finding was that these spike-like voltage deflections started at a membrane potential near -40 mV (Fig. 3B). With time, the amplitude of the voltage deflections became smaller and finally the action potentials ceased (Fig. 3B). If the same maneuver was performed in the presence of ouabain, we observed neither action potentials nor voltage oscillations (n = 3).

The addition of Ba²⁺ (10 mmol/l) under resting conditions depolarized the membrane by 7.4 ± 3.5 mV (n = 65). In 5 out of 65 experiments Ba²⁺ (10 mmol/l) induced repetitive depolarizations (Fig. 4A). After a few minutes, the membrane excitations subsided and ended. Basically, two different types of repetitive voltage deflections were observed with about the same frequency. The first type showed fast, spike-like depolarizations, resembling action potentials. These depolarizations were composed of three phases—a slower depolarization was followed by a fast depolarization and a fast repolarization (Fig. 4B). The second type was characterized by smoother, more sinus wave-like voltage oscillations (Fig. 4C). Transitional forms between these two types of repetitive depolarizations were commonly observed. Both types had similar amplitudes, varying from about 3–35 mV, and frequencies varying from about 5–60 per min.

The incidence of evoking action potentials by application of Ba²⁺ was increased to nearly one half when Ba²⁺ was added to the cells after K⁺ depletion. The effect of simultaneous readdition of K⁺ and Ba²⁺ to K⁺-depleted cells is shown in Figure 5. After an initial hyperpolarization, the membrane potential depolarized. In 13 out of 28 experiments of this kind, repetitive depolarizations as shown in Figure 5 were observed. These began when the membrane voltage had reached -40 mV. In two experiments, we could demonstrate that cells that were not excitable after application of Ba²⁺ alone or by readding K⁺ to K⁺-depleted cells showed repetitive action potentials after simultaneous readdition of K⁺ and Ba²⁺ to K⁺-depleted cells (not shown). The Ba²⁺-induced repetitive voltage deflections were effectively blocked by 10⁻⁶ mol/l nifedipine (n = 4, Fig. 5).

Effect of Vasoactive Substances

The alpha-adrenergic agonist norepinephrine (10⁻⁵ mol/l) depolarized the membrane potential by 7.4 ± 3.5 mV (n = 49, Fig. 6). The norepinephrine-induced voltage effect was not uniform in all cells. In some cells, the depolarization was sustained, and in other cells it was followed by a repolarization after 1–2 min that sometimes reached values above the resting potential (not shown). The alpha₁-antagonist prazosin (10⁻⁵ mol/l) blocked the norepinephrine-induced potential changes (n = 3, Fig. 6).

In 11 out of 49 experiments in which we added norepinephrine to pericytes, we observed repetitive
depolarizations after application of norepinephrine (Fig. 7). We also observed spike-like depolarizations as well as more regularly shaped voltage oscillations upon addition of norepinephrine. Both were absent in nominally Ca2+-free solution (n = 2, Fig. 7) and were inhibited in the presence of the Ca2+-antagonist nifedipine (10^{-5} mol/l, n = 3). If norepinephrine was added to K+-depleted cells simultaneously with readdition of K+ we observed repetitive action potentials or oscillations in 7 out of 18 experiments. The time course of the membrane potential was similar to the recording shown in Figure 5 for simultaneous readdition of K+ and Ba^{2+} to K+-depleted cells (not shown).

Superfusing the monolayer with Ringer’s solution that contained histamine (10^{-5} mol/l) led to an immediate and sustained depolarization (Fig. 8). This depolarization averaged 8.6 ± 2.8 mV in 11 recordings. The H_{3}-histamine receptor blocker diphenhydramine (10^{-5} mol/l) completely blocked the histamine-induced potential response (Fig. 8).

Acetylcholine (10^{-4} mol/l) led to a minimum depolarization of 1-4 mV in four recordings. In three experiments no potential changes were observed with acetylcholine. Endothelin (10^{-7} mol/l, n = 11) and vasopressin (10^{-6} mol/l, n = 8) had no significant or reproducible effect on membrane voltage in pericytes under our experimental conditions.

**Discussion**

Retinal microvascular pericytes can be successfully impaled with microelectrodes in vitro, and intracellular voltage can be recorded for up to 2 hr. Membrane potentials in cultured retinal pericytes averaged -31 mV, a low resting potential compared to other cultured ocular cells.11-15 The low membrane potential goes along with a low relative K+-conductance. Other conductances that contribute to the resting potential are not yet well understood, and no attempts were made to further characterize these membrane conductances.

Ouabain, a relatively specific inhibitor of the Na^{+}/K^{+}-ATPase, depolarized the resting potential by 4 mV. Therefore, we can assume that the electrogenic pump contributes a few millivolts to the membrane potential under resting conditions. We recently have shown that the Na^{+}/K^{+}-ATPase can be markedly stimulated by readding K+ to K+-depleted cells.12 Upon removal of extracellular K+, the pump lacks its substrate and its operation ceases. This leads to a depolarization. Consequently, Na+ is not being pumped out of the cell anymore, and the intracellular Na+ concentration rises. Upon readdition of K+, the ATPase is reactivated. Because the intracellular Na+ concentration regulates the activity of the pump, the electrogenic Na^{+}/K^{+} transport operates with increased activity until the intracellular Na+ concentration reaches its resting level. This activation of the electrogenic pump can be seen in membrane voltage record-
An important finding of the present study is that cultured retinal pericytes are excitable cells. Although electrically quiescent under resting conditions, repetitive spike-like depolarizations could be induced by Ba\textsuperscript{2+} and norepinephrine, and after stimulation of the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase. These repetitive depolarizations were blocked by the Ca\textsuperscript{2+} antagonist nifedipine and inhibited in the absence of extracellular Ca\textsuperscript{2+}. Thus, the excitability seems to involve Ca\textsuperscript{2+} channels. Ba\textsuperscript{2+} probably induces action potentials by passing through Ca\textsuperscript{2+}-channels, thereby depolarizing the membrane. This hypothesis is supported by the fact that Ba\textsuperscript{2+} could induce action potentials even in the absence of extracellular Ca\textsuperscript{2+} (1 mmol/l EGTA added, data not shown). After stimulation of the electrogenic Na\textsuperscript{+}/K\textsuperscript{+}-pump, repetitive voltage deflections were observed starting in the slow depolarizing phase at a potential near -40 mV. The voltage near -40 mV is the apparent threshold for voltage-dependent Ca\textsuperscript{2+} channels at which these channels are most sensitive to voltage changes.\textsuperscript{16} Norepinephrine might act in part via receptor-operated Ca\textsuperscript{2+} channels. That we observed fast spike-like voltage deflections as well as slower sinus wave-like voltage oscillations in response to Ba\textsuperscript{2+} and norepinephrine might reflect the predominance of rapidly or slowly inactivating Ca\textsuperscript{2+} channels in the respective cells. Several experimental procedures reproducibly led to a depolarization of the membrane potential (e.g., increasing extracellular K\textsuperscript{+} concentration, addition of histamine) without inducing oscillatory potentials. These results suggest that depolarization alone is not sufficient for inducing action potentials in pericytes. Other, not yet identified mechanisms likely contribute to the activation of calcium channels, mediating action potentials after application of Ba\textsuperscript{2+} or norepinephrine, or after stimulation of the electrogenic Na\textsuperscript{+}/K\textsuperscript{+} pump.

Excitability is a prominent feature of cell membranes of muscular origin, and action potentials have been recorded from a variety of different vascular smooth muscle cells.\textsuperscript{17,18} The finding that retinal pericytes have smooth muscle-like excitable membranes with voltage-dependent Ca\textsuperscript{2+}-channels further supports the assumption that pericytes are closely related to vascular smooth muscle cells. Therefore, pericytes could largely contribute to regulation of blood flow at the microvascular level.

A major factor in the regulation of contractility of vascular smooth muscle cells, and most likely of pericytes, is the intracellular Ca\textsuperscript{2+} concentration.\textsuperscript{16} Ca\textsuperscript{2+} induces phosphorylation of myosin chains and hence development of contractile force. Knowledge of the regulation of intracellular Ca\textsuperscript{2+} concentration therefore is important in the understanding of control mechanisms of vascular smooth muscle (and pericyte) tone. Vasoactive drugs may influence intracellular Ca\textsuperscript{2+} concentration by directly acting on Ca\textsuperscript{2+}.
channels. On the other hand, the membrane potential can control intracellular Ca$^{2+}$ concentration through the voltage dependence of Ca$^{2+}$ channels. Substances that depolarize the membrane would indirectly increase the open-state probability of Ca$^{2+}$ channels, intracellular Ca$^{2+}$ concentration would rise, and the contractile force would be enhanced. Hyperpolarization would have an opposite, relaxing effect. Hormones that act on the membrane potential therefore can be expected to alter contractility of vascular smooth muscle cells (and pericytes) and modulate vascular resistance and blood flow.

Norepinephrine depolarized pericycle membranes and induced action potentials. This effect could be blocked by the alpha, antagonist prazosin. Alpha, agonists have been well characterized as vasoconstrictors in many vessels, including bovine retinal arteries. This would agree with a constrictive norepinephrine effect on retinal pericytes. In cerebral pericytes, mainly alpha$_1$ receptors have been found. Thus, differences seem to exist in retinal and cerebral pericytes.

Endothelin is a recently discovered peptide-hormone with strong vasoconstrictive potency. In vascular smooth muscle cells, endothelin induced a rise in intracellular Ca$^{2+}$ activity and depolarized the membrane potential. In retinal pericytes, receptors for endothelin have been described, and endothelin has been shown to increase intracellular Ca$^{2+}$ and contract pericytes in vitro. We did not find a significant effect of endothelin on membrane potential in retinal pericytes. However, in our hands, too, endothelin led to a rise in intracellular Ca$^{2+}$ concentration (data not shown). These results suggest that in pericytes, endothelin acts on intracellular Ca$^{2+}$ without significant changes in membrane potentials.

Vasopressin and acetylcholine have been shown to induce marked depolarization and action potentials in aortic vascular smooth muscle cells. In retinal pericytes, we did not observe a major effect of these hormones on membrane potential. Thus, although retinal pericytes share some electrical membrane properties with vascular smooth muscle cells of larger vessels, there are some important differences.

Histamine is a vasoactive hormone that has been shown to exert relaxing and contracting effects on various vascular and nonvascular smooth muscle cells. Two classes of histamine-receptors have been described. In most cases, muscle contraction has been shown to be due to activation of H$_2$ receptors, while H$_1$ receptors often mediate muscle relaxation. In our experiments, we found a significant depolarization induced by histamine that could be blocked by the H$_1$ antagonist diphenhydramine, indicating the presence of H$_1$ receptors in retinal pericytes. In an in vitro study, histamine has been shown to contract retinal pericytes. These data would argue for a vasoconstrictive effect of histamine in the retinal microvasculature.

In summary, retinal capillary pericytes are excitable cells that show Ca$^{2+}$-mediated oscillatory potentials. This is a typical feature of cells of muscular origin. Membrane depolarizations were observed in response to several vasoactive substances. Pericycle membrane potential therefore may be an important control element in pericycle contractility and regulation of retinal blood flow at the microvascular level.

Key words: pericytes, membrane potentials, retinal capillaries, action potentials, tissue culture

Acknowledgments

The excellent technical assistance of Marianne Koch and Astrid Krolik is gratefully acknowledged.

References


