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Retinovascular physiology and pathophysiology: new experimental approach/new insights

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Abstract

An important challenge in visual neuroscience is understand the physiology and pathophysiology of the intra-retinal vasculature, whose function is required for ophthalmocception by humans and most other mammals. In the quest to learn more about this highly specialized portion of the circulatory system, a newly developed method for isolating vast microvascular complexes from the rodent retina has opened the way for using techniques such as patch-clamping, fluorescence imaging and time-lapse photography to elucidate the functional organization of a capillary network and its pre-capillary arteriole. For example, the ability to obtain dual perforated-patch recordings from well-defined sites within an isolated microvascular complex permitted the first characterization of the electrotonic architecture of a capillary/arteriole unit. This analysis revealed that this operational unit is not simply a homogenous syncytium, but has a complex functional organization that is dynamically modulated by extracellular signals such as angiotensin II. Another recent discovery is that a capillary and its pre-capillary arteriole have distinct physiological differences; capillaries have an abundance of ATP-sensitive potassium (K_{ATP}) channels and a dearth of voltage-dependent calcium channels (VDCCs) while the converse is true for arterioles. In addition, voltage transmission between abluminal cells and the endothelium is more efficient in the capillaries. Thus, the capillary network is well-equipped to generate and transmit voltages, and the pre-capillary arteriole is well-adapted to transduce a capillary-generated voltage into a change in abluminal cell calcium and thereby, a vasomotor response. Use of microvessels isolated from the diabetic retina has led to new insights concerning retinal vascular pathophysiology. For example, soon after the onset of diabetes, the efficacy of voltage transmission through the endothelium is diminished; arteriolar VDCCs is inhibited, and there is increased vulnerability to purinergic vasotoxicity, which is a newly identified pathobiological mechanism. Other recent studies reveal that K_{ATP} channels not only have an essential physiological role in generating vasomotor responses, but their activation substantially boosts the lethality of hypoxia. Thus, the pathophysiology of the retinal microvasculature is closely linked with its physiology.

Keywords

Arteriole; Capillary; Diabetes; Hypoxia; K_{ATP} channels; Purinergic vasotoxicity; Voltage-dependent calcium channels

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1. Introduction

1.1. Retinal vasculature: unique task/unique adaptations

The circulatory system of the retina has the unique task of supplying oxygen and nutrients to a tissue whose translucency is essential for function. Throughout much of evolution, the metabolic needs of the vertebrate retina were met by the diffusion of nutrients and oxygen from the choriocapillaris, which is a dense vascular complex located beneath the retina and thus, not in the path of light passing to the photoreceptors. However, because an exclusive dependence on diffusion limits how far away neurons of the inner retina can be from the choriocapillaris, avascular retinas must be relatively thin (Chase, 1982; Buttery *et al.*, 1991).

In contrast to sub-mammalian retinas, the retinas of most mammals and all primates contain blood vessels. By eliminating the exclusive dependence on the choriocapillaris, vascularized retinas are able to process visual information with a significantly thicker inner synaptic layer (Chase, 1982; Buttery *et al.*, 1991). On the other hand, because intra-retinal blood vessels deflect incoming photons, they can compromise visual function. As a likely adaptation to limit blood vessel-induced image degradation, the density of capillaries in the retina is particularly low (Funk, 1997). However, this paucity of microvessels leaves little functional reserve for the vital task of adjusting local perfusion to meet the stringent metabolic demands of retinal neurons. Thus, it is particularly important that blood flow in the retina be especially tightly coupled to local needs.

Specialized adaptations enhance the ability of the retinal vasculature to effectively match blood flow to meet local metabolic needs despite having a low capillary density. One important adaptive feature of the retina's circulatory system is its functional independence. Another important feature is its highly decentralized functional organization.

The ability of the retinal vasculature to function independently is established, in part, by its tight blood-retinal barrier, which prevents circulating vasoactive molecules from directly affecting the contractile cells located on the blood vessel wall. Independence is also enhanced by the retinal vasculature's lack of autonomic innervation (Ye *et al.*, 1990), which in other vascular beds, conveys CNS oversight of local blood flow. As a consequence of these adaptations, retinal blood flow is largely autoregulated and thus, is not subject to being diminished due to the metabolic demands of non-retinal tissues.

In addition to being autoregulated, the retinal vasculature has a highly decentralized functional organization that facilitates the efficient coupling of local perfusion to local needs. Indicative of its decentralized organization, the retina's circulatory system, unlike that of other tissues, consists exclusively of microvessels, i.e., arterioles and capillaries. Also consistent with a highly developed decentralized operation, the capillaries of the retina are reported to have the highest density of pericytes (Shepro & Morel, 1993), whose contractions and relaxations are thought to regulate local perfusion by altering the capillary lumen (Anderson, 1996; Funk, 1997; Peppiatt *et al.*, 2006; Puro, 2007; Hamilton *et al.*, 2010; Bonkowski *et al.*, 2011). In addition, evidence is emerging that a capillary network plus its pre-capillary tertiary arteriole constitute an operational unit that is well adapted to regulate local perfusion at this decentralized location within the retinal vasculature (Oku *et al.*, 2001; Matsushita *et al.*, 2010; Zhang *et al.*, 2011).

1.2 Research questions

Because visual function in humans, sub-human primates and most mammals is dependent on the proper functioning of the intra-retinal vasculature, an important research quest is to understand the physiology and pathophysiology of this portion of the circulatory system. Importantly, due to the specialized adaptations of the retinal vasculature, an understanding

of how it functions in health and disease cannot be derived simply by extrapolating findings from the study of non-retinal blood vessels. Rather, the circulatory system of the retina must be analyzed in its own right.

Due to the importance the retinal vasculature, it has received the attention of many talented investigators whose efforts have resulted in a substantial literature that has significantly advanced understanding (Schonfelder *et al.*, 1998; Clermont & Bursell, 2007; Metea & Newman, 2007; Scholfield *et al.*, 2007; Pournaras *et al.*, 2008; Hamilton *et al.*, 2010; Hein *et al.*, 2010). However, because this review is a highly selective assessment focusing exclusively on physiological and pathophysiological studies of the most distal portion of the retinal microvasculature, i.e. the capillary/pre-capillary arteriolar complex, many studies of the retina's circulatory system do not receive attention. In addition, the breadth of this overview is further limited by our emphasis on advances facilitated by the use of a newly developed experimental preparation, i.e., tissue print isolation of the retinal microvasculature.

Although the capillary/arteriole complex serves as the most decentralized site of blood flow regulation in the retina, the application of powerful investigational techniques such as patch-clamping to elucidate the physiology and pathophysiology of this operational unit remained very limited until recently because of the lack of a suitable experimental preparation. However, this problem has been addressed by the development of a method to isolate vast microvascular complexes from the retina of the adult rat, whose intra-retinal vasculature shares important similarities with that of humans (Zhang, 1994). The ability to study freshly isolated living retinal microvessels now permits the application of the patch-clamp technique, as well as fluorescent-imaging, time-lapse photography and cell viability assays, to the study of the distal most portion of the retinal microvasculature.

Here, we chiefly emphasize progress made since the appearance of an earlier review (Puro, 2007). Here, we summarize recent progress made by using isolated capillary/arteriolar complexes to address two key questions: (1) How is the distal microvasculature of the retina functionally organized? and (2) How does the physiology of the retinal microvasculature influence its vulnerability to pathophysiological conditions?

2. New experimental approach: the tissue print preparation of retina microvascular complexes

A tissue print method for isolating vast complexes of rat retinal microvessels has provided an experimental preparation that makes it relatively straight forward to quantify ion channel activity, cell-to-cell voltage transmission, intracellular calcium, endogenous oxidants and cell death in order to better understand the physiology and pathophysiology of the capillary/arteriole unit.

2.1 Methodology

To facilitate the widespread utilization of the tissue print technique for the preparation of retina microvascular complexes, methodological details are provided here that go well beyond what is possible to present in other publications. This method has been optimized for the isolation of microvascular complexes from the retinas of Long-Evans rats (Charles Rivers, Boston, MA), although it has also proven effective for isolating microvessels from the mouse.

At 5 weeks to 6 months of age, rats that have been provided food and water *ad libitum* and maintained from birth under a 12-h low light (0.05 – 0.25 lux)/dark cycle are euthanized with carbon dioxide. Immediately after death, a modification of the Winkler technique is

used to rapidly (< 3 s) remove each retina. This technique begins with a #11 scalpel blade being used to vertically hemisect the cornea and also to incise the conjunctiva. A curved forceps (catalog 11009-13, Fine Science Tools, Inc., Foster City, CA) is then used to grasp the optic nerve at the site where it exits the eyeball. With the forceps nearly closed, it is brought anteriorly, and the eye is squeezed by the forceps' arms. When the forceps exits the orbit, the retina along with attached vitreous is found to be draped over the forceps' arms.

The isolated retina with attached vitreous is promptly placed in a 60 mm Petri dish containing 10 ml of a solution (solution A), which consists of 140 mM NaCl, 3 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 10 mM Na-Hepes, 15 mM mannitol, and 5 mM glucose at pH 7.4 with osmolality adjusted to 310 mosmol l⁻¹, as measured by a vapor pressure osmometer (Wescor, Inc., Logan, UT, USA). With the aid of fine forceps (Dumont #5, Fine Science Tools, Inc.), adherent vitreous is fastidiously, but efficiently, removed from the retina; of note, this step is critical in order to successfully obtain large microvascular complexes consisting of a secondary arteriole, a tertiary arteriole and a capillary network (Fig. 1). Next the retinas are incubated for ~24 min at 30°C in a 20-ml scintillation vial (03-337-4, Fisher Scientific, Pittsburgh, PA, USA) containing 2.5 ml Earle's balanced salt solution (E2888, Sigma, St.Louis, MO, USA) supplemented with 0.5 mM EDTA, 6 U papain (2X crystalline, catalog # LS003124, Worthington Biochemicals, Freehold, NJ) and 2 mM cysteine; immediately prior to placement of the retinas into this solution, its pH is adjusted to 7.4 by bubbling 5% carbon dioxide. Of note, for each new lot of papain, the duration of incubation is empirically adjusted to optimize the yield of microvascular complexes; in testing dozens of lots of papain, we have found optimal incubation durations to be between 23 and 25 minutes.

After incubation in the papain-containing solution, the retinas are transferred to a 60 mm Petri dish containing 10 ml of solution A at room temperature. Each retina is then cut into quadrants so that each piece contains one of the four primary arterioles extending from the optic disc. Subsequently, one of the retinal quadrants is placed in a diamond-shaped glass-bottomed chamber (similar to the RC-16 bath chamber produced by Werner Instrument Corporation, Hamden, CT). With 1 ml of solution A in the chamber, a retinal quadrant is positioned on the glass bottom with the vitreal (inner) retinal surface facing up. Then, with the aid of a Dumont #5 forceps, a 15 mm diameter #1 glass coverslip (CS-15R, Warner Instrument Corporation) is extremely gently positioned onto the retinal quadrant. If necessary, effort is made during this compression step to uncurl the piece of retina so that it is not folded as it becomes gently compressed. Of note, prior to their use, coverslips have been cleaned by exposure for 5 to 15 min in a 50% ethanol solution followed by a 5 min wash in distilled water; no special coating is applied to the coverslips. After approximately 30 s of the retinal quadrant being gently sandwiched between the coverslip and the bottom of the chamber, the coverslip is carefully removed and promptly placed in a Petri dish containing solution A. Of note, care is taken to quickly, but as non-traumatically as possible, pass the microvessel-containing coverslip through the meniscus of the solution and position it, with its vessel-containing surface up, on the bottom of the Petri dish. For each quadrant, this tissue print step is repeated with up to three coverslips.

A pair of retinas typically generates 4 or 5 coverslips containing microvascular complexes. Most of the isolated microvascular complexes consist of: (1) a network of 6 µm diameter capillaries with abluminally located pericytes whose somas appear as "bumps on a log" and have a density of 4 per 100 µm, (2) a ~400 µm long pre-capillary tertiary arteriole whose diameter is 7 to 10 µm and whose abluminal surface has 5 "dome-shaped" mural cell somas per 100 µm and (3) a 12 to 18 µm diameter secondary arteriole that is encircled by a layer of "doughnut-shaped" smooth muscle cells and that may be many millimeters in length. Of note, as *in vivo*, capillaries occasionally exit directly from a secondary arteriole.

Microvascular complexes isolated by this tissue print method bear a striking physical resemblance to trypsin digest preparations of the retinal vasculature; however, very importantly, the complexes isolated by this tissue print technique are alive with ~98% of the cells in studied microvascular complexes capable of excluding the vital dye, trypan blue (Nakaizumi & Puro, 2011). Microvascular complexes isolated by this tissue print technique are shown in Figure 2, as well as in a series of recent publications (Matsushita & Puro, 2006; Puro, 2007; Zhang *et al.*, 2011). In addition, time-lapse videos showing vasomotor responses of isolated retinal microvessels are available as supplemental material to half a dozen papers (Kawamura *et al.*, 2003; Wu *et al.*, 2003; Kawamura *et al.*, 2004; Yamanishi *et al.*, 2006; Ishizaki *et al.*, 2009; Matsushita *et al.*, 2010).

2.2 Experimental advantages and caveats

The ability to isolate vast complexes of the retinal microvasculature has permitted the use of the patch clamp technique, calcium-imaging and time-lapse photography to assess the effects of extracellular vasoactive molecules on the ionic currents, intracellular calcium and abluminal cell contractility (Kawamura *et al.*, 2003; Kawamura *et al.*, 2004; Yamanishi *et al.*, 2006). Furthermore, the ability to easily distinguish capillaries and pre-capillary tertiary arterioles allows the monitoring of currents, calcium and contractions at specific microvascular locations (Matsushita & Puro, 2006; Ishizaki *et al.*, 2009; Matsushita *et al.*, 2010). Also, it is not technically challenging to use mini-perfusion (Ishizaki *et al.*, 2009) to apply pharmacological agents, such as a K_{ATP} channel activator, focally onto specific sites within the retinal microvasculature. It is also feasible to transect an isolated microvascular complex at its capillary/arteriolar junction in order to determine whether an ionic current is generated in the capillary network and/or in the pre-capillary arterioles (Ishizaki *et al.*, 2009). In addition, it is relatively straightforward to quantify the efficacy of electrotonic transmission within the retinal microvasculature by simultaneously obtaining dual perforated-patch recordings from abluminal cells located at precisely defined microvascular locations (Wu *et al.*, 2006; Zhang *et al.*, 2011; Nakaizumi *et al.*, 2012). Also with isolated microvascular complexes, it is easy to quantitatively compare the amount of cell death occurring in capillaries and arterioles under pathophysiological conditions such as hypoxia (Nakaizumi & Puro, 2011). An additional advantage of studying isolated microvessels is that the effects of exogenously applied chemicals can be assessed in the absence of potentially confounding responses of non-vascular cells.

However, despite the experimental advantages of isolated retinal microvascular complexes, it is clear that conclusions based on studies of these microvessels ultimately require *in vivo* validation, although utilizing patch-clamping, calcium-imaging and time-lapse photography at precise locations within the retinal microvasculature appears at present to be technically unfeasible *in oculo*. Clearly, the possibility of species differences also warrants caution in extrapolating conclusions based on the study of rodent retinal microvessels to the microvasculature of the human retina. In addition, because the tissue print preparation is glia- and neuron-free, it is of limited use for directly determining how neuronal activity alters blood flow or how an increase in glial cell calcium elicits vasomotor responses, as has been observed in the intact retina (Metea & Newman, 2006). Furthermore, since internal perfusion of the tertiary arterioles and capillaries has proven to be technically challenging, the effects of internal pressure- and flow-related shear stress, as well as the effects of the intra-luminal application of putative vasoactive molecules, have not been systematically evaluated. However, despite caveats, this experimental preparation has helped to reveal a number of previously unappreciated mechanisms that are likely to play important roles in the physiology and pathophysiology of the retinal microvasculature.

3. Retinal microvascular physiology: new insights

The availability of isolated retinal microvascular complexes consisting of secondary and tertiary arterioles, as well as a capillary network, has greatly facilitated the use of the patch-clamp technique to help elucidate the functional organization of the retinal microvasculature.

3.1. Electrotonic architecture of the retinal microvasculature

Since voltages induced by extracellular signals play a key role in generating vasomotor responses, it is important to characterize how voltage is transmitted within the retinal microvasculature. Use of isolated retinal microvessels allowed the first characterization of the electronic architecture of a capillary/arteriolar complex of any vascular bed (Zhang *et al.*, 2011). This was done by obtaining nearly a hundred simultaneous dual perforated-patch recordings from defined microvascular sites.

To measure the efficacy of electrotonic transmission within the retinal microvasculature, voltages are simultaneously monitored via perforated-patch pipettes located at two sites on a microvessel as a current step was injected via one of the pipettes. Recent publications have illustrated this dual recording technique for assessing electrotonic transmission in the retinal microvasculature (Kawamura *et al.*, 2003; Wu *et al.*, 2006; Zhang *et al.*, 2011). For each pair of recordings, the ratio of the voltage change detected at the non-stimulated site ($\Delta V_{\text{responder}}$) to the voltage step induced at the site of current-injection ($\Delta V_{\text{stimulator}}$) is plotted against the inter-pipette distance. From these data, the efficacies by which voltages are transmitted axially through the endothelium and radially between abluminal cells and endothelial cells can be calculated based on conclusions of a detailed assessment of various models of the retinal microvasculature's electrotonic architecture (Wu *et al.*, 2006; Zhang *et al.*, 2011). From this assessment, it was ascertained that the linear regression fit of a semi-log plot of $\Delta V_{\text{responder}}/\Delta V_{\text{stimulator}}$ ratios *versus* the inter-pipette distance yields key information. Namely, because the decay of an axially spreading voltage is a first-order process, the slope of the linear regression fit is the rate at which voltage decays as it is transmitted axially. Additional important information is derived from the y-intercept value, which is the extrapolated $\Delta V_{\text{responder}}/\Delta V_{\text{stimulator}}$ ratio at 0 μm . As explored in substantial detail (Wu *et al.*, 2006; Zhang *et al.*, 2011), a y-intercept value of less than 1.0, as is observed for each region of the retinal microvasculature (Zhang *et al.*, 2011), reflects the total amount of voltage dissipation that occurs during radial abluminal/endothelial cell transmission both at the current-injection site and at the distantly recorded site. Consequently, the extrapolated $\Delta V_{\text{responder}}/\Delta V_{\text{stimulator}}$ ratio at 0 μm is the product of the radial transmission efficacies at the stimulated and distal sites, and thus, the square root of the y-intercept value of the linear regression fit is the efficacy of radial transmission.

An analysis of nearly 100 dual recording experiments revealed that the retinal microvasculature is not simply a homogeneous syncytium, but possesses a complex electrotonic architecture (Zhang *et al.*, 2011).

3.1.1. Axial transmission—Dual recording experiments using isolated retinal microvascular complexes showed that voltage spreading axially through capillaries, tertiary arterioles and secondary arterioles is transmitted with a decay rate of only $\sim 2\%/100 \mu\text{m}$ (Table 1). Detailed analysis yielded strong support for a model of the electrotonic architecture in which axial transmission is almost exclusively via the gap junction pathway linking endothelial cells rather than via abluminal cell-to-abluminal cell communication (Zhang *et al.*, 2011).

In contrast to the highly efficient transmission within capillaries and the arterioles, voltage decreases by ~40% as it passes through a capillary/arteriole branch point and also as it is transmitted from a tertiary arteriole to a secondary arteriole (Table 1). On the other hand, although there is a significant attenuation of voltage at branch points between these microvascular regions, transmission at branches within the capillaries and arterioles does not cause a detectable voltage loss. While an explanation for the differing effects of microvascular branch points remains uncertain, modeling studies based on data obtained from larger non-retinal arterioles suggest that the relative diameters and capacitative loads of the pre- and post-branch vessels determine how much, if any, voltage dissipation occurs at a branch point (Segal & Neild, 1996). At present, it is unclear what the functional consequence is of the ~40% dissipation that occurs at the junction of a capillary and a tertiary arteriole and at the junction of tertiary and secondary arteriole. One proposal is that a transmission efficacy that is well below 100% enhances the ability of the proximal vessel to integrate inputs from multiple distal branches (Zhang *et al.*, 2011).

3.1.2. Radial transmission—Dual perforated-patch recordings from isolated retinal microvascular complexes also revealed that significant voltage dissipation occurs during radial transmission between an abluminal cell and the endothelium (Zhang *et al.*, 2011). Interestingly, the efficacy of radial abluminal cell-to-endothelial cell transmission is not uniform in the retinal microvasculature. Namely, the efficacy of radial transmission is 76% in the capillaries while abluminal cell/endothelial cell transmission in the tertiary and secondary arterioles is ~45% (Table 1).

The observation that radial transmission is most efficient in the capillaries supports a model of the retinal microvasculature in which the capillary network is particularly well adapted for the transmission of voltages generated in response to vasoactive signals (Matsushita *et al.*, 2010). Due to the relative efficiency of radial transmission in the capillaries, most of the voltage generated by an abluminal pericyte is transmitted to the underlying endothelium, which then provides a pathway for proximal transmission.

3.1.3. Transmission velocity—Dual recordings in isolated retinal microvascular complexes permitted the first determination of the velocity at which a voltage is transmitted within the capillaries and small arterioles (<10 μm diameter) of any vascular bed. These experiments showed that the conduction velocity is ~50 mm s^{-1} throughout the capillary/arteriole complex (Zhang *et al.*, 2011). Interestingly, similar transmission velocities are reported in studies of larger (> 50 μm diameter) blood vessels of non-retinal tissues (Tsuchiya & Takei, 1990; Stevens *et al.*, 2000; Emerson *et al.*, 2002). Thus, it may be that a conduction velocity of about 50 mm s^{-1} is a general feature of the circulatory system.

3.1.4. Modulation by angiotensin II—A fundamental question concerning the electrotonic architecture of the retinal microvasculature is whether it is static or dynamic. Recent dual recording experiments assessing the effect of angiotensin II on voltage transmission revealed that the electrotonic architecture is dynamic, rather than unchanging. The actions of angiotensin are of interest since this molecule is not only present in the circulation, but can be produced by the renin-angiotensin system of the retina (Kohler *et al.*, 1997; Fletcher *et al.*, 2010). In addition, it is well established that angiotensin II causes retinal arterioles and capillaries to constrict, with the smaller vessels being most sensitive to this vasoactive signal (Schonfelder *et al.*, 1998; Kulkarni *et al.*, 1999).

Electrophysiological studies using freshly isolated retinal microvascular complexes recently revealed for the first time that in addition to causing vasoconstriction (Kawamura *et al.*, 2004), angiotensin II also reversibly inhibits axial transmission (Zhang *et al.*, 2011). A dual perforated-patch recording experiment demonstrating angiotensin's inhibition of electrotonic

transmission is illustrated in Figure 3. Based on quantitative analyses of a large series of dual recordings, the decay rate for voltages passing axially through capillaries and arterioles during exposure to angiotensin is ~50%/100 μm , which is a markedly faster rate of decay than the 2%/100 μm observed under control conditions (Table 1). Although angiotensin II potently inhibits axial transmission, the efficacy of radial abluminal cell/endothelial cell transmission is not significantly affected. In addition, transmission at capillary/arteriolar branch points and at the junction of tertiary and secondary arterioles is also not altered by this vasoactive molecule (Table 1). Thus, axial endothelial cell-to-endothelial cell transmission is selectively inhibited by this molecule. At present, the molecular basis for the differential sensitivity of axial, as compared with radial, transmission to the inhibitory effect of angiotensin II is unknown, although a difference in the type of connexins constituting endothelial cell-to-endothelial cell and abluminal cell-to-endothelial cell gap junctions arterioles is likely to be of importance.

As a consequence of angiotensin's inhibition of axial transmission, the spread of locally generated voltages is markedly limited (Fig. 4). Thus, by inhibiting axial transmission, angiotensin II regulates the geographical extent of the microvasculature's response to voltage-changing inputs (Zhang *et al.*, 2011).

Interestingly, even though >50,000 papers have been published about angiotensin, this vasoactive signal's inhibition of voltage transmission is a newly appreciated action. On the other hand, it has been reported the angiotensin II enhances the spread of a conducted vasoconstriction or a local electrical stimulation within rat mesenteric arterioles (Gustafsson & Holstein-Rathlou, 1999). Whether this enhancing effect on transmission is due to an increase in membrane resistance and/or a change in gap junctions remains unknown. In the retinal microvasculature, a change in membrane resistance does not appear to be the cause of angiotensin's inhibition of electrotonic transmission since this effect is robust in the arterioles whose membrane resistance is not affected by this vasoactive molecule (Zhang *et al.*, 2011).

Because endothelin-1 and ATP also inhibit voltage transmission within isolated retinal microvessels (Kawamura *et al.*, 2002; Kawamura *et al.*, 2003), it is clear that the ability to regulate the spatial extent over which voltages are transmitted is not a unique attribute of angiotensin II. Not only do these new observations reveal a previously unappreciated action for these vasoactive molecules, but they also establish that the electrotonic architecture of the retinal microvasculature is dynamic, not static.

3.2. Functional sub-specialization of the retinal microvasculature

Recent studies of isolated retinal microvascular complexes have yielded new information concerning how the capillary/arteriolar complex is functionally organized.

3.2.1. Topographical heterogeneity of ion channels—Use of the tissue print preparation of retinal microvascular complexes revealed that the physiology of the capillaries and the tertiary arterioles differs significantly. Evidence of the functional sub-specialization within the retinal microvasculature is the recent discovery that functional ATP-sensitive potassium (K_{ATP}) channels are located predominately in the capillaries while voltage-dependent calcium channels (VDCCs) are operative chiefly in the arterioles.

3.2.1.1. Location of voltage-dependent calcium channels: Investigations using isolated retinal microvessels recently determined the topological distribution of functional L-type voltage-dependent calcium channels (VDCCs) in the capillary/arteriole unit (Matsushita *et al.*, 2010). VDCCs are of importance since their voltage-dependent regulation of calcium influx allows the transduction of a change in voltage into a change in cell calcium, which by

regulating the contractile tone of arteriolar mural cells changes the diameter of the arteriolar lumen and thereby, alters blood flow. Multiple lines of evidence support the operational concept that VDCC function is chiefly in the arteriolar portion of the capillary/arteriole unit. For example, as shown in Fig. 5, patch-clamp recordings showed that the density of the VDCC current is markedly greater in tertiary arterioles than in the capillaries (Matsushita *et al.*, 2010). Also indicative of functional VDCCs being predominantly in the tertiary arterioles, calcium-imaging studies demonstrated that depolarization-induced VDCC-dependent calcium increases are substantially larger in the arteriolar mural cells than occurs in the capillary pericytes. Furthermore, depolarization causes a VDCC-dependent contraction of the abluminal mural cells of the tertiary arterioles, but not of the pericytes located on capillaries. Also consistent with VDCCs playing a role in arterioles, but not in capillaries, depolarization induces a VDCC-dependent vasoconstriction in the tertiary arterioles, but not in the capillary network.

Of note, although the endothelium of the circulatory system is generally considered to lack VDCCs, an absence of functional VDCCs in the retinal endothelium has not been established, even though the α_{1C} subunit of the L-type calcium channel is detected by immunocytochemistry almost exclusively in abluminal cells of the retinal microvasculature (Matsushita *et al.*, 2010).

The finding that the localization of functional VDCCs is overwhelmingly in the pre-capillary tertiary arterioles lends further support for the emerging concept of operational sub-specialization within the capillary/tertiary arteriole unit.

3.2.1.2. Location of ATP-sensitive potassium channels: K_{ATP} channels are critically important in the retinal microvasculature because the activation of these channels generates the hyperpolarization required to instigate a vasomotor response to adenosine (Li & Puro, 2001; Ishizaki *et al.*, 2009), which is released by metabolically compromised retinal cells (Roth *et al.*, 1997). Recent study of isolated microvascular complexes showed that K_{ATP} currents are generated almost exclusively in the capillary portion of the capillary/arteriolar complex (Ishizaki *et al.*, 2009). One line of evidence for this topographical distribution was obtained by focally applying the selective K_{ATP} channel activator, pinacidil, at sites along isolated retinal microvascular complexes while voltage was monitored via a perforated-patch pipette. As illustrated in Figure 6, the amplitude of the hyperpolarization induced by pinacidil varied markedly depending on the microvascular site at which it was mini-perfused. Numerous similar experiments consistently demonstrated that the largest K_{ATP} channel-induced hyperpolarizations are generated in the capillaries (Ishizaki *et al.*, 2009).

Additional evidence for the heterogeneous distribution of functional K_{ATP} channels was derived from experiments taking advantage of the ease by which it is possible to transect isolated microvascular complexes at their capillary/arteriolar junctions (Ishizaki *et al.*, 2009). Specifically, the tip of a glass micropipette that is identical in shape to our usual recording pipette is moved in a controlled fashion by use of a piezoelectric-based micromanipulator while the microvascular complex of interest is viewed at X400 magnification with an inverted microscope equipped with phase-contrast optics. With the micropipette tip just barely touching the bottom of the microvessel-containing coverslip, a microvessel is transected at the tertiary arteriolar/capillary junction. Although a transient depolarization often occurs as a microvessel is transected, we found that the resting membrane potentials of the arteriolar and capillary portions to the microvascular complex were not significantly affected (Ishizaki *et al.*, 2009).

After transection of a microvascular complex at its capillary/arteriolar junction, the arteriole portion of the microvasculature failed to respond to pinacidil (Fig. 7). In contrast, a robust

hyperpolarizing current was detected by a perforated-patch pipette sealed onto the capillary that was no longer connected to the arterioles. Consistent with functional K_{ATP} channels being located predominately in the capillaries, transection experiments also established that the K_{ATP} conductance activated during exposure to adenosine is generated almost exclusively in the capillaries (Ishizaki *et al.*, 2009).

Of note, although K_{ATP} current has been detected in individual retinal pericytes that have been made electrically isolated from the endothelium (Sakagami *et al.*, 2001), there is no evidence excluding K_{ATP} channels from also being located in the plasma membrane of the endothelial cells of the retina.

Experiments using the patch-clamp to quantify K_{ATP} currents, dichlorofluorescein fluorescence to detect endogenous oxidants and time-lapse photography to assess vasomotor responses in freshly isolated retinal microvessels recently yielded information that provides for a more complete understanding of the physiology of the capillary K_{ATP} channels (Ishizaki *et al.*, 2009). Namely, these ion channels are redox-sensitive; their functionality is established by endogenous oxidants, and the oxidized redox status required for capillary K_{ATP} channels to be activatable depends on endogenous polyamines whose catabolism is known to generate H_2O_2 and other potent oxidants (Wang & Casero, 2006). Taken together, these findings and other recent observations (Ishizaki *et al.*, 2009) revealed for the first time that the ability of a capillary/arteriole unit to generate a vasomotor response to adenosine is dependent on the polyamine-driven oxidation that occurs in capillary cells. Thus, a newly proposed idea concerning retinal microvascular physiology is that the level of endogenous oxidants plays a key role in establishing the functional organization of the retinal microvasculature.

With an abundance of functional K_{ATP} channels, retinal capillaries are well adapted for generating voltages in response to adenosine. One proposal is that the generation of voltages at sites within the capillary network, as opposed to more proximal locations, tightens the coupling of capillary perfusion to the local metabolic demand by enhancing the spatial resolution for the detection of adenosine inputs (Ishizaki *et al.*, 2009).

3.2.1.3. Operational implications: Due to the paucity of functional VDCCs in the capillaries, voltages generated within the capillary network must be transmitted proximally via gap junction pathways (see section 3.1.1.) to the tertiary arteriole where the abundance of VDCCs makes this portion of the capillary/arteriole unit especially well equipped to convert a change in voltage into a change in intracellular cell calcium and thereby, a change in mural cell contractility, lumen diameter and blood flow.

4. Retinal microvascular physiology: new insights

The quest to better understand the pathophysiology of the retinal microvasculature is now facilitated by the ability to study microvascular complexes freshly isolated from the retinas of diabetic rats. The effects of diabetes are of keen interest since key features of diabetic retinopathy, which is a major sight-threatening disorder, are the early dysregulation of the retinal blood flow (Kohner *et al.*, 1995; Clermont & Bursell, 2007; McGahon *et al.*, 2007; Curtis *et al.*, 2009) and, ultimately, the death of microvascular cells (Cogan *et al.*, 1961; Mizutani *et al.*, 1996; Gardiner *et al.*, 2007).

Study of microvessels isolated from the diabetic retina recently revealed a number of previously unappreciated actions of diabetes. Newly made observations are that diabetes significantly affects the transmission of voltages through the endothelium, the function of

arteriolar VDCCs and the vulnerability of the retinal microvasculature to purinergic vasotoxicity, which is a recently identified pathophysiological mechanism.

Analysis of how isolated retinal microvessels respond to hypoxia has also led to the appreciation that in addition to the important role of K_{ATP} channels in microvascular physiology, these channels also exert a potent pathophysiological effect. These studies revealed that the activation of K_{ATP} channels significantly boosts the lethality of hypoxia, which is an important pathophysiological condition that occurs in a number of retinal vascular disorders.

4.1. Electrotonic architecture: diabetes-induced alterations

Since blood flow dysregulation occurs in the diabetic retina well before the onset of microvascular cell death and may play a role in the progression of diabetic retinopathy (Kohner *et al.*, 1995; Clermont & Bursell, 2007), there is considerable interest in determining how diabetes affects microvascular function. With the ability of a capillary/arteriolar complex to function effectively as an operational unit being dependent on the cell-to-cell transmission of voltage, it is important to determine how diabetes affects the electrotonic architecture of the retinal microvasculature.

In microvascular complexes freshly isolated from the retinas of rats made diabetic by streptozotocin, quantification of the efficacy by which voltage is transmitted between pairs of perforated-patch pipettes sealed onto abluminal cells located at well defined locations in the capillaries and arterioles demonstrated that the electrotonic architecture is significantly altered. Namely, soon after the onset of hyperglycemia, a voltage spreading axially through the endothelium of the diabetic retinal microvasculature decays at a rate that is 5-fold faster than in non-diabetic microvessels (Nakaizumi *et al.*, 2012). As a result of this attenuation in axial transmission, locally generated voltages remain geographically delimited; the capillary/arteriolar complex would be a less interactive unit, and it appears likely that the ability of the retinal vasculature to regulate blood flow may be compromised.

4.2. Voltage-dependent calcium channels: diabetes-induced inhibition

Another new finding made from the study of retinal microvessels freshly isolated from the diabetic retina is that the activity of VDCCs in the tertiary arterioles is markedly diminished (Matsushita *et al.*, 2010). Patch-clamp studies showed that the density of the VDCC current generated in the tertiary arterioles is decreased by ~60% soon after the onset of diabetes. Complementing this electrophysiological evidence of decreased VDCC function, the VDCC-dependent rise in mural cell calcium during depolarization is also ~60% smaller in diabetic tertiary arterioles. Also, consistent with the loss of VDCC function, the depolarization-induced contraction of arteriolar mural cells and the constriction of arteriolar lumens are significantly less in the diabetic microvasculature. Similarly, in the diabetic microvasculature, a K_{ATP} channel-induced hyperpolarization causes only a minimal VDCC-dependent relaxation of tertiary arteriolar mural cells (Matsushita *et al.*, 2010).

By what mechanism does diabetes cause VDCC function to decrease? Recent experimental work with isolated retinal microvessels indicates that the diabetes-induced attenuation of VDCC function is dependent on the polyamine, Spermine (Matsushita *et al.*, 2010). Consistent with this possibility, this ornithine-derived molecule is reported to inhibit VDCCs in non-retinal cells by allosterically modulating the channel's dihydrophyridine binding site (Schoemaker, 1992). In agreement with the possibility of spermine playing a role in ocular complications of diabetes, its concentration is elevated in the diabetic eye (Nicoletti *et al.*, 2003), and its inhibition of potassium efflux via inwardly rectifying K^+ channels is boosted in diabetic retinal arterioles (Matsushita & Puro, 2006).

Indicative of spermine having a role in the diabetes-induced inhibition of arteriolar VDCCs, treatment of diabetic microvessels with the polyamine synthesis inhibitor, difluoromethylornithine (DFMO), reverses the inhibitory effect of diabetes on the VDCC function. Furthermore, exposure of non-diabetic microvessels to spermine mimics the inhibitory effect of diabetes on VDCCs (Matsushita *et al.*, 2010). Thus, new experimental evidence derived from studies of freshly isolated retinal microvessels support a working model in which the polyamines mediate the diabetes-induced inhibition of arteriolar VDCCs. As a consequence, the ability of the capillary/arteriolar unit to generate voltage-driven vasomotor responses is lost (Matsushita *et al.*, 2010), and almost certainly the effectiveness of this operational unit to exert decentralized control over retinal blood flow is diminished.

4.3. K_{ATP} channels: pathophysiological role

Study of isolated retinal microvessels led to the recent discovery that capillary K_{ATP} channels not only have an important operational role, but their activation boosts the lethality of hypoxia (Nakaizumi & Puro, 2011). The response of the retinal microvasculature to hypoxia is of considerable interest because a relative oxygen deficiency occurs in a variety of retinal vascular disorders.

Findings from a recent series of experiments using isolated retinal microvascular complexes (Nakaizumi & Puro, 2011) led to the formulation of a working model (Fig. 8) in which an hypoxia-induced decrease in intracellular ATP causes the activation of K_{ATP} channels. The opening of these channels results in a hyperpolarization that increases the electrochemical gradient for an influx of calcium via the non-specific cation channels, which are in the retinal microvasculature (Sakagami *et al.*, 1999). In addition, calcium-induced calcium release (CICR) causes an additional rise in cytoplasmic calcium, whose increased concentration boosts the amount of hypoxia-induced cell death in retinal capillaries (Nakaizumi & Puro, 2011).

Interestingly, the activation of the K_{ATP} channel/Ca²⁺ influx/CICR pathway not only boosts the vulnerability of retinal microvessels to hypoxia-induced cell death, but also causes pericytes located on the capillaries to contract (Nakaizumi & Puro, 2011). *In vivo*, the contraction of pericytes could exacerbate retinal hypoxia since the constriction of the capillary lumen by these abluminal cells is likely to impede the inflow of oxygenated blood.

The newly appreciated role of K_{ATP} channels in hypoxic retinal microvessels illustrates the emerging concept that the physiology of the retinal microvasculature is closely linked with its pathophysiology. In addition, the identification of the pathophysiological role of the K_{ATP} channel/Ca²⁺ influx/CICR pathway provides new potential targets for pharmacologically limiting microvascular damage in the hypoxic retina.

4.4. Purinergic vasotoxicity: a new concept

Experiments assessing how the extracellular vasoactive signal, ATP, affects isolated retinal microvessels led to the recent discovery that the activation of certain purinoceptors, i.e., the P2X₇ receptor/channels, not only has a physiological role (Kawamura *et al.*, 2003), but their activation may cause vascular cell death, which is termed, purinergic vasotoxicity (Sugiyama *et al.*, 2004; Sugiyama *et al.*, 2005). Although purinergic vasotoxicity is a new concept, this pathophysiological scenario is similar to the well-known excitotoxicity that occurs in the CNS when the neurotransmitter, glutamate, causes neuronal cell death.

4.4.1. P2X₇ purinoceptor function in the retinal microvasculature—Studies of microvessels freshly isolated from the adult rat retina helped elucidate how microvascular

function is affected by extracellular ATP, which is of interest since this nucleotide is a putative glia-to-vascular signal, as well as a molecule released by the endothelium, activated platelets and injured cells (Queiroz *et al.*, 1997; Cotrina *et al.*, 2000; Wang *et al.*, 2000; Newman, 2001, 2003; Wurm *et al.*, 2011). In experiments using freshly isolate retinal microvessels, exposure to ATP caused the intracellular calcium concentration of the abluminal cells to increase (Kawamura *et al.*, 2003). Further by a calcium-dependent mechanism, abluminal cells contract and microvascular lumens constrict (Kawamura *et al.*, 2003). A variety of experiments revealed that the ATP-induced rise in cell calcium is the result of a P2Y₄ receptor-mediated release of stored calcium, as well as an influx of calcium via activated P2X₇ receptor/channels (Kawamura *et al.*, 2003).

Electrophysiological experiments demonstrated that the activation of P2X₇ receptors cause a functional uncoupling of retinal microvascular cells. We found in simultaneous dual perforated-patch recordings from pairs of abluminal cells located on freshly isolated microvessels that the P2X₇ agonist, benzoylbenzoyl-ATP, reversibly reduced electrotonic transmission between the recording micropipettes by nearly 90% (Kawamura *et al.*, 2003). At present, the precise sequence of molecular events linking P2X₇ receptor activation with the closure of microvascular gap junction closure remains to be defined.

4.1.2. Pathophysiology of the P2X₇ purinoceptors—Discovery that functional P2X₇ purinoceptors are in the retinal microvasculature was unexpected since other than in varicose veins (Cario-Toumaniantz *et al.*, 1998), they had not previously been detected in blood vessels. In addition, these purinoceptors were unexpected since studies done chiefly with cells of the immune system had shown that P2X₇ activation results in the formation of pores permeable to 900 Da molecules and also results in the death of these cells (Cockcroft & Gomperts, 1979; Surprenant *et al.*, 1996; Coutinho-Silva *et al.*, 1999). How can these potentially lethal purinoceptors be in retinal microvessels where cell death is almost non-existent? This apparent paradox led to formulation of the hypothesis that there must be a mechanism that prevents the vasoactive signal, ATP, from triggering purinergic vasotoxicity.

Supporting the hypothesis of there being a mechanism to prevent purinergic vasotoxicity, exposure of isolated retinal microvessels to ATP fails to cause pore formation or cell death (Sugiyama *et al.*, 2005), even though exposure to the synthetic P2X₇ agonist, benzoylbenzoyl-ATP (BzATP), does cause pores to form and microvascular cells to die (Fig. 9) (Sugiyama *et al.*, 2004). Additional study revealed that it is ATP's simultaneous activation of the G-protein coupled P2Y₄ receptors that prevents the activation of P2X₇ purinoceptors from causing pore formation and cell death. This P2Y₄-mediated protection is dependent on the release of stored calcium and the activation of an isoform of phospholipase A₂ (Sugiyama *et al.*, 2005). Although a more detailed understanding of this preventive mechanism awaits elucidation of the events linking P2X₇ receptor/channel activation with the opening of transmembrane pores, it is now clear that despite ATP's activation of P2X₇ receptors, its concomitant activation of P2Y₄ receptors prevents purinergic vasotoxicity.

4.4.3. NAD⁺: a selective P2X₇ activator—Because purinergic vasotoxicity requires that P2X₇ activation occur in the absence of the P2Y₄-dependent mechanism that prevents pore formation and cell death, a search was undertaken to identify an extracellular signaling molecule that, unlike ATP, selectively activates P2X₇, but not P2Y₄, receptors. One candidate molecule evaluated in experiments using freshly isolated retinal microvascular complexes is nicotinamide adenosine dinucleotide (NAD⁺), which is thought to be an extracellular signaling molecule in the retina (Esguerra & Miller, 2002) and which is known to initiate a ribosylation reaction in lymphocytes that results in P2X₇ activation, pore formation and cell death (Seman *et al.*, 2003; Aswad *et al.*, 2005). Recent studies of isolated

retinal microvessels showed that by a mechanism dependent on ecto-ribosylation, extracellular NAD^+ causes the activation of P2X_7 purinoceptors, the formation of large transmembrane pores and the death of microvascular cells (Liao & Puro, 2006). Thus, it appears that due to its selective activation of P2X_7 purinoceptors, NAD^+ is a putative candidate for triggering purinergic vasotoxicity in the retinal microvasculature.

4.4.4. Effect of diabetes on purinergic vasotoxicity—Because microvascular cell death is a hallmark of diabetic retinopathy (Cogan *et al.*, 1961; Mizutani *et al.*, 1996; Hammes *et al.*, 2002; Gardiner *et al.*, 2007), there is considerable interest in ascertaining how diabetes affects the vulnerability of the retinal microvascular to purinergic vasotoxicity. Study of microvessels isolated from rats made diabetic by streptozotocin (Sugiyama *et al.*, 2004) revealed that there is a ~100-fold decrease in the half-maximally effective concentration at which the P2X_7 purinoceptor agonist, BzATP, causes pores to form and microvascular cells to die (Fig. 9). Likewise, the sensitivity of the diabetic microvasculature to P2X_7 -dependent pore formation and cell death triggered by extracellular NAD^+ is also boosted ~100-fold (Liao & Puro, 2006). The increased vulnerability of diabetic microvessels to purinergic vasotoxicity appears not to be associated with an increase in the number of functional P2X_7 purinoceptors, but rather diabetes appears to enhance the process by which P2X_7 activation results in the formation of large pores (Sugiyama *et al.*, 2004; Liao & Puro, 2006).

In vivo, additional mechanisms may be involved in increasing the vulnerability of diabetic retinal microvessels to purinergic vasotoxicity. For example, hyperglycemia's up-regulation of ecto-ribosylation (Di Giulio *et al.*, 1999) would facilitate NAD^+ 's activation of P2X_7 purinoceptors. Also, it is known that diabetics can generate autoantibodies that block CD38 (Antonelli & Ferrannini, 2004), which is an ecto- NAD^+ -glycohydrolase that inhibits the ribosylation reactions (Krebs *et al.*, 2005) required for the activation of P2X_7 purinoceptors by NAD^+ .

Although additional study is required, experiments using isolated retinal microvascular complexes not only have provided the first evidence of purinergic vasotoxicity, but also have shown that diabetes markedly increases the vulnerability of the retinal microvasculature to this pathophysiological process.

5. Summary and future directions

In the quest to elucidate how the visual system works and how diseases disrupt its function, there is considerable interest in the intra-retinal vascular system since ophthalmocception in humans, all sub-human primates and most other mammals is dependent on this highly specialized portion of the circulatory system. Recently, use of a tissue print method to isolate vast microvascular complexes from the rat retina has permitted the application of techniques such as patch-clamping, fluorescence imaging and time lapse photography to gain better insight into the physiology and pathophysiology of the retinal microvasculature. Since voltages induced by extracellular signals play a key role in generating vasomotor responses, an important objective is to characterize cell-to-cell transmission of voltage within microvascular complexes isolated from the retina by the tissue print technique recently permitted the first characterization of the electrotonic architecture of a capillary/arteriolar complex. From this analysis, it was discovered that the retinal microvasculature is not simply a homogeneous syncytium, but has a complex electrotonic architecture. Finding that vasoactive signals such as angiotensin II inhibit axial transmission in retinal microvessels revealed their previously unappreciated capability to regulate the spatial distribution of locally generated voltage changes and also established that the electrotonic architecture is dynamic, not static.

Additional study of freshly isolated retinal microvessels showed that capillaries and pre-capillary arterioles have distinct physiological differences. For example, capillaries have an abundance of functional K_{ATP} channels and a paucity of functional VDCCs while the converse is true for the pre-capillary tertiary arterioles. Also, radial transmission is much more efficient in the capillaries than in the pre-capillary arterioles. These new observations support the operational concept of functional sub-specialization within the capillary/arteriolar unit. Namely, capillaries are well equipped to effectively generate and transmit voltages, and arterioles are well-adapted to transduce a change in voltage into a change in abluminal cell calcium and thereby, changes in abluminal cell contractility, lumen diameter and blood flow.

Use of retinal microvascular complexes isolated from the retinas of diabetic rats has led to new pathophysiological insights into the early functional effects of diabetes. For example, soon after the onset of diabetes, the efficacy of voltage transmission through the endothelium is significantly diminished, VDCC function in the arterioles is markedly decreased and the vulnerability of the retinal microvasculature to purinergic vasotoxicity is markedly increased. Other studies of isolated retinal microvessels showed that in addition to an essential physiological role in the generation of a vasomotor response to adenosine, the activation of capillary K_{ATP} channels results in a substantial boost in the lethality of hypoxia. This sort of observation supports the idea that the pathophysiology of the retinal microvasculature is closely linked with its physiology.

Clearly much remains to be learned about the physiology and pathophysiology of the retinal microvasculature. It seems likely that the tissue print method for isolating large microvascular complexes from the rodent retina will continue to aid in this endeavor. For example, this experimental preparation will facilitate efforts to determine the topological distribution and operational role of ion pumps and exchangers, which unlike ion channels, have received scant attention other than a study showing that Na^+/H^+ exchangers mediate the vasomotor effects of extracellular lactate in the retinal microvasculature (Yamanishi *et al.*, 2006). Also in the future, as additional quantitative data of the electrophysiological characteristics of the retinal microvasculature become available, a better understanding of the physiology of the capillary/arteriole unit will be gained by the formulation of a detailed computational model, as has been done for larger blood vessels (Diep *et al.*, 2005; Takahashi *et al.*, 2009). Another need for future study is to elucidate how the inflammatory mediators up-regulated in the course of diabetic retinopathy (Tang & Kern, 2011) may alter the functional organization and operational capabilities of the retinal microvasculature, as well as the vulnerability of retinal microvessels to oxidative stress, hypoxia and purinergic vasotoxicity. In addition, although this review highlighted diabetes as an intensively studied pathophysiological condition, it is likely that in the future many of the other important clinical disorders affecting the retinal microvasculature will receive much needed attention.

In the future, it will be necessary to perform assays *in vivo* in order to validate the conclusions derived from studies of isolated retinal microvessels, although at present it appears unfeasible to perform patch-clamping, calcium-imaging and time-lapse photography in the retinal microvasculature *in oculo*. Also, future studies will ultimately be needed to assess the relevance to the human retinal vasculature of physiological and pathophysiological insights gained from the study of the circulatory system of the rodent retina. Finally, although this review has focused on the role of ion channels and voltage-regulated functions, it is well appreciated that non-voltage related mechanisms also play a key role in regulating the function of the retinal microvasculature. Clearly, an important area of future research will be to better understand the complex interrelationships of intracellular metabolic pathways, receptors, ion channels and gap junctions in the circulatory system of the retina under physiological and pathophysiological conditions.

Certainly, a hope for the future is that characterization of previously unappreciated pathophysiological mechanisms will provide new targets for pharmacologically limiting sight-threatening complications of diabetic retinopathy and other retinal disorders. It appears likely that use of the tissue print technique for obtaining living microvascular complexes from the normal and diabetic retina will aid in this process of drug discovery.

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Figure 1. Schematic showing the portion of the rat retinal vasculature isolated by a newly developed tissue print procedure. Modified from Zhang *et al.* (Zhang *et al.*, 2011) with permission of the *Journal of Physiology*.

Figure 2.

A microvascular complex freshly isolated from the retina of an adult rat. **A**, low magnification differential interference contrast photomicrograph showing from proximal to distal, the smooth muscle-encircled secondary arteriole, the tertiary arteriole and the capillary network. **B**, enlarged view of a portion of the tertiary arteriole with its dome-shaped mural cells (arrowheads). **C**, enlarged view of the capillary with abluminal pericytes whose somas (arrowheads) have a “bump on a log” appearance. Modified from Matsushita and Puro (Matsushita & Puro, 2006) with permission of the *Journal of Physiology*.

Figure 3.

An experiment assessing the effect of angiotensin on electrotonic transmission in the retinal microvasculature. **A**, Phase-contrast photomicrograph showing the sites at which perforated-patch pipettes were sealed onto a capillary pericyte and a mural on the tertiary arteriole. Bar scale: 50 μm . **B**, Plot of the ratio of the change in the voltage detected by the pipette at the arteriolar site ($\Delta V_{\text{responder}}$) and the change in voltage induced by the injection of current via the pipette at the capillary site ($\Delta V_{\text{stimulator}}$) versus time. Bar shows when 500 nM angiotensin was added to the perfusate. **C**, Voltage traces from a current-injected capillary pericyte and from a mural on the tertiary arteriole when the bathing solution lacked angiotensin. **D**, Voltage traces during angiotensin exposure. From Zhang *et al.* (Zhang *et al.*, 2011) with permission from the *Journal of Physiology*.

Figure 4.

The decay of voltage as it spreads through the retinal microvasculature under control conditions and during exposure to angiotensin II. Based on the experimentally determined transmission efficacies listed in Table 1, the vertical axis shows the relative voltage change in abluminal cells located along a microvessel during the spread of a voltage that was generated in the capillary endothelium at a site 400 μm distal to the capillary/tertiary arteriole junction. From Zhang *et al.* (Zhang *et al.*, 2011) with permission of the *Journal of Physiology*.

Figure 5.

VDCC currents in tertiary arterioles and capillaries of isolated retinal microvascular complexes. Top panels show plots of the peak inward current density *versus* test potential, as well as typical current traces. A holding potential (h.p.) of -5 mV is known to fully inactivate the retinal microvascular VDCCs (Sakagami *et al.*, 1999). Bottom panels show similar electrophysiological data, but were obtained from capillaries. Modified from Matsushita *et al.* (Matsushita *et al.*, 2010) with permission from *Investigative Ophthalmology & Visual Sciences*.

Figure 6.

Use of focal application of the K_{ATP} activator, pinacidil, to map the distribution of functional K_{ATP} channels in the retinal microvasculature. The numbers located adjacent to the voltage trace indicate when the pinacidil-containing micropipette was re-positioned to the similarly labeled location in the drawing (Inset). Pinacidil application onto the capillaries (locations 1 and 4) induced the largest hyperpolarizations. Modified from Ishizaki *et al.* (Ishizaki *et al.*, 2009) with permission from the *Journal of Physiology*.

Figure 7.

Use of the microvessel transection technique to determine where within the retinal microvasculature the functional K_{ATP} channels are located. **A**, Sketch of an isolated microvascular complex. Bold arrows point to the locations of the perforated-patch recordings. **B**, I-V relations recorded from the tertiary arteriole (site 1 in panel A) of the intact microvascular complex before and after addition of the K_{ATP} channel activator, pinacidil, to the perfusate. **C**, I-V relations recorded at the same arteriolar site as in panel B, but after the microvessel was transected near its arteriole/capillary junction. **D**, I-V relations recorded from the capillary (site 2) of the transected microvessel. Modified from Ishizaki *et al.* (Ishizaki *et al.*, 2009) with permission from the *Journal of Physiology*.

Figure 8.

Model of how the polyamine/K_{ATP} channel/Ca²⁺ influx/CICR pathway boosts the lethality of hypoxia in retinal capillaries. In this model, an hypoxia-induced decrease in ATP results in the activation of capillary K_{ATP} channels, which are redox-sensitive channels whose function has been found to require polyamine-dependent oxidation (Ishizaki *et al.*, 2009). With the opening of K_{ATP} channels, the membrane potential (V_m) of capillary cells increases, and as a consequence, there is an increase in the electrical gradient for the influx of calcium via the non-specific cation channels expressed in retinal microvessels (Sakagami *et al.*, 1999); the paucity of functional voltage-dependent calcium channels in retinal capillaries (Matsushita *et al.*, 2010) limits their role. In turn, the rise in capillary cell calcium caused by the hyperpolarization-induced increase in calcium influx is amplified by calcium-induced calcium release (CICR), and the resulting high level of intracellular calcium is proposed to boost the lethality of hypoxia. Also shown is that the activation of the polyamine/K_{ATP} channel/Ca²⁺ influx/CICR pathway causes pericytes to contract, the capillary lumen to narrow and thereby, the perfusion of oxygenated blood to decrease. Sites of action of various pharmacological inhibitors are shown. Abbreviations: DFMO,

difluoromethylornithine; Vm, membrane potential; NSC, non-specific cation; CICR, calcium-induced calcium release. Modified from Nakaizumi and Puro (Nakaizumi & Puro, 2011) with permission from *Investigative Ophthalmology & Visual Sciences*.

Figure 9. Cell death induced in non-diabetic and diabetic retinal microvessels by the P2X₇ agonist, BzATP. From Sugiyama *et al.* (Sugiyama *et al.*, 2004) with permission from *Investigative Ophthalmology & Visual Sciences*.

Table 1

Efficacies of electrotonic transmission at sites within the retinal microvasculature. Asterisks indicate that the efficacy of axial transmission within the capillaries and arterioles was significantly decreased by angiotensin II. Standard errors are shown. Modified from Zhang et al. (Zhang *et al.*, 2011) with permission from the *Journal of Physiology*.

Microvascular site	Efficiency of transmission under control conditions	Efficiency of control In angiotensin II
Capillary		
Axial transmission	$98 \pm 3\%/100\mu\text{m}$	$50 \pm 7\%/100\mu\text{m}^*$
Radial transmission	$76 \pm 5\%$	$76 \pm 5\%$
Capillary/arteriole junction	$57 \pm 8\%$	$62 \pm 8\%$
Tertiary arteriole		
Axial transmission	$97 \pm 3\%/100\mu\text{m}$	$52 \pm 3\%/100\mu\text{m}^*$
Radial transmission	$51 \pm 3\%$	$59 \pm 8\%$
3° /2° arteriole junction	$62 \pm 5\%$	$54 \pm 12\%$
Secondary arteriole		
Axial transmission	$98 \pm 2\%/100\mu\text{m}$	$55 \pm 11\%/100\mu\text{m}^*$
Radial transmission	$44 \pm 2\%$	$45 \pm 2\%$