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Microcirculation: Electrophysiological Basis for the Response of Endothelial Cells to Inflammatory Mediators-bradykinin

Kai Miao
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Microcirculation: Electrophysiological basis for the response of endothelial cells to inflammatory mediators-bradykinin

Miao, Kai, Ph.D.
East Tennessee State University, 1994
MICROCIRCULATION: ELECTROPHYSIOLOGICAL BASIS FOR THE RESPONSE OF ENDOTHELIAL CELLS TO INFLAMMATORY MEDIATORS-BRADYKININ

A Dissertation
Presented to
the Faculty of the Department of Physiology
James H. Quillen College of Medicine
East Tennessee State University

In partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

By

Kai Miao

December 1994
APPROVAL

This is to certify that the Graduate Committee of

Kai Miao

met on the

11st day of November, 1994.

The committee read and examined his dissertation, supervised his defense of it in
an oral examination, and decided to recommend that his study be submitted to the
Graduate Council and the Associate Vice-President for Research and Dean of the
Graduate School, in partial fulfillment of the requirements for the degree of Doctor of
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the Graduate Council

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Associate Vice-President for
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ABSTRACT

MICROCIRCULATION: ELECTROPHYSIOLOGICAL BASIS FOR THE RESPONSE OF ENDOTHELIAL CELLS TO INFLAMMATORY MEDIATORS - BRADYKININ

by

Kai Miao

The transmembrane potential \((V_m)\) of vascular endothelial cells (EC's) is proposed to regulate endothelial and vascular responses. However, due to the technical inaccessibility of EC's, in situ, a hypothesized role of electrical activities of EC's in vascular responses has scarcely been investigated. Using conventional microelectrodes, I studied the electrical basis for determining the resting \(V_m\) in intact EC's from hamsters. The resting \(V_m\) were found to be -40 mV for aortic EC's and -43 mV for vena caval EC's. The contributions of ions to the resting \(V_m\) of aortic EC's were compared in terms of the transference number \(t_{(x)}\). The results suggest that \(K^+\) has a smaller contribution (15–20%) than that reported for cultured EC's; whereas, both Na\(^+\) and Cl\(^-\) determine (9–16%) the resting \(V_m\). To develop a technique for in situ monitoring changes in \(V_m\) of postcapillary venular EC's in the hamster mesentery, a voltage-sensitive fluorescent probe, bisoxonol, was used to load the cells and the fluorescence signals were analyzed under an intravital microscope by recording the fluorescence intensity \((I_f)\) and processing fluorescent images of the bisoxonol-loaded cells. Calibrations were conducted by simultaneously measuring changes in \(V_m\) with microelectrodes and bisoxonol from aortic EC's and by varying extracellular Na\(^+\) in microvessels. Both calibrations yielded the linear relationship between \(V_m\) and bisoxonol \(I_f\), showing the slope of 5.7%/mV for aortic EC's and 5.2%/mV for microvascular EC's. The resting \(V_m\) of microvascular EC's was -76 mV. Altering extracellular K\(^+\) to 25, 50, and 100 mM in the suffusate depolarized microvascular EC's by 5, 8, and 10 mV; whereas, the same alterations via both suffusion and perfusion induced the depolarization by 18, 30, and 42 mV, indicating that the K\(^+\) conductance has an asymmetric distribution. Ba\(^{2+}\) (1 mM) produced a depolarization by 70 mV, suggesting that the activity of K\(^+\) channels dominates the resting \(V_m\). To correlate the bradykinin-induced increase in microvascular permeability to the changes in \(V_m\), the albumin flux \((J_A)\) was measured using TRITC-albumin along with monitoring \(V_m\). Bradykinin (1 \(\mu\)M) induced a hyperpolarization of EC's by 8 mV and a biphasic increase in \(J_A\) from the basal level of 1.00 \(\times\) 10\(^{-6}\) to a transient peak of 9.17 \(\times\) 10\(^{-6}\) followed by a sustained level of 3.05 \(\times\) 10\(^{-6}\) cm/s. The linear correlations of net increases in both the peak and the sustained values of \(J_A\) to changes in \(V_m\) indicate that the hyperpolarization determines the peak in part and the sustained level in all. Under high K\(^+\) (50 mM), bradykinin produced a repolarization from a depolarized \(V_m\) of -54 mV to -66 mV and a smaller increase in \(J_A\) from the basal level of 0.38 \(\times\) 10\(^{-6}\) to the peak of 5.51 \(\times\) 10\(^{-6}\) followed by a significantly lowered, sustained level of 1.11 \(\times\) 10\(^{-6}\) cm/s. The repolarization under high K\(^+\) indicates that besides the activation of Ca\(^{2+}\)-dependent K\(^+\) channels, other electrical events may be implicated. The correlation between the repolarization and the lowered value of \(J_A\) at the peak implies that this variation in \(V_m\) also mediates the bradykinin-induced increase in \(J_A\) under high K\(^+\) condition. In conclusion, our study have demonstrated that intact or in situ EC's have distinct electrical properties which are involved in the regulation of vascular responses to inflammatory agonists such as bradykinin.
DEDICATION

This dissertation is dedicated to my wife for her faithful love and tremendous sacrifices, and to my beloved parents for their encouragement and support. Without all these, my dissertation would not have been possible.
ACKNOWLEDGEMENTS

The author would like to express his sincere appreciation to Dr. William L. Joyner, Chairman of the supervisory committee, for his superb guidance, important assistance, and most preciously his direct participation in the experiments that have made this study successful. The author feel deeply indebted to Dr. Robert Wondergem, one of the committee members. His incredible expertise in Physiology, particularly in Electrophysiology, and his brilliant ideas and suggestions have always been indispensable to the overall development of this study. The author would also like to thank the other members of the committee, Dr. Carole A. Williams, Dr. Fred E. Hossler, and Dr. Michael D. Miyamoto, for their valuable advice, critiques, and support throughout this study.

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Chapter I

GENERAL INTRODUCTION

**Endothelium-Dependent Vascular Functions**

The vascular endothelium lining the inside of blood vessels forms the primary interface between the circulating blood and the surrounding tissue. Endothelial cells (EC's), constituting this innermost layer, are not merely a passive blood-compatible structural unit, but rather a metabolically active tissue that exerts a wide range of physiological functions related to vascular homeostasis. These functional properties include anticoagulation and antithrombosis, procoagulation and prothrombosis, participation in inflammation and immunological responses, vessel growth and repair, and control of vascular tone and microvascular permeability. Vascular EC's play a vigorous role in these physiological and pathophysiological processes by synthesizing and secreting numerous humoral factors or presenting specific molecules on the surface of the plasma membrane. Consequently, the humoral factors or expressed molecules react with circulating substances or cells, e.g. neutrophils, or diffuse into underlying cell components, e.g. smooth muscle cells, to initiate, mediate or fulfill those processes. For relatively detailed information about the biological and biochemical properties of vascular EC’s, several review papers can be resorted to (Pearson, 1991; Børum, 1991, Bagby Jr. and Heinrich, 1991).

**Endothelium-Derived Vasoactive Factors**

One of the important features of the microcirculation is the endothelium-dependence of vascular tone and vascular permeability. A great body of evidence
supports the idea that vascular EC's play an essential role in locally controlling the tone of the vessel wall via the synthesis and release of a host of vasoactive factors that modulate the contractility of smooth muscle cells (Rubanyi, 1991; Shepherd and Katush, 1991; Katush and Shepherd, 1991).

**Endothelium-Derived Vasodilators**

1) *Prostacyclin* In 1976, Moncada and colleagues reported that prostaglandin endoperoxidase was transformed by a microsomal enzyme isolated from arteries into an unstable substance that inhibits platelet aggregation (Moncada, *et al.*, 1976). A year later, this substance, produced from vascular EC's, was demonstrated to have a vasodilation property and was termed prostacyclin (PGI₂) (Moncada, *et al.*, 1977). The formation of prostacyclin in vascular EC's can be stimulated by thrombin, bradykinin, or shear stress on the endothelium (Vane, *et al.*, 1990). Prostacyclin is the major product from the metabolism of arachidonic acid which is released from membrane phospholipids by phospholipase A₂. Upon its generation, prostacyclin is released from EC's and enters underlying smooth muscle cells. Thereafter, it induces the production of cyclic 3',5'-adenosine monophosphate (cAMP), which finally, via poorly-defined mechanisms, triggers the relaxation of smooth muscle cells and thus dilates the blood vessel (Gryglewski, *et al.*, 1988).

2) *Endothelium-Derived Relaxing Factor* In 1980, Furchgott and Zawadzki demonstrated that vasodilation evoked by the muscarinic agent, acetylcholine, depended on the presence of the functionally intact endothelium, and they postulated that a diffusible, labile, nonprostanoid substance designated endothelium-derived relaxing factor (EDRF) was released from the endothelium (Furchgott and Zawadzki, 1980).
Since this initial study, a variety of agonists including bradykinin, thrombin, serotonin, ATP, ADP, and the calcium ionophore, A23187, have been shown to induce the release of EDRF from the endothelium of various vascular beds and cultured EC's (Moncada, et al., 1986; Vanhoutte, et al., 1986; Moncada, et al., 1988; Vane, et al., 1990). Strong evidence suggests that the EDRF generated from EC's in blood vessels or in culture by acetylcholine or bradykinin is nitric oxide (NO) (Ignarro, et al., 1986; Furchgott, et al., 1987; Palmer, et al., 1987). This is also supported by pharmacological similarities between nitric oxide and EDRF (Ignarro, et al., 1987), by inhibition of the EDRF action with hemoglobin and methylene blue (Martin, et al., 1984), and by inhibition of the EDRF production from L-arginine with N\textsuperscript{O}-monomethylene-L-arginine (L-NMMA) (Rees, et al., 1989). Similar to prostacyclin, EDRF released from EC's diffuses into smooth muscle cells, but there it simulates soluble guanylate cyclase to produce a rise in cGMP instead of cAMP. The effect of increased cGMP is to induce smooth muscle relaxation by the inhibition of both Ca\textsuperscript{2+} release from intracellular stores and Ca\textsuperscript{2+} influx via receptor-operated channels. cGMP may also activate cGMP-dependent protein kinases which lead to vasodilation through undefined mechanisms (Shepherd and Katush, 1991).

3) Endothelium-Derived Hyperpolarizing Factors In addition to prostacyclin and EDRF, the existence of other chemically different and nonprostanoid relaxing factors such as the endothelium-derived hyperpolarizing factor (EDHF) has been proposed (Rubanyi and Vanhoutte, 1987; Chen, et al., 1988). In bioassay experiments, the different effect of acetylcholine on the relaxation of vascular rings was demonstrated by comparing to catecholamines and by applying inhibitors of phospholipase A\textsubscript{2} and
arachidonic acid metabolism and blockers of EDRF synthesis, i.e. hemoglobin and methylene blue. The blockade of the acetylcholine action on the tension of vascular rings was performed by using atropine (non-selective muscarinic receptor antagonist), pirenzepine (selective antagonist to M₁-muscarinic receptors) and a compound, McN-A-343 (selective antagonist to M₂-muscarinic receptors), suggesting that the vascular endothelium possesses two muscarinic receptor subtypes (M₁ and M₂), and the M₂ receptor may mediate the release of EDHF. Acetylcholine, via M₁-muscarinic receptors, induces a transient relaxation of isolated arteries by a brief hyperpolarization of the membrane of smooth muscle cells. Hemoglobin and methylene blue inhibited vasorelaxation by acetylcholine but did not alter the endothelium-dependent hyperpolarization of smooth muscle cells. After removal of the endothelium or application of tetraethylammonium (proposed to block Ca²⁺-activated K⁺ channels), the hyperpolarization was converted to a depolarization and the relaxation was reduced by 20-30%. Although increasing evidence supports the existence of EDHF, the nature and the intracellular mechanism(s) of this substance still remain unclear.

**Endothelium-Derived Vasoconstrictors**

Besides endothelium-derived vasodilators, EC's can also synthesize and release a number of vasoconstrictors (Katulić and Shepherd, 1991). The observation that removal of the endothelium from canine femoral arteries significantly reduced the contractions induced by several agonists and hypoxia was the first demonstration that the endothelium not only mediates the relaxation of the underlying smooth muscle, but also facilitates its contraction (De Mey and Vanhoutte, 1982).

1) **Endothelin** Among those putative endothelium-derived vasoconstrictors,
endothelin is the only well-identified substance that is a unique polypeptide containing 21 amino acids (Yanagisawa, et al., 1988). Three endothelin isopeptides, endothelin-1, -2, and -3, have been found in human and other mammalian species. The only one produced by endothelial cells is endothelin-1. The release of this peptide could be induced by arginine vasopressin, epinephrine, thrombin, and calcium ionophore A23187. The stimulation of its receptors on the membrane of smooth muscle cells raises inositol trisphosphate and diacylglycerol by activating phospholipase C. The elevation in inositol trisphosphate triggers the release of $\text{Ca}^{2+}$ from intracellular stores, thereby leading to a strong contraction of vascular smooth muscle cells.

2) Other Endothelium-Derived Vasoconstrictors The vascular endothelium also releases several other vasoconstrictors including angiotensin II, thromboxane $\text{A}_2$, prostaglandin $\text{H}_2$, and superoxide anions by a variety of agonists and stimuli such as hypoxia, stretch of the EC's membrane, the increase in the transmural pressure of the vessel, and the rise in extracellular $\text{K}^+$ (Katuč and Shepherd, 1991). Interestingly, most of these substances are the cyclooxygenase products from arachidonic acid metabolism during platelet aggregation or are generated under noxious conditions. Therefore, the release of these vasoconstrictors may play an active role in pathological states of blood vessels such as hypertension, diabetes, vasospasm, and reperfusion injury.

Role of Calcium in the Release of Prostacyclin and EDRF

Irrespective of unclarified intracellular mechanisms for producing EDHF, an enormous number of investigations have demonstrated that $\text{Ca}^{2+}$, an intracellular second messenger, plays a pivotal role in the synthesis and release of prostacyclin and EDRF from vascular EC's.
Evidence for this hypothesis was derived from the observation that the Ca\(^{2+}\) ionophore, A23187, strongly induced the release of prostacyclin and EDRF (Peach, et al., 1987). In the absence of extracellular Ca\(^{2+}\), bradykinin-stimulated EC's (in the isolated vessels or in culture) release EDRF in an attenuated and transient manner, indicating that some factor other than extracellular Ca\(^{2+}\), presumably intracellular Ca\(^{2+}\), is involved. No subsequent release of EDRF was detected until extracellular Ca\(^{2+}\) was restored. The dose-dependent, bradykinin-stimulated release of EDRF showed that the bradykinin concentration for a half-maximal release of EDRF mobilized only about 18% of available intracellular Ca\(^{2+}\) (Freay, et al., 1989). These results indicate: 1) the release of EDRF depends primarily on extracellular Ca\(^{2+}\) influx; and 2) intracellular store-released Ca\(^{2+}\) is not recycled. Thus, replenishment of intracellular Ca\(^{2+}\) stores needs extracellular Ca\(^{2+}\).

The requirement for extracellular Ca\(^{2+}\) in the release of EDRF applies to not only the stimulation of receptors by bradykinin, acetylcholine, substance P, thrombin, and ATP, but also the action of receptor-unrelated substances including A23187, saturated fatty acids, and thimerosal (Adams, et al., 1989).

The release of prostacyclin from vascular EC's is dependent upon intracellular Ca\(^{2+}\). The production of EDRF from thimerosal-treated cultured EC's was found to be completely abolished; whereas, the release of prostacyclin was not altered (Lückhoff, et al., 1988). Trifluoperazine, a compound that inhibits calmodulin-mediated events, and TMB-8, an inhibitor of intracellular Ca\(^{2+}\) mobilization, reduced the bradykinin-stimulated release of prostacyclin but had a negligible effect on the bradykinin-induced release of EDRF (Whorton, et al., 1984; Suttorp, et al., 1985; Peach, et al., 1987). The dependence of prostacyclin release on intracellular Ca\(^{2+}\) mobilization may be attributed to a different
intracellular pathway for its synthesis which involves phospholipase A₂.

Control of Calcium Influx by the Membrane Potential of EC's

Vascular EC's are nonexcitable cells. However, evidence suggests that electrical activities of the EC's membrane are involved in vascular responses and in some cases may play an essential role in mediating vasoactivities by controlling extracellular Ca²⁺ influx that is related to the release of EDRF and prostacyclin (Adams et al., 1989). As I discussed earlier, the release of EDRF depends primarily on extracellular Ca²⁺ influx whereas the release of prostacyclin requires Ca²⁺ mobilization from intracellular stores. However, this is not to neglect the importance of extracellular Ca²⁺ influx in the release of prostacyclin. The simple reason is that after the initial mobilization of intracellular Ca²⁺, Ca²⁺ stores need to be refilled by Ca²⁺ from extracellular space for a sustained release of Ca²⁺ upon continued stimulation to EC's (Hallam et al., 1989). Thus, the control of extracellular Ca²⁺ influx becomes a critical step in completing the whole process of a vascular response. This mechanism involves electrical activities of the EC's membrane, i.e. ion transport pathways and the membrane potential, an electrical driving force favoring extracellular Ca²⁺ entry.

Transmembrane Pathways for Calcium Influx

There are basically three ion transport processes across the cell membrane: the passive transport process, i.e. ion channels; the facilitated transport process, i.e. ion transporters; and the active transport process, i.e. ion pumps. The active transport process for Ca²⁺ found in the membrane of vascular EC's is the Ca²⁺-ATPase (Ogawa et al., 1986) that helps to extrude Ca²⁺ from the cell rather than to cause Ca²⁺ influx. Therefore, candidates for Ca²⁺ influx are from either ion channels or ion transporters.
1) Ion Channels

The existence of selective voltage-gated Ca\textsuperscript{2+} channels in the EC's membrane is still controversial. Bossu and colleagues reported three types (T, SB, and L) of voltage-gated Ca\textsuperscript{2+} channels in bovine capillary EC's (Bossu et al., 1992). These channels were activated by the membrane depolarization or application of a Ca\textsuperscript{2+}-channel agonist, Bay K8644, and were sensitive to either amiloride or dihydropyridines. This finding is somewhat provocative because most other investigators tend to argue against the existence of these channels in vascular EC's although these channels may serve as an important pathway for Ca\textsuperscript{2+} entry (Adams et al., 1989).

Despite this selective type of Ca\textsuperscript{2+} influx pathway, at least three types of non-selective cation channels have been proposed to mediate Ca\textsuperscript{2+} entry: they are i) the receptor-operated cation channel; ii) the stretch-activated cation channel; and iii) the Ca\textsuperscript{2+} leak channel.

Direct evidence for a receptor-operated Ca\textsuperscript{2+} influx pathway comes from the studies in which \textsuperscript{45}Ca\textsuperscript{2+} influx into EC's was increased after using bradykinin, histamine, or thrombin. These results are consistent with agonist-induced changes in ionic currents and membrane conductances of voltage-clamped EC's measured by other investigators. Buffering intracellular Ca\textsuperscript{2+} with EGTA did not eliminate the agonist-induced current. The I-V curve of this current yielded a linear relationship with a reversal potential at about 0 mV and the current amplitude depended on the extracellular concentration of Na\textsuperscript{+} or Ca\textsuperscript{2+}. The ionic conductance was described to be 35-40 pS and 15-20 pS with isotonic CsCl (140 mM) and BaCl\textsubscript{2} (100 mM) in the patch pipette, respectively, and could be blocked by La\textsuperscript{3+} and Ni\textsuperscript{2+}. All of these characteristics of the Ca\textsuperscript{2+} influx pathway suggest
that agonist-induced Ca\textsuperscript{2+} influx is via the receptor-operated, Ca\textsuperscript{2+}-independent, non-selective cation channel, \textit{i.e.} the channel that is activated by intracellular second messenger(s) other than Ca\textsuperscript{2+} after the stimulation of the receptor by its agonist. However, the mechanism of intracellular signal transduction from the receptor to the channel still remains to be clarified.

Radioisotopic study (Johns \textit{et al.}, 1987) demonstrated that \textsuperscript{45}Ca\textsuperscript{2+} leaked into unstimulated EC's at a rate of 16 pmol/106 cells/sec, although the rate for \textsuperscript{22}Na\textsuperscript{+} leak was 50-fold higher than \textsuperscript{45}Ca\textsuperscript{2+}. This result indicates that there is a \textsuperscript{45}Ca\textsuperscript{2+} leak channel which is another type of the non-selective cation channel. This channel may be important in maintaining the increased cytosolic Ca\textsuperscript{2+} concentration at a sustained level and replenishing intracellular Ca\textsuperscript{2+} stores after the cell is challenged.

The stretch-activated cation channel may also provide a pathway for Ca\textsuperscript{2+} influx. A Ca\textsuperscript{2+}-permeable ion channel in cultured EC's was described to have a conductance of 40 pS in a physiological salt solution. The open probability of the channel increased while the patch of the cell membrane was mechanically stretched by applying a negative pressure to the inside of the pipette and hence, these channels were named the stretch-activated channel (Lansman \textit{et al.}, 1987). The relative permeability of the channel to Ca\textsuperscript{2+} and Na\textsuperscript{+}, \textit{i.e.} \(P_{Ca}/P_{Na}\), was estimated to be 1.2-8.4, favoring Ca\textsuperscript{2+} influx, but the channel is still not Ca\textsuperscript{2+}-selective. Oike \textit{et al.} (1994) reported a Ca\textsuperscript{2+} transient increase in human umbilical vein EC's induced by mechanical stretch due to osmotic cell swelling. Extracellular Ca\textsuperscript{2+} influx was suggested, but they were not able to find any current during Ca\textsuperscript{2+} influx. The possible reason was that the current of Ca\textsuperscript{2+} influx might be too weak to be detected or an electroneutral mechanism was involved.
2) Ion Transporters

At least two ion transporters have been found in vascular EC's, *i.e.* the Na*-Ca2* exchanger (Winquist *et al.*, 1985) and the Na*-H* exchanger (Escobales *et al.*, 1990), and these exchangers may be related to extracellular Ca2* influx and EDRF release.

The measurement of intracellular Ca2* with Fura-2 demonstrated that intracellularly loading Na* with a Na* ionophore, monensin, resulted in an internal Na*-dependent Ca2* influx (Adams *et al.*, 1989), suggesting that the Na*-Ca2* exchanger is involved in this process. However, bradykinin-induced Ca2* influx in bovine aortic EC's (Schilling *et al.*, 1988) or bovine atrial EC's (Laskey *et al.*, 1990) was not affected by extracellular substitution of Na* with N-methylglucamine or with Li* or mannitol. These results do not support the postulate that the Na*-Ca2* exchanger plays a major role in the regulation of Ca2* influx.

The primary function of Na*-H* exchangers maintains intracellular osmolality and pH homeostasis; but, an indirect effect of these exchangers could be associated with changes in intracellular Ca2* concentration via modulating intracellular pH and Na* level (Escobales *et al.*, 1990).

**The Membrane Potential: An Electrical Driving Force for Ca2* Influx**

Besides the above described ion channels and transporters which permit the Ca2* influx into vascular EC's, a more negative membrane potential (V_m) would provide a stronger electrical driving force for Ca2* entry across the membrane. In this case, voltage-gated Ca2* selective channels are not considered since most investigations to date do not support the existence of this type of channels which are activated upon a membrane depolarization. Assuming the activity of ion exchangers are not directly
affected by changes in \( V_m \), then those different cation channels would become the preferential candidates for \( Ca^{2+} \) influx under the influence of \( V_m \). The gross rate of \( Ca^{2+} \) influx (\( I_{Ca} \) or \( I_{Ca}^* \)) via these passive conductance pathways is determined by the product of the \( Ca^{2+} \) membrane conductance (\( G_{Ca} \)) multiplied by the subtraction of the \( Ca^{2+} \) equilibrium potential (\( E_{Ca} \)) from \( V_m \) (Hagiwara and Byerly, 1981), \( i.e. \ I_{Ca} = G_{Ca}(V_m - E_{Ca}) \). Supposing the \( Ca^{2+} \) membrane conductance (\( G_{Ca} \)) and the \( Ca^{2+} \) equilibrium potential (\( E_{Ca} \)) are at their steady states, obviously the increase in \( V_m \), \( i.e. \) the hyperpolarization of the cell membrane, will favor \( Ca^{2+} \) influx since the electrical driving force (\( V_m - E_{Ca} \)) for \( Ca^{2+} \) entry is augmented. Likewise, when the cell membrane is depolarized, the \( Ca^{2+} \) influx will be attenuated because of a decreased electrical driving force. Thus, \( V_m \) can be recognized as an essential modulator in the control of \( Ca^{2+} \) homeostasis in vascular EC's. It needs to be pointed out that \( G_{Ca} \) upon the stimulation by some agonists is, in fact, increased, for example the activation of receptor-operated cation channels as I described earlier. The increased \( G_{Ca} \) works synergistically with the elevated \( V_m \) to cause a strong \( Ca^{2+} \) influx which then mediates vascular responses.

1) \textit{Changes in \( V_m \) Coupled with Changes in Intracellular \( Ca^{2+} \)}

The effect of a change in \( V_m \) on \( Ca^{2+} \) influx in vascular EC's was first observed by Johns and colleagues (1987). In cultured bovine pulmonary artery EC's, the resting \( V_m \) was measured by whole-cell recording of the patch-clamp technique and the \( Ca^{2+} \) influx was detected by \( ^{45}Ca^{2+} \) uptaking. Thrombin and bradykinin were shown to increase \( ^{45}Ca^{2+} \) influx and induce an inward current; whereas, the high \( K^+ \) (140 mM) solution significantly reduced \( ^{45}Ca^{2+} \) uptaking by depolarizing the cell membrane from the resting \( V_m \) of -56 mV to near zero. In Schilling's study (1989) using cultured bovine aortic EC's
the concentration of cytosolic Ca\textsuperscript{2+} was monitored by Fura-2 fluorescence and the $V_m$ level was measured by $[^3\text{H}]\text{TPP}^+$ uptake. The results showed that bradykinin induced a biphasic increase in cytosolic Ca\textsuperscript{2+} consisting of a rapid transient peak followed by a slowly declined plateau and elicited a membrane hyperpolarization; membrane depolarization by high K\textsuperscript{+} lowered the plateau level of the bradykinin-induced increase in cytosolic Ca\textsuperscript{2+} but did not significantly affect the basal or transient level. The release of Ca\textsuperscript{2+} from internal stores and influx of Ca\textsuperscript{2+} from extracellular space were proposed to form the transient peak and the plateau, respectively. Hence, he concluded that bradykinin-stimulated influx of Ca\textsuperscript{2+} from the extracellular space decreased with membrane depolarization. In 1990, Lückhoff and Busse investigated the relationship between intracellular Ca\textsuperscript{2+} concentration and $V_m$ in cultured bovine aortic EC's using Indo-1, a fluorescent probe for Ca\textsuperscript{2+}, and the whole-cell patch-clamp for $V_m$. Bradykinin and ATP evoked an initial six-fold increase in intracellular Ca\textsuperscript{2+} followed by a gradually declined but still elevated phase over several minutes tested. When the cells were depolarized by high K\textsuperscript{+} (70-90 mM) or TEA, a K\textsuperscript{+} channel blocker, the initial peak of Ca\textsuperscript{2+} remained unchanged but left no sustained phase behind. In patch-clamped EC's, bradykinin induced a transient hyperpolarization by 13 mV which was significantly prolonged by BRL34915, a K\textsuperscript{+} channel activator. The time course of the bradykinin-induced hyperpolarization was compared to be similar to that found for the Ca\textsuperscript{2+} response. These effects were abolished by High K\textsuperscript{+}. Based on these data, the authors proposed that the initial rise in intracellular Ca\textsuperscript{2+} in response to receptor-binding agonists, caused by mobilization of Ca\textsuperscript{2+} from intracellular stores, activates K\textsuperscript{+} channels, thereby inducing hyperpolarization. This hyperpolarization provides the driving force for transmembrane
Ca\textsuperscript{2+} influx into vascular EC's. Interestingly also in this study, applying acetylcholine to the freshly isolated cells elicited a smaller response of intracellular Ca\textsuperscript{2+} but in a similar biphasic manner as exhibited in response to bradykinin or ATP. High K\textsuperscript{+} completely abolished this response to acetylcholine rather than left the transient peak unaffected, indicating that extracellular Ca\textsuperscript{2+} is the predominant source for elevating cytosolic Ca\textsuperscript{2+} in this cell preparation in response to acetylcholine. Using Fura-2 to measure intracellular Ca\textsuperscript{2+} and diSC\textsubscript{3}(5) to measure V\textsubscript{m} of cultured porcine aortic EC's, Cowan et al. (1992) demonstrated that bradykinin and ionomycin hyperpolarized EC's by 6 and 14 mV, and transiently increased intracellular Ca\textsuperscript{2+} by about 100 and 300 nM, respectively. A recent study by Sharma and Davis (1994) has shown that substance P induces a hyperpolarization in cultured porcine coronary artery EC's from the resting V\textsubscript{m} of -42 mV to a peak of -79 mV measured by whole-cell current-clamp recording, and the time course of a substance P-induced increase in intracellular Ca\textsuperscript{2+} measured with Fura-2 is closely correlated with the membrane hyperpolarization.

In some other studies, measurements of V\textsubscript{m} for vascular EC's were not conducted with a detection of intracellular Ca\textsuperscript{2+}. However, hyperpolarizations of the cell membrane in response to vasoactive agonists were observed in various cell preparations. The amplitude of a membrane hyperpolarization varied from 6 mV (Cowan et al., 1992) to 40 mV (Mehrke and Daut, 1990) depending on the cell origin, animal species, recording technique, and type and concentration of the agonist tested. Nonetheless, these studies suggest that V\textsubscript{m} variations of the EC's membrane are important events during the process of an endothelium-dependent vascular response to a vasoactive agonist.

2) Ion Channels Responsible for Membrane Hyperpolarization
Since the $K^+$ equilibrium potential ($E_K = -90 \text{ mV}$) is higher than the values reported for the resting $V_m$ of vascular EC's and the $K^+$ membrane conductance is predominant in establishing a resting $V_m$ (Adams et al., 1989), the membrane hyperpolarization upon stimulation is generally thought to be due to the activation of $K^+$ channels. These channels have been documented to include: i) the $Ca^{2+}$-dependent $K^+$ channel; ii) the acetylcholine-activated $K^+$ channel; and iii) the shear stress-activated $K^+$ channel.

The isotopic ion, $^{86}$Rb$, is used as a tracer to study the transmembrane flux of $K^+$. In cultured bovine aortic EC's, $^{86}$Rb$^+$ efflux was observed when the cells were subjected to bradykinin or ATP (Gordon and Martin, 1983; Schilling et al., 1988). This agonist-induced $^{86}$Rb$^+$ efflux was parallel to the change in intracellular $Ca^{2+}$ measured with Fura-2. The application of the calcium ionophore, A23187, duplicated this $^{86}$Rb$^+$ efflux which was abolished by La$^{3+}$ (a blocker of non-selective cation channels). These results suggest that this agonist-induced $^{86}$Rb$^+$ efflux is activated by intracellular $Ca^{2+}$. Colden-Stanfield et al. (1987) confirmed an outward $K^+$ current evoked by bradykinin, which appeared to be dependent on an increase in intracellular $Ca^{2+}$, as it was observed only when the solution in the whole-cell pipette did not contain EGTA. Using the patch-clamp method in either the cell-attached or the inside-out configuration, Sauve and colleagues (1988, 1990) demonstrated that in cultured bovine aortic endothelial cells, ATP or bradykinin activated a $Ca^{2+}$-dependent $K^+$ channel that was highly selective for $K^+$ ($P_N/P_K < 0.01$) and had a single channel conductance of 40 pS. The open probability of the channel was voltage-insensitive. The time-course of the channel activation corresponded to the time-dependent change in intracellular $Ca^{2+}$ and the bradykinin-induced hyperpolarization was directly correlated to the activation of this channel. They proposed that the membrane
hyperpolarization produced by bradykinin or ATP was a result of the activation of Ca\(^{2+}\)-
dependent K\(^+\) channels and a more negative \(V_m\) might, in turn, enhance Ca\(^{2+}\) influx
involved in the Ca\(^{2+}\) signalling process. In cultured pig aortic EC's, Cowan et al. (1992)
examined the effect of several Ca\(^{2+}\)-dependent K\(^+\) channel blockers on the membrane
hyperpolarization elicited by bradykinin and ionomycin. To varying degrees, the
bradykinin-induced hyperpolarization was inhibited by apamine, charybdotoxin, quinine,
and TEA, and all blockers but TEA inhibited the hyperpolarization to ionomycin. In
bovine aortic EC's, the Ca\(^{2+}\)-dependent K\(^+\) current with a single channel conductance of
38 pS induced by bradykinin was blocked by tetrabutylammonium (TBA) and by the
scorpion toxin, noxius toxin, but not by TEA (Colden-Stanfield et al., 1990). This
channel conductance was similar to that found by Sauve et al. (1990). With regard to the
single channel conductance, the Ca\(^{2+}\)-dependent K\(^+\) channel can be classified into three
types: the small conductance channel (6-14 pS); the intermediate conductance channel
(18-60 pS); and the large conductance channel (100-250 pS). Besides this 38-40 pS
intermediate conductance channel, there were two subtypes of large conductance Ca\(^{2+}\)-
dependent K\(^+\) channels, i.e. 150 pS and 250 pS channels, reported in bovine and rabbit
aortic EC's, which could be activated to produce the membrane hyperpolarization by
bradykinin and blocked by charybdotoxin, TEA, and TBA but with varied sensitivities
(Fichtner et al., 1987; Rusko et al., 1991). In the study by Sharma and Davis (1994)
using cultured porcine coronary artery EC's, substance P induced a membrane
hyperpolarization by activating Ca\(^{2+}\)-dependent K\(^+\) channels which had an intermediate
conductance of 23-25 pS and was blocked only by \(d\)-tubocurarine.

In freshly isolated rabbit aortic EC's, acetylcholine produced a transient,
concentration-dependent hyperpolarization and an increase in intracellular Ca\(^{2+}\) (Busse et al., 1988; Danthuluri et al., 1989). It was distinct in the sense that both of these responses appeared dependent on extracellular Ca\(^{2+}\) influx rather than initiated by the Ca\(^{2+}\) mobilization from intracellular stores as proposed for responses induced by most other agonists. Changes in V\(_m\) and intracellular Ca\(^{2+}\) were blocked by the muscarinic receptor antagonist, atropine. The acetylcholine-induced whole-cell outward currents carried selectively by K\(^+\) were increased in a dose-dependent manner and were independent of intracellular Ca\(^{2+}\). Therefore, the channels were termed acetylcholine-activated K\(^+\) channels.

With confluent bovine aortic EC's grown inside a glass capillary, a specific type of K\(^+\) channel was directly activated by applying laminar shear flow in the tube. The force- and time-dependent activation of a whole-cell current was shown in a graded manner by elevating amounts of shear stress in the range of 0.2-17 dynes/cm\(^2\) (Olesen et al., 1988). The activation of these channels produced a hyperpolarization ranging from 0 to 6 mV when the resting V\(_m\) was about -77 mV. As to the functional role of the shear stress-activated K\(^+\) channels, the membrane hyperpolarization induced by the activation of these channels would increase the electrical driving force for Ca\(^{2+}\) influx as demonstrated by Ando et al. (1988) and this electrical signal might also be transmitted directly to underlying smooth muscle cells via electrically coupled gap junctions (Nakache and Gaub, 1988). Both of these two actions may account for the mechanisms of flow-induced vasodilation (Furchgott, 1984).

**Importance of the Membrane Potential in EC's for Regulating Vascular Responses**
As I discussed above, $V_m$ of vascular EC’s and its variations play an active role in regulating $Ca^{2+}$ influx that affects the synthesis and release of endothelial autacoids, such as prostacyclin and EDRF, thereby mediating vascular responses.

In addition to the functional property associated with intracellular messengers and humoral factors, a variation in $V_m$ itself may act directly by propagating this electrical signal into neighboring EC’s and underlying smooth muscle cells via inter-endothelial and myo-endothelial gap junctions (Bény and Gribi, 1989; Spagnoli et al., 1982). The topical application of acetylcholine to a single arteriole in the hamster cheek pouch induced a rapid retrograde vasodilation that was not transmitted by diffusion or convection of a humoral factor or by nerve. It was proposed to be produced by a wave of the membrane hyperpolarization transduced via gap junctions (Segal and Duling, 1986, 1987). Electrical signals generated in smooth muscle cells may also propagate to surrounding EC’s via gap junctions. In pig coronary artery, superfusion with TBA induced electrical and mechanical fluctuations in the smooth muscle, which were transmitted and synchronized into the endothelium showing action potential-like fluctuations (von der Weid and Bény, 1993). The electrical communication between vascular EC’s and smooth muscle cells emphasizes that a variation in $V_m$ itself may function as an independent modulator in regulating vascular responses as well (Daut et al., 1994).

To date, the role of endothelial $V_m$ in regulating microvascular permeability has virtually not been explored yet. However, many inflammatory agonists, such as bradykinin, histamine, thrombin, and substance P, that stimulate the rise in intracellular $Ca^{2+}$ and the release of EDRF also induce the increase in microvascular permeability
(Joyner and Kern, 1990). Studies conducted on isolated venular microvessels (He et al., 1990; Yuan et al., 1993) and endothelial monolayers (Oliver, 1992) have demonstrated that the increase in permeability is related to the elevation in cytosolic Ca\textsuperscript{2+} and the formation of EDRF. These findings imply that $V_m$ and its variations could be involved in the process of an increase in microvascular permeability.

In addition to the involvement in vascular responses, the electrical activities of vascular EC's have also been suggested to participate in the regulation of intracellular pH and cell volume of EC's and e\textsuperscript{−} a in amino acid transport across the membrane (Bussolati et al., 1993). By and large, vascular EC's are not an electrically quiescent cell type, rather their membrane electrical properties, particularly $V_m$, have active implications in the process of vascular functions under physiological and pathological conditions.

**Specific Aims and Significance of This Study**

As I discussed above, $V_m$ of vascular EC's and its variations play an important role in regulating vascular functions. At present, however, the understanding of electrical activities of vascular EC's is still inadequate. Furthermore, due to the technical inaccessibility of vascular EC's, in situ, most investigations to date on EC's membrane electrical properties and their involvement in vascular responses have been confined to cultured EC's. Serious concerns exist about the homogeneity of regulatory process of EC's isolated or cultured compared to normal, in situ, cells and about the physiological environment around the cells, for example, the contact and communication between vascular EC's and smooth muscle cells or pericytes, that is critical for the performance of normal cellular functions but is deprived of in the culture medium. In the study by Sturek et al. (1991), the response of intracellular Ca\textsuperscript{2+} to either bradykinin or acetylcholine
differs greatly between subcultured EC's and freshly dispersed EC's from the coronary artery. Even in the studies using cultured EC's, controversies still exist among reported data with regard to basic electrical properties of EC's such as the value of \( V_m \), and the presence of voltage-gated calcium and chloride channels which are important in determining electrical behaviors and other functions of the cells. Irrespective of variations in the animal species, cell origin, and techniques applied, the lose of membrane components and the inhibition of channel protein expression are likely to occur during the enzymatic digestion of a vessel or the adaption of cultured cells in the medium. Inasmuch as these substantial diversities resulting from varied cell conditions, the observation of intact, or \textit{in situ} vascular EC's is demandingly important. Moreover, information about microvascular EC's electrophysiology is virtually unavailable let alone the functional role of \( V_m \) in regulating microvascular permeability.

Therefore, this study is designed: 1) to investigate the resting \( V_m \) of intact aortic and vena caval EC's from hamsters and contributions of major ions including \( K^+ \), \( Na^+ \), and \( Cl^- \) to the resting \( V_m \), using conventional intracellular recording with microelectrodes; 2) to develop a fluorescence technique for measuring the resting \( V_m \) from hamster mesenteric microvascular EC's, \textit{in situ}, with a voltage-sensitive probe, bisoxonol and to determine the contribution of \( K^+ \) membrane permeability to the resting \( V_m \); and 3) to correlate changes in \( V_m \) with changes in microvascular permeability in response to an inflammatory agonist, bradykinin.

The central hypothesis for this study is: The resting \( V_m \) of intact aortic EC's has a similar value as compared to vena caval EC's and this resting \( V_m \) is contributed primarily by \( K^+ \). The resting \( V_m \) of microvascular EC's is different from intact EC's
In large vessels and $K^+$ contribution to the $V_m$ is predominant. The bradykinin-induced increase in microvascular permeability is mediated by the membrane hyperpolarization of EC's which may be due to the activation of $Ca^{2+}$-dependent $K^+$ channels.

The hamster is a unique mammalian animal model suitable for microcirculation studies. Because of its easy accessibility and good visualization, the vasculature in the cheek pouch and the mesentery has been used intensively to investigate the regulation of blood flow and microvascular permeability. However, lack of any electrophysiological information to date about hamster vascular EC's leaves an undeniable gap that precludes a thorough understanding of mechanisms underlying vascular responses. This study will provide important information characterizing basic electrical properties of hamster EC's from both large vessels and microvessels. The development of a fluorescence technique for measuring changes in $V_m$ of microvascular EC's, in situ, will open a completely new area for observing electrical behaviors of vascular EC's under nearly physiological conditions. Monitoring changes in $V_m$ of microvascular EC's while detecting microvascular permeability will enable us to directly determine the functional role of $V_m$ in mediating the responses of microvessels to bradykinin and thus, to achieve a better understanding of implications of endothelial electrophysiology in the process of inflammatory reactions.
CONTRIBUTIONS OF K⁺, Na⁺, AND Cl⁻ TO THE MEMBRANE POTENTIAL OF INTACT HAMSTER VASCULAR ENDOTHELIAL CELLS

INTRODUCTION

Because of technical difficulties, few attempts have been made to measure the resting $V_m$ from vascular EC's using conventional microelectrodes. Only recently, as a result of the development of patch-clamp techniques and the availability of vascular EC's in culture, have studies progressed in endothelial electrophysiology. Primarily based on reported data from cultured EC's, the ionic basis determining the resting $V_m$ is described as follows.

The Value of the Resting $V_m$

The difference in values of the resting $V_m$ of vascular EC's may suggest a difference in the ionic basis for establishing the resting $V_m$. The values reported for the resting $V_m$ of bovine aortic EC's have a big range from -3 to -85 mV (Richter et al., 1981; Mehrke and Daut, 1991). For bovine pulmonary artery EC's, the resting $V_m$ is about -60 mV (Johns et al., 1987). Pig aortic and coronary artery EC's have the resting $V_m$ of -9 mV (Richter et al., 1986) and -40 to -47 mV (Bény and Gribi, 1989; Bény, 1990), respectively. Guinea-pig aortic and coronary artery EC's have the resting $V_m$ about -41 mV (Northover, 1980) and -35 mV (Mehrke and Daut, 1990). Porcine coronary artery and cerebral capillary EC's showed the resting $V_m$ of -42 mV (Sharma and Davis, 1994).
and -24 mV (Hoyer et al., 1991), respectively. The resting $V_m$ for human umbilical vein EC's is -27 mV (Bregestovski et al., 1988). All of these reported values of the resting $V_m$ for vascular EC's exhibit great variations. The difference could be attributed to an interspecies variation, to a large-small vessel or arterial-venous difference, or perhaps even to differences in techniques performed and in cell culture conditions. In addition, the resting $V_m$ recorded from preconfluent EC's showed a higher value than that of confluent cells. For example, bovine pulmonary artery preconfluent EC's had a resting $V_m$ of -74 mV while confluent EC's had a $V_m$ of -65 mV; human umbilical vein preconfluent EC's had a value of -35 mV and confluent cells had a $V_m$ of -27 mV (Bregestovski and Ryan, 1989). These differences might be due to different cell conditions. The cells at confluence are electrically more synchronized via interendothelial gap junctions and more quiescent with regard to the cell cycle.

Ion Channels Related to the Control of the Resting $V_m$

As I know, the ionic basis of the resting $V_m$ in living cells is a Donnan equilibrium of different ions (primarily $K^+$, $Na^+$, and $Cl^-$) across the cell membrane and in some cases includes a small portion of the contribution of electrogenic ion pumps, for instance, the $Na^+-K^+$ ATPase. The equilibrium potential of an ion is determined not only by the electrochemical gradient of that ion across the membrane but also by the overall permeability or conductance of the membrane to the ion. Basically, the membrane conductance is provided by ion channels which serve as a transmembrane pathway for ion fluxes. In vascular EC's, the documented ion channels which may contribute to the establishment of the resting $V_m$ are as follows:

1) $K^+$ channels
Using whole-cell recording, an inwardly rectifying K⁺ current was revealed in cultured bovine aortic and pulmonary EC's (Adams et al., 1989; Takeda and Klepper, 1990). The I-V curve showed a hyperpolarization-activated strong inward rectification with the zero current potential equal to the K⁺ equilibrium potential, indicating a very high selectivity for K⁺. This conductance (25 pS) could be blocked by Ba²⁺ and Cs⁺ and was changed to a small outward conductance (<5 pS) at potentials positive to the resting $V_m$. These inwardly rectifying K⁺ channels are predominant in the plasma membrane and have been proposed to serve the primary conductance that controls the resting $V_m$ of vascular EC's.

A voltage-gated, transient, outward, A-type, K⁺ current has been found at depolarizing voltage steps in one-third of cultured bovine aortic and coronary artery EC's (Takeda et al., 1987; Bregestovski and Ryan, 1989). This current was blocked by 4-aminopyridine. The physiological role of the voltage-gated, A-type, K⁺ channels remain unidentified. This type of K⁺ channel may regulate the resting $V_m$ in certain populations of vascular EC's in the presence of a depolarizing stimulation (Bregestovski and Ryan, 1989).

2) Na⁺ channels

A relatively Na⁺ selective channel ($P_{Na}/P_K = 1.5$) has been described in rat and pig brain microvascular EC's (Vigne et al., 1989). The single-channel current showed a linear relationship (non-rectifying) with a slope conductance of 23 pS. The channel had a long-lasting open time and could be blocked by amiloride and phenamil. The functional role of these channels may be, in part, to influence Na⁺ and K⁺ membrane conductances which contribute to the resting $V_m$. 
3) Cl\textsuperscript{−} channels

A 'maxi' Cl\textsuperscript{−} channel (382–400 pS) in bovine aortic EC's (Hansen and Olesen, 1989; Olesen and Bundgaard, 1992) and other two types of Cl\textsuperscript{−} channels in bovine pulmonary EC's (Shapiro and DeCoursey, 1991) have been revealed in the whole-cell and cell-attached patch-clamp configurations. These channels were activated by depolarizing potentials and closed at the $V_m$ over -60 mV. Oike et al. (1994) also described an inward Cl\textsuperscript{−} current activated at a holding potential of -40 mV in human umbilical vein EC's and this current was completely blocked by 1,9-dideoxyforskolin. The physiological significance of these channels may be to help repolarize the EC's membrane following depolarizing stimuli.

Specific Aims and Significance of This Study

The resting $V_m$ of vascular EC's is an important electrical property associated with cell functions. Studies using cultured EC's have characterized some unique electrical activities of the cell membrane including the resting $V_m$ and ion channel currents, which have greatly advanced our knowledge in the functional role of endothelial membrane electrophysiology in regulating vascular responses. However, available data describing the ionic basis of the resting $V_m$ were mostly obtained from cultured EC's. These cells have been deprived of both their intact property and physiological environment. To varying degrees, their electrical behaviors or responses to a stimulus may not reflect the authenticity of cell functions. In addition, considerable differences and controversies still exist among studies, which tend to confuse or even preclude the ionic basis of the resting $V_m$ in vascular EC's from elucidation. Regardless of a big range of resting $V_m$ values and the argument about the presence of voltage-gated Ca\textsuperscript{2+} channels,
the contribution of Cl\textsuperscript{-} to the resting $V_m$ is still in question. In the study by Johns et al. (1987) using bovine pulmonary artery EC's, the replacement of extracellular Cl\textsuperscript{-} with methylsulphate did not affect the resting $V_m$ and thereby a conclusion that Cl\textsuperscript{-}
permeability of the membrane plays a negligible role in determining the resting $V_m$ was reached (Bregestovski and Ryan, 1989). Whereas, as I introduced earlier, several types of Cl\textsuperscript{-} channels activated by depolarization have been demonstrated in bovine pulmonary artery EC's. Through these channels, the Cl\textsuperscript{-} equilibrium potential obviously contributes to the resting $V_m$. Furthermore, no systematic observation of the effects of different ions on the resting $V_m$ has been undertaken although it is important. On the other hand, hamsters are recognized to be a great animal model for studying the regulations of vasoactivities including vascular tone, blood flow, permeability, but electrophysiology of hamster vascular EC's is still in the dark.

For all of these reasons, I feel an urgent need to carry out a set of experiments by which the resting $V_m$ of hamster intact vascular EC's and a systematic comparison of ion contributions to the $V_m$ can be scrutinized. The hypothesis for this set of study is:

Hamster intact vascular EC's have the resting $V_m$ that is different from those reported for cultured EC's. The resting $V_m$ is higher in aortic EC's than in vena caval EC's. The resting $V_m$ is determined primarily by membrane permeability to K\textsuperscript{+} but Na\textsuperscript{+} and Cl\textsuperscript{-} contribute to the $V_m$ as well.

Using intracellular recording with conventional microelectrodes, I will:

(1) record and compare the resting $V_m$ of intact vascular EC's from the aorta and the vena cava in hamsters;

(2) verify that the resting $V_m$ is originated from EC's rather than other cell types;
(3) determine the effects of altering extracellular $K^+$, $Na^+$ and $Cl^-$ on the resting $V_m$.

(4) and postulate ion membrane conductances by calculation of their contributions to the overall membrane conductance in vascular EC's.
METHODS

Animals and Preparation of Vascular Tissues

Young adult golden Syrian hamsters of either sex, weighing 100-150 gm, were anesthetized with sodium pentobarbital (60 mg/100 gm, b.w., i.p.), followed by thoracolaparotomy. A segment of the thoracic aorta or inferior vena cava about 0.5 cm in length was dissected and quickly removed from the thoracic cavity. After opening the vessel longitudinally and rinsing away the remaining blood with Krebs solution, the vascular strip with the luminal surface upwards was pinned immediately onto the bottom of a tissue chamber. The tissue was suffused continuously in the chamber with oxygenated (95% O₂/5% CO₂) Krebs physiological salt solution containing (in mM): 103 NaCl, 4.7 KCl, 2.6 CaCl₂, 1.3 MgCl₂, 25 NaHCO₃, 1.2 NaH₂PO₄, 2.8 glucose, 4.9 Na-pyruvate, 4.9 Na-glutamate, 2.7 Na₂-fumarate, pH 7.46 at a constant temperature of 37.0±0.2 °C.

Preparation of Suffusates with Altered Ionic Concentrations

To examine the contribution of overall K⁺, Na⁺ and Cl⁻ conductances to the resting Vₘ of hamster vascular endothelial cells, the Krebs solution was modified for tissue suffusion by increasing K⁺ or decreasing Na⁺ and Cl⁻ concentrations, respectively. 

K⁺ solutions of 50 mM and 100 mM.

High K⁺ conditions in the suffusate were created by elevating KCl from 4.7 mM in the Krebs to either 50 mM or 100 mM through equimolar substitution of NaCl.

Na⁺ solutions of 95.5 mM and 45.5 mM.

Lower concentrations of Na⁺ in the suffusate were prepared by equimolar
substitution of 50 mM and 100 mM tetramethylammonium chloride for sodium chloride in the Krebs, thereby reducing Na⁺ from 145.5 mM to 95.5 mM and 45.5 mM in the suffusate, respectively.

*Cl solutions of 65 mM and 15 mM.*

Altering Cl⁻ concentrations was accomplished by replacing sodium chloride equimolarly with 50 mM or 100 mM sodium gluconate, thereby reducing Cl⁻ from 115 mM in the Krebs to 65 mM or 15 mM in the modified suffusate.

The osmolality of each modified solution, besides the Krebs, was assessed with an automatic osmometer (Precision Systems) monitored by the depression of the freezing point. Osmolality of the solutions varied within a small range of 276-284 mOsm. All chemicals used for preparation of the suffusates were purchased from Sigma chemical company or Fisher Scientific.

**Technique of Intracellular Recording for Vᵦ on Aortic and Vena Caval Strips**

As shown in Figure 1, conventional electrophysiological techniques were employed in this study (Northover, 1980). Briefly, a Ag/AgCl reference electrode was inserted into an agar bridge (4% in Krebs solution) bathed in the tissue chamber and an Ag/AgCl half-cell was inserted into a glass microelectrode containing 0.5 M KCl. The resulting electrode had a tip resistance of about 4-8 MΩ. Both were connected through a high input impedance (>10⁸ MΩ) preamplifier with unit gain (515L Analog Devices) to a storage oscilloscope (Tektronix) and a digital voltmeter (Keithly). A pulse current of 0.05 or 0.5 nA (300 ms duration) was injected intermittently through the KCl-filled electrode to detect changes in electrode resistance during the course of an intracellular
Fig. 1. The working system for measuring the resting $V_m$ of hamster vascular EC's.
recording. The traces of $V_m$ were recorded on a chart recorder (Grass) simultaneously. For $V_m$ measurements, the resulting electrode was moved slowly under the guidance of a light microscope (Olympus) by a mechanical micromanipulator (Aus Jena) into the chamber suffused with the Krebs solution where the appearance of a negative potential was recorded as a tip potential. Then, as the electrode was moved slowly towards the surface (the endothelium) of the aortic or vena caval strip, a resting $V_m$ was categorized by a sudden shift of the potential to a more negative value (>20 mV) and the maintenance of a stable recording for at least one minute. These criteria established the cellular nature of the recording. Furthermore, in most experiments, the response of the electrical recording to shifts in $[K^+]_o$ was used to critique the intra- versus extra-cellular location of the microelectrode. One to four impalements in each individual vascular strip were obtained during most experiments. The total number of recordings depended on the success in impalement and viability of the tissue.

Identification of $V_m$ Recordings as to Cellular Origin, e.g. the Endothelial Cell

Denudation of the endothelium from an aortic strip.

An aortic strip was prepared and mounted onto the bottom of the suffusion chamber following the same procedure as described above. Mechanical denudation of the endothelium was achieved by gently wiping across the surface of the aortic strip with a soft brush-pen.

Electrical recordings from the endothelium-denuded aortic strip.

After the endothelium was denuded from an aortic strip, suffusion was begun with the Krebs solution and electrical recordings were attempted in the manner described
above by microelectrode impalement. Any recording with a sudden shift in the potential and stable for one minute would be described as a $V_m$ from an underlying cell. The features of the voltage tracing from this prepared strip were compared to those from a normal aortic strip using the same protocol, except brushing.

*Specimen preparation of normal and endothelium-denuded aortic strips for scanning and transmission electron microscopy (SEM and TEM).*

Normal and endothelium-denuded vascular strips, prepared as described above, were fixed by immersion in Krebs solution containing 2% glutaraldehyde for 2 hours at room temperature. Strips then were washed with several changes of Krebs solution and post-fixed by immersion in 2% OsO$_4$ for 2 hours at room temperature. After rinsing first in distilled water and then in Krebs solution, the strips were prepared for either SEM or TEM. For scanning electron microscopy, tissue segments were dehydrated in a graded ethanol series, then critical point dried using liquid CO$_2$ as an intermediate fluid (Samdri PVT-3B drier, Tousimis Research Corp.). Tissue segments, mounted on stubs with colloidal carbon and sputter-coated with gold-palladium (Desk-1, Denton Vacuum, Inc.), were viewed and photographed with conventional scanning electron microscope (DSM-940, Carl Zeiss, Inc.). For transmission electron microscopy, tissue segments were dehydrated in a graded ethanol and propylene oxide series, embedded in epox-araldite (Ernst F. Fullam, Inc.), cut into ultrathin sections, stained with uranium and lead, and then viewed and photographed by conventional transmission electron microscopy (JEOL 100-C, JEOL, Inc.).

*The Contributions of $K^+$, $Na^+$, and $Cl^-$ to the $V_m$: Effect of Altering $K^+$, $Na^+$, and $Cl^-$ in the Suffusate on $V_m$ of Aortic EC's and the Calculation of Ion*
Transference Numbers ($t_m$)

In the next series of experiments, while recording the $V_m$ from the same individual EC's, the extracellular ionic composition, i.e. $[K^+]_o$, $[Na^+]_o$ or $[Cl^-]_o$, was altered in a stepwise manner. Each of the three modified suffusates was applied to the aortic segment sequentially at two different concentrations, while a stable $V_m$ was recorded for 3 minutes at each step. Only successful experiments, determined by completed application of the modified suffusates with two altered concentrations, were taken as paired data for this series of experiments. The liquid junction potential was detected by recording changes in the tip potential before, during and after application of each of the three modified ionic solutions to the suffusion chamber while the electrode was in the suffusate bath.

Based on the original value of $V_m$ and its change in response to alterations of extracellular ion concentrations, the transference number ($t_m$), an index of membrane conductance, was calculated and compared for each specific ion tested. The formula for the calculation of $t_m$ is expressed as follows:

$$t_m = \frac{\Delta V_m}{\left(\frac{RT}{zF}\right)\left(\ln C_1/C_2\right)} \times 100\%$$

where: $\Delta V_m$ represents the change in $V_m$ from its original value in the Krebs solution to the value after exposure to the modified ionic suffusate; $C_1$ is the concentration of a specified ion in the original Krebs solution; $C_2$ is the altered concentration of this ion in the modified suffusate and $RT/zF$ has its usual value of 26.7 mV at 37 °C. The value of the $t_m$ reflects the relative contribution of the membrane conductance for a specific ion ($g_{m,i}$) to the overall $V_m$ and plasma membrane conductance ($g_p$). The formula is derived from the Nernst equation, assuming that the intracellular concentration of the specific ion remains constant while the extracellular concentration is altered. This approach is, at
best, a reliable approximation of the specific ion membrane conductance as achieved by
the present technique.

The Response of $V_m$ Recorded from Endothelial Cells (EC's) of Both Aorta
and Vena Cava to Changes in Extracellular $K^+$ ($[K^+]_o$)

Intracellular recordings of $V_m$ were conducted in aortic and vena caval EC's
using the technique described above before and after altering $[K^+]_o$. After the recorded
resting $V_m$ was stabilized for at least 1 minute in normal Krebs solution, the extracellular
concentration of potassium was switched to either 50 mM or 100 mM by using a
manifold device which prevents abrupt shifts in the velocity of flow around the tissue so
that the voltage tracing could be recorded continuously. After a new stable value was
reached usually for 3 minutes, the suffusate was switched back to the normal Krebs
solution. If the $V_m$ did not return to within 2 mV of the original $V_m$ value, the impalement
was considered faulty due to a leakage of the cell membrane, and thereby disregarded.
The resting $V_m$ and changes in $V_m$ after application of these $K^+$ solutions were compared
in aortic and vena caval EC's.

Data Analysis

All data were summarized and expressed as means ± SE. The difference between
means of unpaired samples was tested using the Student’s t-test. The paired t-test was
used to compare the difference between the two transference numbers ($t_{ma}$) for each of the
ions, e.g. $[K^+]_o$, $[Na^+]_o$ and $[Cl^-]_o$. The two-way analysis of variance was used to test
differences among the resting $V_m$ and changes in $V_m$ after altering $[K^+]_o$, $[Na^+]_o$ and $[Cl^-]_o$,
respectively, and multiple comparisons were conducted to determine the differences
between the resting $V_m$ and each changed $V_m$ by the one-sided Dunnett’s test. P-levels
less than 0.05 were considered statistically significant. Analysis of linear correlation and regression was executed according to the method of least-squares computation. The difference between two slopes of the regression lines also were determined by the t-test.
RESULTS

Electrical Recordings of $V_m$ from Normal and Endothelium-Denuded Aortic Strips

Figure 2 represents a typical result from my experiments concerning the cellular origin of $V_m$ from endothelium-intact (left side panels) and endothelium-denuded (right side panels) aortic strips. The electron micrographs shown in panel A and D are SEM’s and those shown in panel B and E are TEM’s. Electrical recordings are shown in panel C and F, respectively. These experiments were repeated in four hamsters. Electron micrographs (SEM’s) of normal aortic strips revealed that the vessels were lined with continuous sheets of endothelial cells, oriented in "cobblestone" arrays parallel to their long axes and direction of blood flow (Fig.2, panel A). In corresponding strips, TEM’s revealed that the endothelium and other underlying cells and structures appeared normal (panel B), the endothelium (E) adhered closely to the underlying elastic lamina (L), cellular ultrastructure and junctional morphology appeared normal, and the smooth muscle cells were observed below a thick cell-free elastic lamina. In contrast, both SEM’s (panel D) and TEM’s (panel E) of endothelium-denuded aortic strips revealed the endothelium to be completely removed, leaving only remnants of cell debris and exposing the underlying elastic lamina.

As depicted in the bottom panels, when the endothelium was present, the electrical signal became more negative upon impalement of an endothelial cell and a continuous trace with a stable transmembrane potential of -38 mV was obtained for 10 minutes (panel C). Furthermore, a relatively low input resistance was obtained as indicated by a small increase in the amplitude of current pulses after impalement. These effects were reversed when the
Fig. 2. A composite of the vascular morphology and electrophysiology from one representative aortic segment in a hamster. In the left panels, a scanning (A) and a transmission (B) electron micrograph and an electrical recording (C) from a normal, endothelium-intact aortic segment are shown; the same morphological (D, E) and electrophysiological (F) data are depicted in the right panels for an aortic segment that had the endothelium removed. E: endothelial cell; L: elastic lamina; arrowheads: endothelial remnants.
electrode was removed from the endothelial cell. On the other hand, about one hundred attempts to electrically record $V_m$ from endothelium-denuded aortic segments ($n=4$) were unsuccessful; only large electrical fluctuations with no stable electrical potential detected as the electrode was advanced into the surface of the aortic segment (panel F). Persistent attempts to record membrane potentials from these endothelium-denuded aortic segments led to the breakage of the microelectrode (indicated as an arrow head in panel F). However, in the endothelium-intact aortic segments ($n=4$), 10 successful stable (5-10 min) electrical recordings of the membrane potentials were obtained in the same number of attempts.

**Intracellular Recording of $V_m$ in Aortic and Vena Cava EC's**

Based on the criteria for $V_m$ measurement, successful electrical recordings of $V_m$ were performed on 15 aortic and 5 vena caval strips. The accumulation of $V_m$, obtained from 55 recordings of aortic EC's and from 15 recordings of vena cava EC's, approximated a normal distribution as shown in the frequency plots in Figure 3. Mean values were $-40\pm1$ mV and $-43\pm1$ mV for aortic EC's and vena caval EC's, respectively, and these two values were not significantly different ($p>0.05$). The recorded values gave a range of -22 to -56 mV for the $V_m$ of aortic EC's and a range of -36 to -49 mV for the $V_m$ of vena caval EC's. The input resistance was measured based on the difference between the height of current pulses in the Krebs and the height of these electrical pulses after impalement. The measured input resistance varied between 10-240 MΩ. The differences were minimal between neighboring cells while great variations could be obtained from cells in outlying regions on any single aortic or vena caval strip.

**The Contributions of $K^+$, $Na^+$ and $Cl^-$ to the Resting $V_m$: The Effect of Altering $[K^+]_o$, $[Na^+]_o$, and $[Cl^-]_o$ on the $V_m$ of Aortic EC's**
Fig. 3. The frequency distribution of the resting $V_m$ as a function of recordings from EC's in the aorta (A) and the vena cava (B).
The effect of altering the external concentration of K⁺, Na⁺, and Cl⁻ on the membrane potential (V_m) from aortic EC's is depicted in Figure 4. As shown in panel A, after a stable resting V_m was recorded (-40 mV) in the normal Krebs solution (4.7 mM K⁺), the [K⁺]_o was switched to 100 mM which caused a decrease in V_m to -30 mV within 1 minute. Concomitant with V_m depolarization, the input resistance decreased from 190 MΩ to 50 MΩ, indicated by the decrease in the height of the intermittent current pulses (I=0.05 nA). When the suffusate was switched back to the normal Krebs solution, the V_m returned to -40 mV and the input resistance increased to 130 MΩ.

The effect of altering the external concentration of Na⁺ from 145.5 mM to 45 mM on V_m in another aortic EC's is shown in panel B. After a stable V_m was recorded at -34 mV, the suffusate was switched to a modified Krebs solution containing 45.5 mM Na⁺. The V_m began to hyperpolarize and within minutes it reached a stable value of -40 mV. When [Na⁺]_o was returned to its original level (145.5 mM), the V_m returned to the resting value (-34 mV).

In the next voltage tracing (panel C), the effect of reducing [Cl⁻]_o from 115 mM to 15 mM by gluconate substitution on V_m is depicted in another aortic EC's. After [Cl⁻]_o was reduced, the V_m began to hyperpolarize from the original value of -32 mV until it reached a new stable value of -43 mV. When the [Cl⁻]_o was returned to 115 mM, the V_m returned to its original value.

In all of these experiments, the variations of the electrode tip potential caused by alterations in each ion concentration of the suffusate were determined as the liquid junction potential. No such potential was observed after altering K⁺ or Na⁺ concentrations; however, when Cl⁻ was reduced from 115 mM to 65 mM and 15 mM in the suffusate by gluconate
Fig. 4. Continuous recordings of $V_m$ responses to altering $[K^+]_o$, $[Na^+]_o$, and $[Cl^-]_o$. A: A $V_m$ tracing before, during, and after application of the high $K^+$ suffusate to the aortic strip. B: A $V_m$ tracing before, during, and after application of low $Na^+$ to the aortic strip. C: A $V_m$ tracing before, during, and after applying the low $Cl^-$ suffusate to the aortic strip. Those intermittent pulses were generated by applying a pulse current of 0.05 nA to the recording microelectrode.
substitution, the liquid junction potentials were recorded to be -4 mV and -6 mV, respectively. In all subsequent analysis of the data for alterations in \([\text{Cl}^-]_o\), the \(V_m\) values were adjusted correspondingly with these junction potentials.

All of the above paired experiments are summarized in Table 1. Also, the transference numbers \((t_{\text{K+}})\) were calculated and compared for each ion at each step of altering concentrations. In response to step-changes in \([\text{K}^+]_o\) from 4.7 mM to 50 mM and from 4.7 mM to 100 mM, the mean \(V_m\) values \((n=6)\) significantly decreased from -43 mV to -34 mV and to -30 mV, respectively; these results yielded significantly different \(t_{\text{K+}}\) values of 11.6% and 14.3%, respectively. In response to step-changes in \([\text{Na}^+]_o\) from 145.5 mM to 95.5 and 45.5 mM, the mean \(V_m\) increased from -36 mV to -38 and -41 mV, respectively. The calculated \(t_{\text{Na+}}\) at each respective concentration of \(\text{Na}^+\) was 16.3% and 16.7%. These values were not significantly different, indicating a relatively constant membrane conductance for \(\text{Na}^+\) at each external concentration. Further, when the external concentration of \(\text{Cl}^-\) was reduced in a stepwise fashion from 115 mM to 65 and 15 mM, the mean \(V_m\) increased significantly from -38 mV to -40 and 43 mV, respectively. The calculated mean \(t_{\text{Cl-}}\) at each step was 15.3% and 9.2% for \([\text{Cl}^-]_o\) of 65 mM and 15 mM, respectively. These \(t_{\text{Cl-}}\) values were significantly different, indicating a decrease in membrane conductance for \(\text{Cl}^-\) at the lower external concentration of 15 mM.

Comparison of the Dependence of the Resting \(V_m\) on \([\text{K}^+]_o\) between Aortic and Vena Caval EC's

In another series of experiments, the determination of the resting \(V_m\) in vena caval EC's \((n=2)\) by \([\text{K}^+]_o\) in comparison with that in aortic EC's \((n=10)\) was tested. In the normal Krebs solution \((4.7 \text{ mM K}^+)\), as shown in Figure 5, the mean \(V_m\) was found to be -44 mV
from vena caval EC's and -42 mV from aortic EC's. After application of 50 mM K\(^+\) to the suffusate, \(V_m\) decreased by 18% to -37 mV in vena caval EC's and by 19% to -34 mV in aortic EC's. When \([K^+]_o\) concentration was raised to 100 mM, further depolarization were achieved: \(V_m\) decreased to -32 mV in vena caval EC's and to -30 mV in aortic EC's (both by 29%). The magnitudes of the membrane depolarization in response to the application of high K\(^+\) at these two concentrations were not significantly different between vena caval EC's and aortic EC's (\(p>0.05\)).

In further analyzing the above data set of aortic EC's (n=10), I noted that the depolarized amplitude of the cell in response to high \([K^+]_o\) appeared to positively correlate with its resting \(V_m\) value. As shown in Figure 6, the resting \(V_m\) ranged from -32 mV to -50 mV and the depolarization of the \(V_m\) correspondingly ranged from 4 to 12 mV at 50 mM \([K^+]_o\) and from 7 to 19 mV at 100 mM \([K^+]_o\). Analysis by linear regression resulted in a positive linear correlation for both the 50 mM \([K^+]_o\) group (\(r=0.69, p<0.05\)) and the 100 mM \([K^+]_o\) group (\(r=0.76, p<0.02\)). No significant difference was found between the slopes of the regression lines for the two concentration groups (\(p>0.05\)). This analysis was not executed for vena caval EC's in the present study due to the limited number of data points (n=2). Notwithstanding, a similar linear correlation might be expected for vena caval EC's because the depolarized amplitudes for vena caval EC's appear to parallel those for aortic EC's as exhibited in Figure 5.
### Table 1. Changes in $V_m$ and $t_{on}$ of aortic EC's after altering $[K^+]_o$, $[Na^+]_o$, and $[Cl^-]_o$.

<table>
<thead>
<tr>
<th>$V_m$ (mV)</th>
<th>$V_m$ (mV)</th>
<th>$V_m$ (mV)</th>
<th>$I_{in}$ (%)</th>
<th>$I_{in}$ (%)</th>
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<tr>
<td>(4.7 mM $[K^+]_o$)</td>
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<td>(4.7 to 50 mM $[K^+]_o$)</td>
<td>(4.7 to 100 mM $[K^+]_o$)</td>
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<td>32</td>
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<td>42 ± 3</td>
<td>34 ± 2*</td>
<td>30 ± 2*</td>
<td>11.6 ± 2.3</td>
<td>14.3 ± 2.6*</td>
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<tr>
<th>$V_m$ (mV)</th>
<th>$V_m$ (mV)</th>
<th>$V_m$ (mV)</th>
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<td>(145.5 mM $[Na^+]_o$)</td>
<td>(95.5 mM $[Na^+]_o$)</td>
<td>(45.5 mM $[Na^+]_o$)</td>
<td>(145.5 to 95.5 mM $[Na^+]_o$)</td>
<td>(145.5 to 45.5 mM $[Na^+]_o$)</td>
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<tr>
<td>36 ± 2</td>
<td>38 ± 2*</td>
<td>41 ± 2*</td>
<td>16.3 ± 1.5</td>
<td>16.7 ± 1.0</td>
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<th>$V_m$ (mV)</th>
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<th>$V_m$ (mV)</th>
<th>$I_{in}$ (%)</th>
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<tr>
<td>(115 mM $[Cl^-]_o$)</td>
<td>(65 mM $[Cl^-]_o$)</td>
<td>(15 mM $[Cl^-]_o$)</td>
<td>(115 to 65 mM $[Cl^-]_o$)</td>
<td>(115 to 15 mM $[Cl^-]_o$)</td>
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<td>38 ± 2</td>
<td>40 ± 2*</td>
<td>43 ± 2*</td>
<td>15.3 ± 1.4</td>
<td>9.2 ± 0.7*</td>
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* $p<0.01$, compared with the original corresponding values.
Fig. 5. Effects of altering $[K^+]_o$ on the resting $V_m$ in aortic and vena caval EC's. The logarithmic plot of the concentration of $[K^+]_o$ vs. $V_m$ represents changes in $V_m$ in response to elevating $[K^+]_o$ from 4.7 mM to 50 and 100 mM for aortic EC's (n=10) and vena caval EC's (n=2). Each point is the mean of $V_m$ values; error bars, SE.
Fig. 6. Correlation between the original resting $V_m$ and the amplitude of depolarization induced by high $[K^+]_o$ in aortic EC's. The data points are divided into two paired groups, 50 mM (n=10) and 100 mM (n=10). Each point is expressed as an individual resting $V_m$ against its depolarized value upon exposure to high $[K^+]_o$. Two points of individual $V_m$ of -32 mV were overlapped at a depolarized value of 4 mV in the 50 mM $[K^+]_o$ group. The analysis of linear correlation and regression was conducted according to the method of least squares for both 50 mM and 100 mM $[K^+]_o$ groups. For the 50 mM $[K^+]_o$ group, $r=0.69$, $P<0.05$; for the 100 mM $[K^+]_o$ group, $r=0.76$, $P<0.02$. No significant difference was found between the two slopes of the regression lines ($P>0.05$).
DISCUSSION

Electrical Recordings of $V_m$ Were from Endothelial Cells

The intracellular recording permits the direct measurement of transmembrane potentials ($V_m$) from intact EC's which avoid making drastic alterations to the structural and functional associations of the cells with their surroundings. However, cellular localization of the microelectrode is a critical point in this study. The microiontophoretic injection of Lucifer yellow was described as an appropriate method for the identification of $V_m$ origin (Bény and Gribi, 1989), but an obvious disadvantage is that this technique cannot be performed for each $V_m$ recording, thus creating the possibility that not all of the $V_m$ recordings were from endothelial cells. Using a combination of electrophysiological and electron microscopic observations in my study, I confirmed that the $V_m$ were recorded from endothelial cells not from cells in the deeper layers of the vascular wall, e.g. smooth muscle cells. Endothelial cells were denuded from the luminal surface of aortic strips with a soft brush-pen while leaving the underlying elastic lamina and other cells intact. The success of this maneuver was documented by scanning and transmission electron microscopy. When an electrical recording of $V_m$ was attempted from the endothelial cell-denuded surface, no stable potential could be obtained and moving the electrode deeper into the aortic strip was not beneficial. In other words, I were unable to record any cell membrane potentials due either to bending or to breaking of the electrode tip because of the toughness of the elastic lamina. This strongly indicated that the $V_m$ recordings in normal, non-denuded vascular strips were from the endothelial cells. These findings also are consistent with the assumption by Northover (1980) that penetration of the elastic lamina with fine-tipped microelectrodes
is impossible. Thus, the method employed in the present study is feasible and reliable for the purpose of electrophysiological investigation of vascular EC's previously thought to be a rather inaccessible cell type for direct intracellular recording.

The Value of the Resting $V_m$ Was Similar between Intact Aortic and Vena Caval EC's

Detected by intracellular recording with conventional microelectrodes, the values of the resting $V_m$ of both aortic EC's (-40 ± 1 mV) and vena caval EC's (-43 ± 1 mV) were not significantly different; the depolarized magnitudes of the resting $V_m$ by high K+ solutions appeared comparable in EC's from the two different origins. These results indicate a similar ionic basis for determining the resting $V_m$ in hamster arterial and venous EC's. The membrane potentials of hamster EC's from these large vessels were similar to those detected from the guinea-pig thoracic aorta EC's, -41.1 mV, (Northover, 1980) and from the pig coronary artery EC's, -40 mV, (Bény, 1990), where their measurements also were performed on intact EC's by intracellular recording with microelectrodes. Relatively comparable values were reported for the resting $V_m$ either in cultured guinea-pig coronary artery EC's using patch clamp techniques, -33 mV to -35 mV, (Daut et al., 1988; Mehrke and Daut, 1990) or in rabbit aortic EC's using conventional microelectrodes, -30 mV, (Venter et al., 1975). However, some reported values for the resting $V_m$ in cultured EC's have been outside the range of my measurements. For example, using microelectrodes, Richter et al. (1986) obtained a mean value of -8.9 mV. Also using the whole-cell recording of the patch clamp technique, Johns et al. (1987) and Olesen et al. (1988) reported values of -56 mV and -77 mV from bovine pulmonary artery and aortic EC's, respectively. These values are not consistent with those observed in my study. Some potential causes for these differences are
probably the variations in employed techniques and/or the heterogeneity of endothelial cells among different animal species and vascular beds. It also is possible that during enzymatic isolation of endothelial cells, followed by cell culture with the artificial surfaces and media, some ion transport systems, intracellular mechanisms and even ion electrochemical gradients are interrupted, leading to alterations in the electrophysiological behavior (Adams, et al., 1989; Taketa and Klepper, 1990). In the present study, the resting \( V_m \) was measured from intact EC's under nearly physiological conditions.

The Intact Vascular EC's Were Electrically Coupled

The input resistance measured using the technique of intracellular recording by microelectrodes comprises the membrane resistance and the intercellular resistance. During my recording of resting \( V_m \), the input resistance was found to be 10-240 M\( \Omega \). Daut, et al. (1988) reported an input resistance of 8 M\( \Omega \) in a monolayer of coronary EC from guinea-pigs but a much higher value of 1.7 G\( \Omega \) in a cultured single vascular EC. The relatively low input resistance observed in this study may imply the presence of electrical coupling between intact endothelial cells in the hamster aorta and vena cava presumably by gap junctions. A considerable decrease in the input resistance was detected during high \( K^+ \) application to the extracellular medium. As exhibited in Fig. 4A, a remarkable decrease in input resistance can be distinguished from a decrease in height of intermittent current pulses (\( I=0.05 \) nA). This characteristic may suggest an increase in membrane conductance, assuming the intercellular resistance was not strongly affected by high [\( K^+ \)]\(_o\). My result is similar to that found in hepatocytes (Moule and McGivan, 1990) and it could be explained by the activation of \( K^+ \) channels. However, a decrease in input resistance may also imply a change in intercellular resistance. In their whole-cell recording, Shapiro and DeCoursey (1991) detected a "pseudo-
cation" conductance attributable to intercellular gap junctions. Thus, I can not rule out the possibility that the input resistance could be reduced by an increase in currents through gap junctions. The presence of electrical coupling between these cells implies that the intact vascular EC's comprises an electrical syncytium which acts in a synchronized manner and can transduce electrical signals to neighboring cells along the vessel wall.

**The Contribution of K⁺ to the Resting Vₘ Was Relatively Small**

It is well known that potassium contributes most to the resting Vₘ in many cell types with its equilibrium potential (Eₖ=-90 mV) near Vₘ. In the studies using cultured bovine aortic by Johns, et al. (1987) and pulmonary artery EC's by Olesen, et al. (1988), changes in Vₘ were shown to be positively correlated with the log of [K⁺]₀ concentration. Ten-fold increases in [K⁺]₀ concentration depolarized Vₘ by 52 mV (resting Vₘ=-56 mV) to 55 mV (resting Vₘ=-77 mV). In my study, however, this was not the case. With a 20-fold increase in [K⁺]₀ (from 4.7 mM to 100 mM), Vₘ decreased only by 29% in both aortic and vena caval EC's (Fig. 6). Considering that the elevation of [K⁺]₀ was achieved by equimolar reduction of [Na⁺]₀ in the present study, the real magnitude of depolarization by high [K⁺]₀ should be recalculated by taking into account the additional effect of lowering [Na⁺]₀ on Vₘ. Increasing K⁺ in the suffusate from 4.7 mM to 50 mM and to 100 mM was created by reducing Na⁺ from 145.5 mM to 100.2 mM and to 50.2 mM, respectively. These reduced concentrations of [Na⁺]₀ were roughly equivalent to 95.5 mM and 45.5 mM of [Na⁺]₀ which was produced by a 2-step replacement with TMA⁺ in another set of experiments. Thereafter, calibrations were conducted simply by adding the average value of net hyperpolarized voltage by low [Na⁺]₀ to each corresponding data point observed for depolarization of Vₘ by high [K⁺]₀ since magnitudes of hyperpolarization by 2-step lowering [Na⁺]₀ were found to
be almost constant (-2 mV at 95.5 mM and -5 mV at 45.5 mM) in each individual \( V_m \) recording in spite of the substantial variation of resting \( V_m \). This adjusted data is expressed in Table 2. After exposure to 50 and 100 mM \([K^+]_o\), the resting \( V_m \) (-42±3 mV, \( n=6 \)) depolarized by 10 mV (22%) and by 17 mV (40%), respectively. Even though high \([K^+]_o\) elicited significant depolarization of \( V_m \) in intact aortic EC, the ratios (22-40%) are still much smaller than those observed by patch clamp techniques in cultured EC. Again indicated by \( t_{\text{m}} \) values (14.8-20.4%), the contribution of \( K^+ \) membrane conductance to the overall \( V_m \) is relatively small. However, my findings are quite close to the observations in intact aortic EC's from guinea-pigs (Northover, 1980) and in intact hepatocytes from mice (Wang and Wondergem, 1991). Several possibilities could be postulated to explain this difference in stoichiometric behavior of intact EC's from that of cultured EC's: 1) specific membrane conductances of other ions, besides \( K^+ \), or nonspecific leak conductance might contribute to the overall membrane conductance; 2) membrane conductances of \( K^+ \) and other ions might function in a voltage-dependent or concentration-dependent fashion during depolarization of \( V_m \) and application of high \([K^+]_o\) (Wang and Wondergem, 1991), for example, the depolarization could activate the membrane conductance of \( Cl^- \) that has been proposed to repolarize the cell membrane following a depolarizing stimulus (Olesen and Bundgaard, 1992); and 3) the exposure of the cell membrane to the high \( K^+ \) solution was confined to the luminal side as the other side attached to the basement membrane and elastic lamina whereas for a cultured cell under patch-clamping approximately the whole plasma membrane would be able to contact the medium. This situation does not invalidate my results; on the contrary, it reflects the response of intact EC's under normal physiological conditions.

It is noteworthy that the net depolarized value of \( V_m \) in aortic EC's appeared to have
Table 2. Adjusted data for changes in $V_m$ and $t_{K^+}$ of aortic EC's after altering $[K^+]_o$.

<table>
<thead>
<tr>
<th>$V_m$ (mV) (4.7 mM $[K^+]_o$)</th>
<th>$V_m$ (mV) (50 mM $[K^+]_o$)</th>
<th>$V_m$ (mV) (100 mM $[K^+]_o$)</th>
<th>$t_{K^+}^*$ (%) (4.7 to 50 mM $[K^+]_o$)</th>
<th>$t_{K^+}^*$ (%) (4.7 to 100 mM $[K^+]_o$)</th>
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<tr>
<td>32</td>
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<td>20</td>
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<td>22.2</td>
<td>29.4</td>
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42 ± 3  32 ± 2$^*$  25 ± 2$^*$  14.8 ± 2.3  20.4 ± 2.6$^*$

* $p<0.01$, compared with the original corresponding values.
a positive correlation with its original resting $V_m$ for both 50 mM and 100 mM $[K^+]_o$ (Fig.5). This phenomenon is consistent with the behavioral characteristic of voltage-gated ion channels and the involvement of other ions in the control of the resting $V_m$; however, further studies are needed to interpret clearly this ionic mechanism.

A significant increase in membrane conductance of $K^+$ was detected during exposure to 100 mM $[K^+]_o$ as compared to 50 mM $[K^+]_o$ using the calculated $t_{K^+}$ values. I did not test the effect of even higher $[K^+]_o$ due to the limitation of maximum replaceable concentration of 103 mM KCl in the Krebs solution. Whether it is possible to find a dramatic increase in $t_{K^+}$ when $[K^+]_o$ approaches its intracellular concentration of about 130 mM (Brock, 1986) remains questionable.

**Na\(^+\) and Cl\(^-\) Were Involved In the Maintenance of the Resting $V_m$**

To ascertain the contribution of membrane conductances for other ions to $V_m$, Na\(^+\) and Cl\(^-\) were investigated in a paired design (n=6) also. Generally, based on an inward electrochemical gradient of Na\(^+\) across the membrane, the equilibrium potential of Na\(^+\) has the positive value that tends to drag the resting $V_m$ away from the more negative-valued equilibrium potential of K\(^+\). If the Na\(^+\) membrane conductance is associated with the resting $V_m$, decreasing the Na\(^+\) electrochemical gradient by lowering $[Na^+]_o$ would bring the resting $V_m$ towards the equilibrium potential of K\(^+\), that is to say the membrane hyperpolarization would occur. As shown in Table 1, reductions in $[Na^+]_o$ hyperpolarized $V_m$ by 2 mV (5%) at 95.5 mM and by 5 mV (14%) at 45.5 mM, respectively. The results of $t_{Na^+}$ calculation demonstrated a constant fraction (16.3-16.7%) of membrane conductance for Na\(^+\) contributing to the overall resting $V_m$, indicating that this conductance may not be voltage- or concentration-dependent. However, the mechanisms underlying the activity of Na\(^+\)
channels for the $V_m$ depolarization by lowering $[\text{Na}^+]_o$ are still not clear. A Na$^+$-selective channel which has a selectivity of 1.5 for Na$^+$ to K$^+$ and is sensitive to amiloride (Vigne, et al., 1989) does not seem to be involved in the control of the resting $V_m$. In a short series of experiments, I found no change in $V_m$ during application of 100 µM amiloride in the suffusate over 15 minutes (unpublished observation). The possibility that the membrane conductance of Na$^+$ is carried by other selective or nonselective cation channels or even leak channels merits further investigation. One reasonable interpretation for the hyperpolarization of $V_m$ by low $[\text{Na}^+]_o$ is that reducing $[\text{Na}^+]_o$ might interfere with some membrane transport systems, such as the Na$^+$-K$^+$-Cl$^-$ cotransporter and the Na$^+$-Ca$^{2+}$ exchanger. If the stoichiometry of Na$^+$-K$^+$-Cl$^-$ influxes is 1:2:2 and of Na$^+$-Ca$^{2+}$ transfluxes is 3:1 in vascular EC, these transport systems are electrogenic and may contribute to the resting $V_m$. A decrease in $[\text{Na}^+]_o$ leading to disengagement of these transport systems may cause a hyperpolarization of $V_m$.

Substitution of Cl$^-$ with equimolar gluconate in the perfusate did produce a liquid junction potential of -4 mV at 65 mM [Cl$^-$_o] and -6 mV at 15 mM; however, the data in the present study (Table 1) was corrected for the potentials and still the endothelial cells demonstrated a remarkable hyperpolarization of $V_m$ when [Cl$^-$_o] was lowered. This result is not consistent with the findings observed in cultured EC using the technology of patch clamping, e.g. Cl$^-$ showed no contribution to the resting $V_m$ of endothelial cells (Johns, et al., 1987; Olesen, et al., 1988). A significant decrease in $I_{\text{Cl}}$ values from 15.3% to 9.2% demonstrates a contribution of Cl$^-$ membrane conductance to the resting $V_m$ in a concentration- or voltage-dependent manner. Several types of Cl$^-$ channels have been found in cultured EC's of bovine aortic and pulmonary artery (Olesen and Bundgaard, 1992;
Shapiro and Decoursey, 1991). Cl⁻ may contribute to the overall resting $V_m$ through these relatively selective pathways. Isosmotic cell shrinkage elicited by the replacement of the permeant anion, Cl⁻, by the impermeant anion, gluconate, (Lewis and Donaldson, 1990) or the disability of Na⁺,K⁺,2Cl⁻-cotransporter of EC's (Brock et al., 1986) after Cl⁻ was removed from the extracellular medium in a single or combined manner could as well result in a hyperpolarization of these endothelial cells in the hamster aorta.

Summary

In conclusion, a feasible and reliable electrophysiological method has been described in the present study for intracellular recording of the resting $V_m$ in intact vascular EC's from the hamster aorta and vena cava. The resting $V_m$ values of aortic EC's (-40±1 mV) and vena caval EC's (-43±1 mV) were not significantly different and close to the values reported for intact guinea-pig aortic EC's and pig coronary artery EC's, suggesting a similarity in the ionic basis determining the resting $V_m$ for EC's from large vessels. The low input resistance indicates the presence of an electrical coupling between the cells. As indicated by $R_{in}$, the membrane conductance of $K^+$ in a concentration-dependent fashion plays a relatively small role (14.8-20.4%) in determining the resting $V_m$ of intact vascular EC's. The amplitude of $V_m$ depolarization was positively correlated with the resting $V_m$ value and this is consistent with the operation of voltage-gated ion channels. The membrane conductances of Na⁺ and Cl⁻ contribute to the overall membrane conductance with fractional contribution relatively less than $K^+$. They may modulate the resting $V_m$ through channels or some electrogenic transport systems in the membrane. Since changes in the resting membrane potential may play an important role in mediating intracellular signal transduction, e.g. Ca²⁺ influx that associates with EDRF release and the synthesis or release of other vascular or inflammatory
substances, the well-defined characteristics of $V_m$ with respect to ionic mechanisms will facilitate and advance our understanding of the regulation of vascular functions.
Chapter III

THE MEMBRANE POTENTIAL IN MICROVASCULAR ENDOTHELIAL CELLS MONITORED BY A FLUORESCENT PROBE, BISOXONOL, IN SITU

INTRODUCTION

Microvessels, particularly capillaries and postcapillary venules, are the most active place to permit exchange of solutes between the blood and interstitial fluid. Microvascular EC's forming the primary barrier play an essential role in controlling this process. As compared to EC's in large vessels, these EC's, to some extent, appear different in terms of cellular structures and functions. However, with regard to their electrophysiology, little is known about whether they have distinct properties and react differently to various stimuli. Due to technical limitations to cell culture and in situ performance of electrophysiological means, basically all of the studies reported to date have been achieved by using EC's from large vessels, either the cultured for patch-clamping or the intact for intracellular recording. Exclusively, there are two documented observations that were conducted in cultured capillary EC's. Using the whole-cell mode for patch-clamping, Hoyer et al. (1991) detected the resting $V_m$ from porcine cerebral capillary EC's to be -24 mV. In isolated capillary EC's from the bovine adrenal gland, Bossu et al. (1992) found the presence of three types (T, SB, and L) of voltage-gated Ca$^{2+}$ channels which were activated by the membrane depolarization and blocked by amiloride or dihydropyridines. These two studies presented different electrical properties of capillary EC's as compared to EC's from large vessels. The resting
$V_m$ of -24 mV is considerably lower than most of those reported for EC's in large vessels. The latter case of Ca\textsuperscript{2+}-selective channels is even more striking because with these channels, extracellular Ca\textsuperscript{2+} can enter the cell without relying on the membrane hyperpolarization as I described before. This would make the hypothesized mechanisms for extracellular Ca\textsuperscript{2+} influx entirely different. However, most research groups favor the notion that vascular EC's lack these Ca\textsuperscript{2+} channels even though their data are based on EC's from large vessels. Nevertheless, these findings somehow suggest that capillary EC's may have distinct electrical characteristics which endow diverse functional engagement for these cells in the regulation of microvascular responses.

Techniques for $V_m$ measurement include intracellular recording with conventional microelectrodes, whole-cell patch-clamping, intracellular uptake of radioactive ions and fluorescent indication with voltage-sensitive dyes. Obviously, the patch-clamp and isotope accumulation can not be introduced into my *in situ* experiments and at present, intracellular recording with microelectrodes appears technically impossible for my study due to the toughness of mesenteric tissue and the small size of a single EC's. Moreover, mechanical perturbing the cell membrane by microelectrode impalement would not only affect cell integrity but also present a sever damage to the vessel wall. For these concerns, fluorescent probes appear to be the most appropriate tool for this set of study.

For the past 20 years, the validity of fluorescent dyes as probes for measuring $V_m$ has been extensively studied and firmly established (Waggoner, 1979; Loew, 1988; Freedman and Novak, 1989; Smith, 1990). In general, these dyes are either positively charged or negatively charged molecules and can be grouped into the cyanine, merocyanine, styryl, oxonol and safranine classes in regard to their molecular structures. According to their
response speed and mechanism of the voltage-dependent optical change, these dyes can also be divided into two classes. The slow dyes (some of the cyanine, merocyanine and oxonol dyes) respond to a change in $V_m$ in times of seconds and work by a mechanism of voltage-sensitive redistribution between the medium and the inside of the cell (altering fluorescence well over 80%); the fast dyes (styryl, safranine, most of merocyanine, and some of cyanine and oxonol dyes) respond to a change in $V_m$ in times of milliseconds and work by a mechanism of voltage-sensitive localization on or very near the membrane (changing fluorescence less than 1%).

Bisoxonol belongs to a family of oxonol dyes that have been well studied (George et al., 1988a; George et al., 1988b). Its negatively charged, lipophilic properties permit free voltage-dependent distribution across the membrane inversely to the change in $V_m$ following the Nernst equation, that is to say, an increase in bisoxonol fluorescence corresponds a $V_m$ depolarization and likewise a decrease in its fluorescence indicates a $V_m$ hyperpolarization or repolarization. Again, because bisoxonol is negatively charged, it does not tend to accumulate in mitochondria that usually possess much higher transmembrane potential, thereby having less intracellular compartmentalization (Bronner and Landry, 1991).

Bisoxonol also has less cytotoxicity than many other dyes. There are three subtypes of bisoxonol dyes and bis-(1,3-dibutylbarbituric acid)trimethine oxonol (DiBAC$_4$(3)) is the one I will employ for this study. This subtype of bisoxonol has the highest voltage-sensitivity of the three dyes and it enhances its fluorescence intensity up to 20-fold by binding to cytosolic and membrane proteins (Rink et al., 1980; Bräuner et al., 1984). In this study, bisoxonol will be continuously perfused through microvessels. After it has been equilibrated across the cell membrane in accordance with a $V_m$ level, its concentration in the perfusate
(D_0) should be maintained relatively constant no matter what this V_m level is and therefore, as shown in Figure 7, its intracellular concentration (D_i) will indicate the V_m level following the equation (Wilson et al., 1985):

\[ D_i = D_0 e^{\alpha x T V} \]

This is the principle I abide by in measuring the change in V_m of microvascular EC's, in situ.

The hypothesis for this set of study is: The fluorescence of bisoxonol can monitor changes in V_m for microvascular EC's, in situ. The resting V_m in these cells has a different value as compared to that in EC's from large vessels and the membrane permeability to K^+ is the primary factor determining the resting V_m. Microvascular EC's have a uniform distribution of the membrane permeability to K^+ across the microvessel wall.

Based on this hypothesis, I aim:

1) to verify the stability of bisoxonol fluorescence under varied medium conditions;
2) to calibrate the change in bisoxonol fluorescence intensity to the change in V_m in intact aortic EC's;
3) to calibrate bisoxonol fluorescence intensity to values of V_m in microvascular EC's, in situ;
4) to measure the resting V_m and the role of K^+ in maintaining the resting V_m of microvascular EC's;
5) to observe the effect of Ba^{2+} in blocking K^+ channel activity on the resting V_m;
6) to compare depolarizing actions of both high K^+ and Ba^{2+} on the luminal and the abluminal side of the EC's membrane.
bis-(1,3-dibutylbarbituric acid)trimethine oxonol

diBA-C₄-(3)⁻

MW 516.4

excitation wavelength: 495 nm

emission wavelength: 517 nm

\[ V_m = \frac{(RT/zF)}{D_0/D_i} \ln \left( \frac{D_0}{D_i} \right) \]

\[ D_i = D_0 e^{F/RTV_m} \]

**Fig. 7.** Bisoxonol, a voltage-sensitive fluorescent probe. Its chemical structure, distribution kinetics, and relationship with \( V_m \).
METHODS

Animals and Solutions

Young golden hamsters of either sex, weighing 80–140 g, were used in this study. All the following solutions were prepared daily:

1) Ringer's solution contained 120 mM NaCl, 4.6 mM KCl, 2 mM CaCl₂·2H₂O, 1.45 mM MgSO₄·7H₂O, 25 mM NaHCO₃, 5.5 mM D-Glucose, 5 mM HEPES·NaOH;

2) high K⁺ solutions (25, 50 and 100 mM K⁺) were prepared by replacing NaCl with equimolar KCl in Ringer's solution, thereby making the final concentration of KCl to be 25, 50 and 100 mM;

3) Ba²⁺-Ringer's and Ba²⁺&K⁺ solutions were prepared by adding 1 mM Ba²⁺ to Ringer's and to the 50 mM K⁺ solution. In these two solutions, MgSO₄ had been substituted by MgCl₂ equimolarly to avoid precipitation.

4) bovine serum albumin (BSA) solutions were made into 0.01, 0.1, 0.5, 1, and 5 g% by dissolving BSA in Ringer's.

5) K⁺-free Na⁺ solutions contained 5, 15, 50, and 146 mM Na⁺ with K⁺ replaced by either Na⁺ or tetramethylammonium (TMA⁺) in Ringer's, depending on the final Na⁺ concentration.

6) other solutions:

i) bisoxonol A stock solution of 2x10⁻³ M bisoxonol in ethanol was prepared and stored in a refrigerator. Before an experiment, it was diluted in application solutions with required concentrations, for instance, 2 μM.

ii) TRITC-albumin A stock solution of TRITC-albumin in Ringer's was made to
have a concentration of 1 g% in advance and stored in the freezer. Before an experiment, the stock solution was diluted in application solutions to obtain required concentrations.

iii) gramicidin D The stock solution (10 mg/ml) was prepared by dissolving gramicidin D in a solvent, dimethyl sulfoxide (DMSO) and stored in the refrigerator. The application concentration of 10 µg/ml was obtained by diluting the stock solution in corresponding solutions.

All of the above chemicals were purchased from Sigma Chemical Co. or Fisher Scientific Inc., except that bisoxonol and TRITC-albumin were from Molecular Probes, Inc.

Examination of Bisoxonol Fluorescence under Varied Medium Conditions

Using a spectrophotofluorometer (LS 50, Perkin-Elmer), I conducted a series of experiments, in vitro, to examine the stability of bisoxonol fluorescence. The fluorescence spectra of bisoxonol were scanned between 450-510 nm for excitation and between 505-600 nm for emission with a 2.5 nm slit width on both sides and the fluorescence intensities at the maximum emission (517 nm) were registered as well under the following varied medium conditions:

1) varied bisoxonol concentrations A series of Ringer’s solutions with varied bisoxonol concentrations of 0, 0.1, 0.5, 1, 2, 5, and 10 µM were tested.

2) varied pH values The variation of pH in Ringer’s was obtained by adding 1 M HCl or 5 M NaOH to the solution and monitored to be 6.5, 7.0, 7.2, 7.4, 7.6, 8.0, and 8.5 by a pH meter (Model 810, Fisher Scientific). Then bisoxonol in the stock solution was added to these Ringer’s solutions with the final concentration of 2 µM.

3) varied ionic strength The above high K⁺ and Ba²⁺ solutions were used to test the effect of ionic strength on the stability of bisoxonol (2 µM) fluorescence.
4) varied BSA concentrations Bisoxonol was diluted in the above series of BSA Ringer's solutions to reach the final concentration of 2 μM. Then, bisoxonol fluorescence in these solutions was measured to detect the effect of an interaction between bisoxonol and protein molecules.

5) varied medium temperature Viability of bisoxonol (0.1–10 μM) to changes in medium temperature was tested by comparing its emission spectrum and intensity at 25 °C and 37 °C.

6) crossover of fluorescence between bisoxonol and TRITC-albumin TRITC-albumin is a fluorescent probe used as a tracer for measuring microvascular permeability to macromolecules. It has the wavelength of maximum excitation at 555 nm and maximum emission at 580 nm. If this fluorescent dye is used along with bisoxonol, there is a potential for crossover of fluorescence since either the excitation or the emission wavelengths of both dyes are relatively close. Therefore, in this set of experiments, the fluorescence crossover was examined by adding these two dyes in the medium. The concentration of TRITC-albumin was varied at 0.001, 0.002, 0.004, 0.006, 0.008, 0.01, 0.1, and 0.5 g% in the following solutions: i) normal Ringer's; ii) Ringer's plus 2 μM bisoxonol; and iii) Ringer's plus 5 μM bisoxonol. At first, as each solution was subjected to the excitation light of 495 nm, the emission of that solution at 517 nm was measured for the fluorescence intensity of bisoxonol. Then, the excitation light was switched to 555 nm, and the maximum emission of the sample was measured at 580 nm for TRITC-albumin. The slit width was broadened in this set of experiments to 15 nm for excitation and to 4.9 nm for emission.

Calibration of Bisoxonol Intensity to the Value of $V_m$ in Intact Aortic EC's

A change in bisoxonol fluorescence intensity ($I_f$) was calibrated to a change in the
voltage value of \( V_m \) using hamster intact aortic EC's. Similar to the procedures I have described before for intracellular recording of \( V_m \) in intact EC's, a segment of the hamster thoracic aorta was removed, opened and pinned with the luminal surface upwards onto the bottom of a tissue chamber attached to the stage of an epifluorescence microscope (Olympus, BH2), as shown in Figure 8. Initially, the tissue was continuously superfused with oxygenated Ringer's solution (37 °C, pH 7.4). Then, a microelectrode was advanced into aortic EC's to record the resting \( V_m \) according to the criteria I followed in my first study (Miao et al., 1993). To load the intact EC's with bis-oxonol, the suffusate was switched to Ringer's solution containing 2 \( \mu \)M bisoxonol in the dark. After 15–20-minutes of loading and equilibrating the EC's with bis-oxonol, the aortic strip was excited repeatedly by a light at 490 nm from a HBO 100-W mercury lamp (Leitz) for 2 seconds at 30-second intervals. At the same time, the fluorescence intensity of bisoxonol emitted from the focused surface of the endothelium was recorded at an emission wavelength of 515 nm through a measuring window (160 x 160 \( \mu \)m²) in the photomultiplier tube compacted with a photometer system (MVP, Leitz). This fluorescence intensity was stabilized within 5 min. and was registered as a baseline of bisoxonol intensity, i.e. \( I_0 \), corresponding to the resting \( V_m \) in these cells which were under simultaneous intracellular recording with a microelectrode. These outputs of both bisoxonol intensity and \( V_m \) voltage were depicted on a 4-channel chart recorder (Grass). Then, high \( K^+ \) solutions (25, 50, and 100 mM) were applied sequentially to the tissue to induce the depolarization of the cell membrane and the responses of both fluorescence intensity (\( I_r \)) and \( V_m \) were recorded continuously in the same manner. Changes in \( I_r (\%\Delta I_r = [(I_r-I_0)/I_0]100\%) \) and in \( V_m (\Delta V_m = \text{the depolarized } V_m - \text{the resting } V_m) \) were
Fig. 8. The working system for measuring changes in \( V_m \) of aortic EC's simultaneously using bisoxonol and a microelectrode.
compared to obtain a calibrated rate of $\% \Delta I_r / mV$ (a percent change in $I_r$ per millivolt).

**Cannulation of Microvessels for Intravital Microscopy**

After general anesthesia with sodium pentobarbital (60 mg/Kg. body weight), the hamster mesentery was spread over a cover-slipped pedestal in a specially-designed tray and suffused with Ringer's solution (37 °C, pH 7.4) which was oxygenated with 95% $O_2$ and 5% $CO_2$). Converging capillaries or post-capillary venules (8–30 μm) with brisk blood flow were cannulated doubly at the site of convergent branches and then the microvessels were perfused constantly with Ringer's solution at a pressure of 20–30 cm $H_2O$ via one of the two cannulated pipettes. These cannulations and subsequent observation and measurement were accomplished with an inverted fluorescence microscope (Leitz) with a 40x (NA 0.7) objective and a 10x eyepiece in a dim red light (>650 nm).

**Measurement of $I_r$ from Microvascular EC's, in situ, Using Bisoxonol**

As shown in Figure 9, the light from a 100-W mercury lamp (Leitz) was passed first through a heat filter (Leitz) and then a band-pass filter (490 nm, Leitz). This excitation light was reflected through the microscope objective to the cannulated vessel. The emission light from the excited EC's lining the vessel wall was passed through a band-pass filter (515 nm, Leitz) to a measuring window (about 50 x 70 μm$^2$) in a photomultiplier tube (Leitz). After autofluorescence of the cells had been registered, Ringer's solution containing 2 μM bisoxonol-was perfused into the microvessel via the cannulated pipette. After 15 min of loading, fluorescence intensity ($I_r$) from the bisoxonol-loaded EC's was measured intermittently for 2 seconds at 30-second intervals until it reached a stable value.

**Calibration of Bisoxonol $I_r$ to the Value of $V_w$ for microvascular EC's, in situ**
Fig. 9. The working system for measuring bisoxonol I, from microvascular EC’s, 
in situ, under the intravital epifluorescence microscope.
After a baseline of bisoxonol $I_r$, i.e. $I_0$, had been obtained, the microvessel was perfused with bisoxonol (2 μM) and gramicidin D (10 μg/ml) in the K*-free/5 mM Na* solution by switching on the other pipette. When the bisoxonol $I_r$ was stabilized, this new value of $I_r$ was registered as the intensity for 5 mM Na* solution. Afterwards, the microvessel was recannulated and perfused with K*-free/15, 50 and 146 mM Na* solutions which also contained bisoxonol (2 μM) and gramicidin D (10 μg/ml). The bisoxonol $I_r$ from microvascular ECs upon application of these solutions with varied Na* concentrations were recorded as a function of each different Na* solution. While the vessel was perfused with these different solutions, the suffusate was maintained identical to the perfusate except it did not contain bisoxonol. After each step of changing the Na* concentration in both suffusate and perfusate, the fluorescence images from microvascular EC’s were detected continuously by a SIT camera (Dage MTI) and digitized (512x512, 16 bit deep) at their steady state by an Image-1 processing system (Universal Imaging) as a 16-frame average and then, stored on an optical disk for subsequent image processing.

Changes in the bisoxonol $I_r$, i.e. $ΔI_r$ at each Na* concentration were compared to the original resting level ($I_0$) and calculated by:

$$\%ΔI_r=(ΔI_r/I_0)100\%$$

Gramicidin D is an ionophore for monovalent cations, mainly Na* and K*, and it creates a membrane conductance predominantly for these ions (Gómez-Puyou and Gómez-Lojero, 1977). Since extracellular K* was replaced equimolarly with tetramethylammonium (TMA+) and TMA+ is an impermeable ion to gramicidin D, the membrane potential could be determined by the electrochemical gradient between extracellular Na* ([Na*]o) and
intracellular Na\(^+\) and K\(^+\) ([Na\(^+\)+K\(^+\)]) following the Nernst equation (Grinstein et al., 1984). Therefore, values of \(V_m\) under varied extracellular Na\(^+\) concentrations ([Na\(^+\)]\(_0\)) were calculated by the equation:

\[
V_m = \frac{RT}{F} \ln \left( \frac{[\text{Na}^+]/([\text{Na}^+]+[\text{K}^+])]_o \right)
\]

assuming that intracellular concentrations of Na\(^+\) and K\(^+\) are 16 and 130 mM, respectively (Brock, et al., 1986).

The means of calculated \(V_m\) values versus the means of measured \(\%\Delta I_r\) obtained at the four different [Na\(^+\)]\(_o\) concentrations were then analyzed for their linear correlation and regression. The resting \(V_m\) of microvascular EC's can be found on the regression line at the "null point" (Hoffman and Laris, 1974) where bisoxonol \(I_r\) equals to \(I_o\).

**Actions of K\(^+\) and Ba\(^{2+}\) on the Resting \(V_m\) of Microvascular EC's, *in situ***

After bisoxonol \(I_r\) stabilized at a resting level (\(I_o\)), high K\(^+\) (25, 50, and 100 mM), 1 mM Ba\(^{2+}\)-Ringer's or 1 mM Ba\(^{2+}\)& 50 mM K\(^+\) solutions were applied to the vessel via suffusion and/or perfusion. During these manipulations, bisoxonol (2 \(\mu\)M) was present constantly in the perfusate. Responses of bisoxonol \(I_r\) were recorded continuously in the same manner as described above. Fluorescent images of the microvessel formed by EC's were meanwhile acquired at their steady state by the Image-1 system before and after applying different solutions.

**Data Analysis**

All data points were expressed as means±SE. The differences between means of paired two-group and three-group samples were tested using the paired t-test and the two-way analysis of variance plus multiple comparisons, respectively. P-level less than 0.05 was considered statistically significant. Analysis of linear regression and correlation was
conducted according to the principle of least-squares computation.
RESULTS

Stability of Bisoxonol Fluorescence under Varied Medium Conditions

1) Effect of Bisoxonol Concentration: With the concentration of bisoxonol varied from 0.1 to 10.0 μM in Ringer's solution, the peak of an emission spectrum was shifted slightly from 517 to 521 nm and the fluorescence intensity at 517 nm was enhanced exponentially from near zero to 37 units (Figure 10A and 10B).

2) Effect of pH Value: The variation of pH (pH 6.5-8.5) in Ringer's solution showed no effect on either the emission spectrum or the fluorescence intensity of bisoxonol (2 μM) as shown in Figure 11.

3) Effect of Ionic Strength: The potassium concentration in normal Ringer's (4.6 mM K⁺) was altered in gradients to 25, 50, and 100 mM; to evaluate a barium effect, 1 mM Ba²⁺ also was added to Ringer's or to 50 mM K⁺ solution. These manipulations of ionic strength or ionic concentration did not produce any significant changes in either the emission spectrum or the fluorescence intensity of bisoxonol as compared to normal Ringer's (Figure 12).

4) Effect of Varying Albumin Concentration: When bovine serum albumin (BSA) was added to the Ringer's solution (0.01-5%), dramatic increases in the fluorescence intensity of bisoxonol (2 μM) were found; the intensity increased exponentially from about 35 units in Ringer's to almost 300 units in 5% BSA solution. A slight shift in the maximum emission to 520 nm also was noticed at a higher BSA concentration (Figure 13A and 13B).

5) Effect of Temperature: Viability of bisoxonol (0.1-10.0 μM) to temperature was tested by comparing its emission spectrum and intensity at 25 °C and 37 °C. A reduction in
the fluorescence intensity was found to be less than 10% after solution temperature was raised from 25 to 37 °C while no shift was detected for the emission spectrum.

6) Crossover of Measurements for Bisoxonol and TRITC-albumin: As shown in Figure 14A, TRITC-albumin did not exhibit considerable fluorescence at bisoxonol emission wavelength; whereas, a decrease in the fluorescence intensity of bisoxonol was found as the concentration of TRITC-albumin was elevated. This phenomenon is opposite to that predicted for fluorescence crossover and it could be due to a quenching effect of TRITC-albumin on bisoxonol or to some other unknown cause(s). With regard to the fluorescent intensity of TRITC-albumin at its own emission wavelength, an exponential rise was observed when TRITC-albumin was increased (Figure 14B). The presence of bisoxonol seemed to have no interference with the fluorescent intensity readings of TRITC-albumin.

The fluorescence crossover also was tested under the epifluorescence microscope connected to a photomultiplier. Significant crossover (10-35%) in the fluorescence intensity was detected within the emission range of bisoxonol (2 µM) in the presence of 0.5% TRITC-albumin either on the perfused mesentery tissue or in a well (14 µl) of Decislide (UriSystem, Fisher Scientific). However, bisoxonol did not produce any considerable interference with the fluorescent intensity readings of TRITC-albumin.

Correlation of Changes in Bisoxonol I_t to Changes in V_m for Intact Aortic EC’s

The resting V_m of intact aortic EC’s was found not to be affected by the bisoxonol-loading process and the mean of the resting V_m was -35±2 mV (n=7). High K⁺ solutions (25, 50 and 100 mM) induced the membrane depolarization by 5.3±0.3, 8.0±1.0 and 14.3±2.0 mV and correspondingly increased I_t by 33.0±6.1, 48.3±11.4 and 81.7±17.8%. As exhibited in Figure 15, the millivolt change in V_m (ΔV_m) was correlated with the percent increase in I_t.
Fig. 10. Effect of raising the concentration of bisoxonol in Ringer’s solution (37 °C, pH 7.4). A: fluorescence spectra of bisoxonol between 505-600 nm excited at 495 nm; B: a logarithmic function of the fluorescence intensity (at 517 nm) of bisoxonol to the concentration of bisoxonol in the medium.
Fig. 11. Effect of varying pH in Ringer's solution (37 °C) on emission spectra of bisoxonol (505-600 nm) excited at 495 nm.
Fig. 12. Effect of varying ionic strength in Ringer's solution (37 °C) on emission spectra (505-600 nm) of bisoxonol (2 μM) excited at 495 nm.
Fig. 13. Effect of raising the concentration of albumin (BSA) in Ringer's solution (37 °C, pH 7.4) on bisoxonol fluorescence. A: fluorescence spectra of bisoxonol (2 μM) between 505–600 nm excited at 495 nm under varied concentrations of BSA; B: an increase in the fluorescence intensity (at 517 nm) of bisoxonol as a logarithmic function of an increase in the concentration of albumin in the medium.
Fig. 14. Crossover of measurements of the fluorescence intensity for bisoxonol and TRITC-albumin in Ringer's solution (37 °C, pH 7.4). A: Effect of raising the concentration of TRITC-albumin on the fluorescence intensity (at 517 nm) of bisoxonol excited at 495 nm; B: Effect of the presence of bisoxonol on the fluorescence intensity (at 580 nm) of TRITC-albumin excited at 555 nm.
a positive and linear correlation ($r=0.855$, $p<0.05$) was obtained with a slope of 5.7%
/mV, that is to say, every 5.7\% change in bisoxonol $I_f$ from measured intact aortic EC's corresponds to one millivolt change in $V_m$ of the cells.

**Correlation of a Change in Bisoxonol $I_f$ to a Change in $V_m$ for Microvascular EC's, *in situ***

In mesentery microvessels, as exhibited in Figure 16, continuous perfusion with Ringer's solution washed out the blood from the lumen of the vessel (panel A) that was within the viewing area under the intravital microscope. The absence of the blood would rule out any influence of blood cells and circulating factors on EC's lining the wall of microvessels, and thereby, the milieu conditions of those EC's were well controlled by the perfusate in the lumen and by the suffusate in the interstitial space. After bisoxonol (2 \( \mu \)M) was applied to the perfusate for 15–20 minutes, those microvascular EC's were loaded and equilibrated with this fluorescent dye. Since bisoxonol dramatically enhances its fluorescence by binding to intracellular proteins, the bisoxonol-loaded EC's showed the bright fluorescent images in the fluorescence field whereas the luminal and abluminal spaces of the microvessel were rather dark (Panel B). Therefore, bisoxonol $I_f$ measured under the intravital epifluorescence microscope was directly from those bisoxonol-loaded EC's.

Under the well-controlled working system, *in situ* experiments (n=5) for calibrating bisoxonol $I_f$ to $V_m$ values in microvascular EC's were conducted by varying the extracellular Na\(^+\) concentration in the presence of Gramicidin D (10 \( \mu \)g/ml). As compared to the resting level of bisoxonol fluorescence ($I_n$), changes in bisoxonol $I_f$ were measured to be -51.9\%, 51.9\%, 232.8\%, and 415\% under varied Na\(^+\) concentrations of 5, 15, 50, and 146 mM, respectively. Correspondingly, these four extracellular Na\(^+\) concentrations would set four
Fig. 15. Correlation of the increase in bisoxonol fluorescence to the depolarization of aortic EC's (n=7). The resting $V_m$ was -35±2 mV. The depolarization was induced by raising the concentration of extracellular $K^+$ from 4.6 mM to 25, 50, and 100 mM in Ringer's solution. Changes in $V_m$ were measured simultaneously with microelectrodes and bisoxonol (2 $\mu$M). The linear correlation was significant ($p<0.05$).
values of $V_m$ to be -90, -60.7, -28.6, and 0 mV, assuming intracellular Na$^+$ is 16 mM and K$^+$ is 130 mM (Brock et al., 1986). Based on these calculated values of $V_m$ and measured changes in $I_n$, my results yielded a linear correlation ($r=0.993$, $p<0.05$) of bisoxonol $I_t$ to $V_m$ levels in a range of 0--90 mV with a slope of 5.2% (Figure 17). This slope suggests that a 5.2% change in the bisoxonol intensity from microvascular EC's corresponds to a 1.0 millivolt change in the resting $V_m$ of the cells. Using the null point on this curve, i.e. the point where $\%\Delta I_t=0$, the resting $V_m$ of microvascular EC's was found to be -76 mV. As compared to the changes in bisoxonol $I_n$, the fluorescent images of the microvascular EC's showed the consistent response to the variation in extracellular Na$^+$ (Figure 18). Images in panel 3, 4, 5, and 6 corresponded, in an increasing manner, to the successive fluorescence profiles of those EC's produced by 5, 15, 50, and 146 mM Na$^+$, respectively. Panel 2 represents the fluorescent image of those EC's at the resting level which could be recognized to be between panel 3 and panel 4 with respect to the brightness of fluorescence from the cells.

**Depolarization of Microvascular EC's, in situ, by High K$^+$**

In a series of experiments on microvascular EC's ($n=4$), the effects of increasing extracellular K$^+$ concentrations (25, 50 and 100 mM) sequentially on $V_m$ were examined by measuring $I_t$ from bisoxonol-loaded EC's. In Figure 19, fluorescent images from a microvessel before, during and after application of high K$^+$ to both the inside and outside of the vessel are shown. As ascertained by bright-field and fluorescence microscopy, the cells lining the inside of the microvascular wall fluoresced the brightest. Altering extracellular K$^+$ produced increases in the fluorescence of these cells and the greater the increase in extracellular K$^+$, the greater the intensity of the fluorescent images. Removing extracellular
Fig. 16. Images of a postcapillary venules (diameter=12 μm) in the hamster mesentery. A: The perfused microvessel in the bright field under the intravital microscope; B: The same perfused microvessel in the bisoxonol (2 μM) fluorescent field.
$V_m = \frac{RT}{zF} \ln \left( \frac{[Na^+]_o}{[Na^+ + K^+]_i} \right)$

**Fig. 17.** Correlation of changes in bisoxonol (2 μM) fluorescence to changes in $V_m$ from microvascular EC's, *in situ* (n=5). Changes in $V_m$ were produced by varying the concentration of extracellular Na$^+$ ([Na$^+$]$_o$) in the presence of Gramicidin D (10 μg/ml) and calculated according to the equation: $V_m = \frac{RT}{zF} \ln \left( \frac{[Na^+]_o}{[Na^+ + K^+]_i} \right)$. Changes in bisoxonol fluorescence was measured in comparison with its resting level and correlated to the calculated $V_m$ values, correspondingly. The resting $V_m$ was obtained from the correlation curve at the "null point" where the change in bisoxonol fluorescence is zero.
Fig. 18. Fluorescent images of bisoxonol-loaded microvascular EC's under varied concentrations of extracellular Na⁺. Panel 1: a microvessel (diameter=16 μm) perfused with bisoxonol (2 μM) in Ringer’s in the bright field; panel 2: the same vessel in the bisoxonol fluorescent field; panel 3, 4, 5, and 6: the same vessel perfused with varied Na⁺ concentrations (5, 15, 50, and 146 mM) in Ringer's solution, respectively.
high K* brought their fluorescence back towards the original level. Using the photomultiplier system for measuring $I_r$ from these microvessels, I obtained results consistent with the observations from the image analysis. As shown in Figure 20, when high K* solutions were applied via suffusion, $I_r$ was elevated to a maximal level within 1-3 min; when high K* was present in both suffusate and perfusate, $I_r$ increased to much higher values within 1-3.5 min. The depolarized $V_m$ upon 25 and 50 mM K* was maintained at a relatively stable level before removing high K* from the perfusate; whereas, fluctuations of $I_r$ were found during 100 mM K*-induced depolarization which might indicate actions of some ion channels activated or inactivated at this depolarized voltage level. To sum the data, as illustrated in Figure 21, raising K* on the abluminal side ([K+]o) increased $I_r$ by 28±4%, 40±7% and 51±4% whereas raising K* on both the abluminal and luminal sides ([K+]o) increased $I_r$ by 94±17%, 157±28% and 219±24%, respectively. The results from these two different maneuvers, i.e. altering [K+]o and [K+]o for each concentration, were significantly different. According to the slope of 5.2%/mV obtained from the in situ calibrated curve, the depolarized amplitudes of $V_m$ in response to high K* (25, 50, and 100 mM) were calculated to be 5±0.8, 8±1.3 and 10±0.7 mV for the K* effect by suffusion and 18±3.3, 30±5.3, and 42±4.6 mV for the K* effect by both suffusion and perfusion. Correspondingly, transference numbers of K* ($t_{K^+}$) were calculated to be 0.12 for the three high K* solutions when suffused and 0.38, 0.48 and 0.50 for this series of high K* solutions when both suffused and perfused, showing significant differences between the two groups and between 25 versus 50 or 100 mM K* within the both suffusion and perfusion group ($p<0.01$).

Depolarizing Actions by Ba²⁺ and by both Ba²⁺ and K* on Microvascular EC's,

*in situ*
Fig. 19. Fluorescent images from a bisoxonol-treated microvessel projecting \( V_m \) changes in microvascular EC's by altering extracellular \( K^+ \). Panel 1, 2, 3, panel 4, 5, 6, and panel 7, 8, 9 exhibit representative and comparative fluorescent images from one microvessel before, during, and after exposure to high \( K^+ \) (25, 50, and 100 mM), respectively.
Fig. 20. Continuous recordings of changes in bisoxonol fluorescence of EC's from a microvessel. After the baseline of bisoxonol fluorescence under Ringer’s (control) condition had been recorded for 2 minutes, high K⁺ (25, 50, and 100 mM) solutions were applied to the microvessel via suffusion and then both suffusion and perfusion. The responses of bisoxonol fluorescence from the EC’s were continuously monitored. In a similar manner, high K⁺ solutions were removed from the vessel after the bisoxonol intensity had reached a stable level.
Fig. 21. Comparison of increases in bisoxonol I, from the measured microvascular EC's between suffusion (o) and both suffusion and perfusion (o/i) of the microvessels with high K⁺ (25, 50, and 100 mM) solutions.
Effects of Ba\textsuperscript{2+} (1 mM) and high K\textsuperscript{+} (50 mM) and their interaction on $V_m$ of microvascular EC's were compared. As illustrated in Figure 22, Ba\textsuperscript{2+} produced brighter fluorescent images of the microvascular wall than those by 50 mM K\textsuperscript{+}; however, this effect was attenuated in the presence of high K\textsuperscript{+}. In this series of experiments, the effects of K\textsuperscript{+} and Ba\textsuperscript{2+} and their interaction on the resting $V_m$ of microvascular EC's were investigated. In Figure 23, for the application of either K\textsuperscript{+} or Ba\textsuperscript{2+}, the increase in I_f (a depolarizing action) was significantly greater when the application was to the inside as compared to the outside of the microvessel (p<0.05). For any given method of application, either inside or outside or both inside and outside, the change in I_f was significantly greater for Ba\textsuperscript{2+} than for K\textsuperscript{+} (p<0.05). However, when Ba\textsuperscript{2+} and K\textsuperscript{+} were applied simultaneously, their interaction significantly attenuated the increase in I_f as compared to either ion alone (p<0.05). Referring to the calibrated rate of 5.2%/mV, membrane depolarizations by [K\textsuperscript{+}]o/i, [Ba\textsuperscript{2+}]o/i, and [Ba\textsuperscript{2+}&K\textsuperscript{+}]o/i were calculated to be 37, 70 and 22 mV, respectively. These results were significantly different from each other (p<0.05).
Fig. 22. Fluorescent images of EC's from a specific segment of the microvessel. Panels 1, 2, and 3 show the images under control, [K⁺]₀ and [K⁺]ₐₒ conditions; panels 4, 5, and 6 are the images under control, [Ba²⁺]₀ and [Ba²⁺]ₐₒ conditions; and panels 7, 8, and 9 are the images under [Ba²⁺]ₐₒ, [Ba²⁺ & K⁺]ₐₒ, and [Ba²⁺ & K⁺]ₐ conditions.
Fig. 23. Comparison and interaction between K⁺ (50 mM) and Ba²⁺ (1 mM) applied via suffusion (o), perfusion (i), or both suffusion and perfusion (o/i) (n=5). Note: *p<0.05, compared to [K⁺] or [Ba²⁺ & K⁺], correspondingly; #p<0.05, compared to corresponding [K⁺].
DISCUSSION

Stability of Bisoxonol Fluorescence under Varied Medium Conditions

Many fluorescent dyes are liable to variations in medium conditions. This characteristic would limit the validity of a fluorescent probe when it is used in an unfavored experimental system. Before bisoxonol was employed to indicate the change in $V_m$ for vascular EC's, I have tested its fluorescence stability under varied medium conditions. My results suggest that bisoxonol is quite stable to variations in the ionic strength of the medium in terms of its fluorescence intensity as well as its emission spectrum. Therefore, the high $K^+$ or $Ba^{2+}$-induced increases in bisoxonol $I_r$ measured in vascular EC's were not caused by a change in the property of bisoxonol fluorescence itself but by a change in the amount of bisoxonol accumulated in the cells. In a range of 6.5–8.5, variations in solution pH did not produce any change in bisoxonol fluorescence, indicating bisoxonol is a pH-insensitive dye and its fluorescence is not affected by any fluctuation in intracellular pH which may occur under certain circumstances. The reduction in bisoxonol intensity by an increase in the medium temperature is rather small with regard to a well-controlled solution temperature in my experiments. The fluorescence intensity of bisoxonol rises exponentially either as the concentration of bisoxonol increases or by the addition of protein, e.g. BSA, to the medium. This is similar to the reported data by Bräuner et al. (1984). In their study, bisoxonol $I_r$ was found increased when either albumin or globulin was added to the medium. This suggests that the bisoxonol working mechanism is related to binding of intracellular protein molecules. Interestingly in my study, the increase in bisoxonol $I_r$ showed a logarithmic function to the increase in either the bisoxonol concentration or the concentration of BSA.
in the media. If this is the real case, then it could be explained nicely why the diffused amount of intracellular bisoxonol inversely follows the exponential change in $V_m$ whereas bisoxonol $I_f$ exhibited a linear relationship with changes in $V_m$. When a logarithmic function ($I_f = a \ln D_1 + b$) comes to an exponential function ($D_1 = D_0 e^{(V_s T)V_m}$), a linear relationship ($I_f = cV_m + d$) will be produced. But, this interpretation should be carefully cited because the logarithmic function of bisoxonol $I_f$ was found in, *in vitro*, condition and only within my tested range of either bisoxonol or protein concentration which is not necessarily the same as that in the cytosolic space. The crossover of TRITC-albumin emission on the fluorescence intensity of bisoxonol will lead to significant errors if TRITC-albumin is present while the bisoxonol $I_f$ is being measured for a change in $V_m$ of microvascular EC's, *in situ*. However, the presence of bisoxonol will not interfere with the readings of TRITC-albumin for monitoring changes in microvascular permeability.

**Bisoxonol Is a Reliable Voltage-Sensitive Probe for Measuring $V_m$ in Vascular EC's**

Over the past 20 years, voltage-sensitive fluorescent probes have been used widely to monitor the transmembrane potential of cellular organelles and small cells, such as mitochondria, red blood cells, mast cells, neutrophils, and lymphocytes. A few attempts have been made to observe $V_m$ in cultured vascular EC's using a cyanine dye, DiOC$_3$(3) (Lerner et al., 1988; Goligorsky, 1988) or bisoxonol (Laskey et al., 1992). However, no calibration of bisoxonol $I_f$ to the $V_m$ value has been achieved in these studies although bisoxonol fluorescence indicated the gross change in $V_m$ for the vascular EC's. My calibration experiments were conducted in two different ways, *i.e.* correlating changes in bisoxonol $I_f$ to changes in $V_m$ that were directly recorded with microelectrodes from intact
aortic EC's; and correlating changes in bisoxonol \( I_f \) to changes in \( V_m \) that were generated by varying extracellular Na\(^+\) in the presence of Gramicidin D for microvascular EC's. Both calibrations yielded the linear relationship between \( V_m \) and bisoxonol \( I_f \), showing the rate of changes at 5.7%/mV for aortic EC's and 5.2%/mV for microvascular EC's. These two values are relatively comparable, indicating that the responding kinetics of bisoxonol during \( V_m \) variations is similar between aortic and microvascular EC's. A lower rate of the change in bisoxonol \( I_f \) per mV has been reported to be 1%/mV for homokaryons of BICR/M1R-\( k \) cells by Brüuner et al. (1984) although the technique used in their study for calibrating the fluorescent response of bisoxonol to \( V_m \) was similar to one of my techniques, that is, the intracellular recording with microelectrodes. The difference in the calibrated rate could be due to the difference in animal species and cell types. In rat thymocytes, the response of bisoxonol \( I_f \) exhibited a relatively linear relationship to the change in \( V_m \) in a range of 0-75 mV calibrated by varying the extracellular Na\(^+\) concentration (Grinstein et al., 1984) as I did for microvascular EC's. In agreement with these studies, my results indicate that bisoxonol is a reliable probe for measuring changes in \( V_m \) of vascular EC's, in situ, and further, the calibrated rate of 5.2-5.7%/mV from linear correlations can be used to quantitatively interpret a change in bisoxonol \( I_f \) as a change in \( V_m \).

According to the "null point" theory (Hoffman and Laris, 1974), the baseline level of bisoxonol \( I_n \) i.e. \( I_{\phi} \), corresponds to the resting \( V_m \) of microvascular EC's that was found to be -76 mV. This value is about twice as high as the resting \( V_m \) (-40-43 mV) in intact aortic or vena caval EC's measured by using microelectrodes in my previous study (see Chapter II). This substantial difference could be explained by a difference in the ionic basis for determining the resting \( V_m \) in microvascular EC's as compared to that in EC's of large
vessels. Obviously, the depolarizing amplitude of the resting $V_m$ by high $K^+$ is considerably different between these EC's from different vascular beds. For example, the 100 mM $K^+$ solution produced a depolarization of 42 mV in microvascular EC's that was significantly greater than a depolarization of 17 mV in aortic EC's. This difference in depolarizing amplitude by high $K^+$ at the same concentration indicates that the contribution of $K^+$ to the resting $V_m$ is greater in microvascular EC's than in EC's from large vessels. Therefore, the resting $V_m$ in microvascular EC's would be drawn more towards the $K^+$ equilibrium potential ($E_K=-90$ mV), thereby generating a resting $V_m$ with more negative value in microvascular EC's as compared to that in EC's from large vessels. The physiological significance of a high resting $V_m$ in microvascular EC's is unclear. This electrical property of the cell membrane may be associated with the unique role of microvascular EC's in regulating local blood perfusion and microvascular permeability. In comparison with EC's lining the inside of conduit vessels, microvascular EC's might be more active in the process of vascular functions. Structurally and metabolically, microvascular EC's are, to varying degrees, different from EC's in large vessels. The greater the resting $V_m$ is, the more room the cell has for electrical responses to external stimuli. In addition to different cellular mechanisms for generating the resting $V_m$, different measuring techniques could result in variations in a value of the resting $V_m$. When a microelectrode is used to detect the resting $V_m$, impalement of a cell would inevitably damage the cell membrane. The leakage of cytosolic contents as well as a direct contact between the interior of the cell and the medium would break down electrochemical gradients of ions or generate leakage currents across the membrane. Thus, the resting $V_m$ in EC's from large vessels would possibly be reduced when it is recorded during an experiment. Whereas, when bisoxonol is used to monitor the resting $V_m$ in
microvascular EC's, integrity of the cell membrane can be maintained and so can the resting $V_m$. However, unlike intracellular recording with microelectrodes, the bisoxonol fluorescence is not a direct measure but an estimate of $V_m$. The resting $V_m$ in microvascular EC's was estimated at the "null point" on the calibrated correlation curve. This calibration is based on several assumptions: 1) concentrations of intracellular $K^+$ and $Na^+$ are known and constant; 2) Gramicidin D makes the membrane conductance entirely for $K^+$ and $Na^+$ and there are no intrinsic activities of either active transport systems, for instance $Na^+-K^+$-ATPase, or other ionic currents, e.g. a $Cl^-$ current; and 3) TMA$^+$ is an impermeant to the membrane in the presence of Gramicidin D. If experimental conditions do not perfectly satisfy these assumptions, an estimated value of the resting $V_m$ would vary to some degree. Nevertheless, the techniques of both intracellular recording and bisoxonol fluorescence were employed at their best in this study. The values of the resting $V_m$ and its variations in response to external stimuli are reliable. In cultured porcine cerebral capillary EC's, the resting $V_m$ was detected to be -24 mV by the whole-cell patch-clamping. The difference in the resting $V_m$ of microvascular EC's could be due to different cell conditions, experimental means, or it may be characteristic of the cells from different organs and animals.

**Differential Depolarizing Actions of High K$^+$ on Microvascular EC's**

The application of high K$^+$ solutions to microvascular EC's, *in situ*, produced significant depolarizations of the cell membrane. In comparison with the effect of high K$^+$ on intact aortic EC's as I described in Chapter II, these depolarizing actions appeared considerably stronger. For example, in aortic EC's, the depolarization induced by 100 mM K$^+$ was about 17 mV (Table 2) whereas in microvascular EC's, it was about 42 mV. Again, indicated by $t_{K^+}$, the contribution of the overall K$^+$ membrane conductance to the resting $V_m$
in microvascular EC's was 0.50 that is 1.5-fold greater than 0.2 found in aortic EC's. This result suggest that the membrane permeability to K⁺ is the primary determinant for the resting Vₘ in microvascular EC's. This could also explain why the resting Vₘ is higher in microvascular EC's than in EC's from large vessels probably because the strong K⁺ membrane conductance tends to bring the resting Vₘ towards its equilibrium potential (Eₚ= -90 mV). However, the contribution of K⁺ to the resting Vₘ in microvascular EC's is still smaller than that reported for cultured vascular EC's, which might suggest a remarkable difference in the ionic basis for regulating the resting Vₘ between EC's, in situ, and EC's in culture.

Another distinct characteristic of the Vₘ response to high K⁺ could also be observed in my in situ experiments. As shown in Figure 21, within ranges of 4.6 to 25, 25 to 50, and 50 to 100 mM, any given 2-fold change in the K⁺ concentration of the suffusate produced a constant change in bisoxonol I, by 12%. In comparison, however, the same alterations in the K⁺ concentration via both suffusion and perfusion produced significantly greater increases in bisoxonol I, by 39%, 63%, and 62% per 2-fold change in K⁺, respectively. The difference in a K⁺-induced depolarizing amplitude between K⁺ in the suffusate and K⁺ in the perfusate could be explained by the possibility that a constant but rather smaller K⁺ conductance of the EC's membrane is on the abluminal side whereas the EC's membrane on the luminal side possesses a greater K⁺ conductance. In other words, the K⁺ membrane conductance of microvascular EC's, in situ, may distribute asymmetrically across the vessel wall. This distinct property was not observed in the study using cultured EC's (Colden-Stanfield et al., 1992). However, my inference does not preclude other potential causes such as a difference in the diffusive capability of potassium ions across the vessel wall and/or a difference in the
structural and functional connections of the EC's membrane to surrounding tissue components, such as the basement membrane, pericytes and mast cells. Nonetheless, the differential depolarizing actions of high K$^+$ observed in my study reflect, at least, the different responsiveness of microvascular EC's, *in situ*.

In terms of a depolarizing amplitude per 2-fold change in K$^+$ concentration, a value of 19 mV (a 99% increase in bisoxonol I$_D$) would be predicted by the Nernst equation if the membrane conductance is due entirely to K$^+$. A similar effect was observed in cultured EC's (Johns *et al.*, 1987). The lower values (62-63% at highest) observed in this study suggest that the contribution of K$^+$ to the resting $V_m$ of microvascular EC, *in situ*, is relatively small particularly when the K$^+$ concentration is below 25 mM and some other ions, such as Na$^+$ and Cl$^-$, also may contribute to the resting $V_m$.

**Comparison and Interaction of the Depolarizing Effects between K$^+$ and Ba$^{2+}$**

The application of Ba$^{2+}$ (1 mM) to the microvessels induced a depolarization of the EC's membrane up to 70 mV that was significantly greater than the depolarized amplitude (37 mV) by 50 mM K$^+$. However, when both Ba$^{2+}$ and high K$^+$ were in the medium, the depolarization was significantly reduced to 22 mV. My results also demonstrated that the above effects were predominantly on the luminal side rather than on the abluminal side of the microvessel, thereby supporting again the asymmetrical distribution of the K$^+$ conductance in the EC's membrane, *in situ*.

Ba$^{2+}$ is a strong blocker of inwardly rectifying K$^+$ channels. In the presence of Ba$^{2+}$, $V_m$ was reduced to only -6 mV supposing the resting $V_m$ of -76 mV obtained at the "null point" holds true in this set of experiments. The dramatic decrease in $V_m$ by the Ba$^{2+}$ blocking action suggest that the ion conductance of the EC's membrane is dominated by
these K⁺ channels. This result is consistent with the observations in cultured EC's (Johns et al., 1987; Colden-Stanfield et al., 1987) but seems to conflict with the relatively smaller depolarizing effect of high K⁺ in my study. The rational explanations could be drawn as: 1) Ba²⁺ not only blocks inwardly rectifying K⁺ channels but passes through nonselective cation channels that serve as Ca²⁺ influx pathways. The interference with Ca²⁺ influx by Ba²⁺ would inhibit the activation of Ca²⁺-dependent K⁺ channels which help to hyperpolarize the membrane; 2) Some other ion channels that contribute to the repolarization may be interfered by Ba²⁺ as well; and 3) the K⁺ conductance appeared increased when the extracellular K⁺ concentration was high as indicated in this study by tₑ values, i.e. 0.50 at 100 mM K⁺ versus 0.38 at 25 mM K⁺. This may imply that if extracellular K⁺ is raised over 100 mM, the K⁺ conductance would continue to increase so that the depolarizing action of high K⁺ would become stronger.

The blocking effect of Ba²⁺ was found to be attenuated by K⁺ interference. The mechanism underlying this action is unknown. By detecting the absorbance of oxonol V, a voltage-sensitive dye, to indicate Vₘ changes, Majander and Wikström (1989) found a blocking action of Ba²⁺ on the Vₘ depolarization induced by high K⁺ in human neutrophils and this effect could be released with a higher concentration of K⁺. However, the presence of Ba²⁺ did not give rise to an increase in the absorbance of oxonol V, a sign for a Vₘ depolarization. Whereas, in my study, a dramatic increase in bis-oxonol I, indicates a strong depolarization of vascular EC's by Ba²⁺.

Summary

In conclusion, bisoxonol is a quite stable fluorescent dye; alterations in the fluorescence intensity of bisoxonol in EC's from both macro- and micro-vessels, in situ, are
correlated linearly with changes in $V_m$; thus, this technique is feasible for studying the
electrophysiological properties of EC's, *in situ*. Microvascular EC's have a resting $V_m$ of -76
mV that can be altered significantly by raising extracellular $K^+$ and the overall $K^+$ membrane
conductance is sensitive to $Ba^{2+}$. Further, the microvascular EC's may have a curtain degree
of polarity or a varied membrane conductance for $K^+$ as indicated by the asymmetric
depolarizing effects of both high $K^+$ and $Ba^{2+}$. 
BRADYKININ-INDUCED INCREASE IN MICROVASCULAR PERMEABILITY IS MEDIATED BY THE MEMBRANE POTENTIAL OF ENDOTHELIAL CELLS

INTRODUCTION

Endothelial Barrier in Microvessels

Among the important functions of microvessels is the control of substance exchange across the vascular wall. Microvascular EC's forming a monolayer inside of the vessel serves, both passively and actively, as the primary restrictive barrier between the circulating blood and the interstitium. Because the EC's membrane is basically a lipid bilayer, the endothelial barrier allows those lipid soluble molecules to freely traverse, either directly pass through EC's themselves or diffuse within and along the cell membrane to the other side of the barrier. Whereas, for those substances that are hydrophilic, such as small-sized ions and large-sized proteins, the transport would take place mostly via water-filled pores across the endothelial barrier (Joyner and Kern, 1990). These pores are provided either by intracellular channels formed with coalescent vesicle chains or by intercellular junctional gaps. Therefore, the size and the number of the pores relative to the size of those hydrophilic molecules would determine the transport kinetics of those molecules. Under normal condition in most microvascular beds, ions, due to their small size, are able to freely diffuse via pores and hence, there is no ion concentration gradient across the barrier, but most macromolecules like proteins, because of their large size, are restricted by the endothelial
barrier and thus, there is an outward concentration gradient of proteins across the vessel wall. During pathological processes, such as inflammatory reactions, the endothelial barrier undergoes the increase in the number and size of the pores which leads to leakage of macromolecules and even diapedesis of inflammatory cells from the vessel, thereby producing edema and injury to the underlying tissue. Thus, one of the final goals in the microcirculation study is to understand the control of endothelial barrier functions under both physiological and pathological conditions.

**Kinetics of Substance Transport Across Endothelial Barrier**

The concept of permeability is mostly introduced to describe the transport kinetics of a substance across the endothelial barrier. Permeability defines the easiness or the rate for a given solute to be transported from one side to the other across a membrane by diffusion. Diffusion is the process in which solutes move from one region of a high concentration to another of a low concentration resulting from random thermal motion (Brownian motion) of each individual particle of those molecules. This process is described by Fick’s First Law:

$$J_t = D_t A \frac{dC}{dX}$$

where $J_t$ is the net flux of the solute across a barrier (moles/s); $D_t$ represents the diffusion coefficient of the solute ($\text{cm}^2/\text{s}$); $A$ is the area of the barrier for diffusion ($\text{cm}^2$); $dC$ is the concentration difference across the barrier (mole); and $dX$ is the thickness of the barrier (cm).

When a barrier for diffusion is the biological membrane that is selective to the diffusive solute, Fick’s First Law is modified as:

$$J_t = P_t A dC$$

where $P_t$ is the permeability coefficient of the solute ($\text{cm/s}$), $P_t = K_t D_t/dX$, in which
$K_*$ is the partition coefficient describing the lipid solubility of the solute, for hydrophobic molecules, $K_*>1$ and for hydrophilic molecules, $K_*<1$, but if hydrophilic molecules diffuse via pores in the endothelial barrier, then presumably $K_*=1$.

From the above equations, for a given concentration difference of the solute ($dC$) across the endothelial barrier, $J_*$ is determined by $P_*$ that depends upon the size of certain number of pores relative to the size of the solute supposing the fluid temperature and viscosity are fixed. Therefore, measuring $P_*$ can detect how leaky the microvessel is. However, in most cases, the accurate measurement of $P_*$ is impossible due to the uncertainty of the diffusion area in a microvascular bed. For this reason, the microvascular permeability is often described in a value of $P_*$ product.

Besides the diffusion movement, the amount of net solute flux across the microvessel wall is affected by another driving force, i.e. convection or ultrafiltration, if there is a difference in hydraulic or osmotic pressure. In the convection movement, the water flow carries the solute along to moves across the endothelial barrier. The water flow can be expressed by Starling's Hypothesis:

$$J_* = L_p A [(P_e - P_f) - \sigma (\pi_e - \pi_i)]$$

where $J_*$ is the net water flux across the barrier (cm$^3$/s), $L_p$ is the hydraulic conductivity of the membrane (cm$^3$/s/cm H$_2$O), $A$ is the area of the barrier for the water flow (cm$^2$), $(P_e - P_f)$ is the difference of the hydrostatic pressure between blood flow interstitial fluid (cm H$_2$O), $\sigma$ is the osmotic reflection coefficient, and $(\pi_e - \pi_i)$ is the difference of the osmotic pressure between the plasma and interstitial fluid. Based on this equation, the net water flux that carries the solute along is determined by the balance of both hydrostatic and osmotic pressures. The size of pores in the endothelial barrier is apparently an important
factor influencing this balance by varying $\sigma$.

Taken both diffusive and convective movements account, the net exchange of the solute across the endothelial barrier can be described by Staverman-Kedem-Katchalsky equation:

$$J_s = P_s A d C + J_c (1-\sigma)C^*$$

where $J_s$ is the solute flux, $C^*$ is the average concentration of the solute within the pore, and other terms have the same meaning as described above.

So far, I can form a perception that the control of solute fluxes across the vessel wall is primarily achieved by the control of pore number and size in the endothelial barrier. There are many hypotheses and experimental models to describe the pore system in the microvascular endothelium. In this study, I will not focus on this issue but the flux of macromolecules, *i.e.* BSA, will be measured to indicate the bradykinin-induced change in microvascular permeability and this change is probably related to the alteration in the structure of the endothelial barrier, for instance, the increase in intercellular gaps.

**Effect of Ca$^{2+}$ Influx on Microvessel Permeability**

Many investigations have confirmed that inflammatory agonists including bradykinin induce the increase in microvascular permeability by forming gaps between EC's (Hulström and Svensjö, 1980; Joris *et al*., 1972; Clough and Michel, 1988). However, the intracellular mechanisms are still unclear. Accumulated evidence suggests that extracellular Ca$^{2+}$ influx in response to a number of agonists are involved during the increase in permeability and most vasodilators that stimulate the release of EDRF and prostacyclin also have the effect to increase in microvascular permeability.

Using Ca$^{2+}$ ionophore, A23187, or ionomycin, several studies have shown a
reversible increase in permeability of hamster and frog microvessels (Curry and Joyner, 1988; Curry et al., 1990; He et al., 1990; He and Curry, 1991). Removing extracellular Ca\textsuperscript{2+} or blocking Ca\textsuperscript{2+} channels prevented the increase in permeability induced by inflammatory mediators such as histamine, bradykinin, and serotonin (Liddel and Simpson, 1980; Mayhan and Joyner, 1984; Olesen, 1985). The time-course of the change in permeability of microvessels to albumin was also measured during action of A 23187 (Joyner and Curry, 1986; Curry and Joyner, 1988; Curry et al., 1990), which is similar to that for LDL and dextran transport (Rutledge et al., 1990). Permeability increased to a transient peak value within 1-3 min. followed by a sustained-lower but elevated value as long as the ionophore was in the perfusate. A direct measurement of changes in intracellular Ca\textsuperscript{2+} (detected by Fura-2) correlated with changes in vascular permeability was performed by He et al. (1990). Using Ca\textsuperscript{2+} ionophore, ionomycin, they demonstrated that intracellular Ca\textsuperscript{2+} increased from the control level (65 nM) to a initial peak of 285 nM after 1-3 min, then gradually decreased to a sustained level of 199-129 nM; after the ionophore was removed, Ca\textsuperscript{2+} fell back to the control level of 77 nM. Similarly, hydraulic conductivity (L\textsubscript{p}) increased to a peak of 9.5 times the control value after 1-3 min, followed by a decrease to 2 times the control after 6 min. and L\textsubscript{p} remained at this level as long as the ionophore was in the perfusate. The detailed investigation in regard to Ca\textsuperscript{2+}-dependent permeability changes induced by histamine has been reported in cultured EC's monolayers (Rotrosen and Gallin, 1986), but the time correlation between intracellular Ca\textsuperscript{2+} and changes in permeability was not achieved in this study. However, some other studies using cultured EC's monolayers showed a steadily increased permeability in the presence of histamine, thrombin or Ca\textsuperscript{2+} ionophores (Alexander et al., 1988; Killackey et al., 1986; Garcia et al., 1986), rather than a biphasic pattern.
observed in intact microvessels. This difference was proposed to be due to a higher basal permeability of cultured EC's monolayers. Mechanisms of changes in permeability mediated by Ca^{2+} entry are still unclear. Most acceptable interpretation could be that Ca^{2+} produces an increase in microvascular permeability by forming intercellular gaps via a direct interaction with the contractile cytoskeleton (Schnittler et al., 1990; Wysolmerski and Lagunoff, 1990) and/or via indirect actions as an intracellular signal on the synthesis and release of EC's autacoids such as endothelium-derived relaxing factor (EDRF) and prostacyclin (PGI_2) (Oliver, 1992; Yuan et al., 1993).

**Transduction Pathway for Bradykinin Action**

Bradykinin is a peptide consisting of nine amino acids (nonapeptide) and it is a member of the kinin family. Bradykinin is formed by the local action of serine proteases, the kallikreins, on a precursor protein, the kininogens (Roberts, 1989). Kallikreins are present in most tissues as well as in the plasma, neutrophils, and other body fluids (Bhoola et al., 1992). Additionally, kallikreins have been found in the vascular wall with higher concentration in small vessels (Nolly et al., 1985; 1992) and different kininogens are present in the blood (Jacobsen, 1966). The major enzyme for bradykinin degradation is angiotensin converting enzyme (Dorer et al., 1974) which is also present in the EC's membrane. Therefore, the circulatory system including both the blood and the vascular wall contains all of the components for the generation and the degradation of bradykinin. Bradykinin has diverse biological effects. In addition to mitogenesis (Roberts and Gullick, 1989), pain perception (Miller, 1987), neurotransmitter-like action (Perry and Snyder, 1984), and mediation of prostaglandin release (Needleman et al., 1975), bradykinin is important in regulating the circulatory functions. In large vessels, bradykinin acts as a potent
endothelium-dependent vasodilator influencing the blood pressure; and in microvessels, it affects the permeability. During inflammatory reactions, the local concentration of bradykinin could increase by over 60 times (Roberts, 1989). The local signs characteristic of inflammation, such as redness, swelling, pain, and hotness, are thought to be related, at least in part, to the effect of locally generated bradykinin. Bradykinin-induced changes in permeability of microvessels have been extensively reported, but mechanisms regarding transduction pathways of the bradykinin action are not fully understood. Based on the literature, I have assembled different known steps into a proposed overall transduction pathway regarding the action of bradykinin on the microvascular permeability as represented in Figure 25.

The B2 receptor of bradykinin is believed to mediate a vascular permeability response (Marceau et al., 1983; Whally et al., 1987). The signal of B2 receptor binding is then coupled to phospholipase C (PLC) (Hong and Deykin, 1982) via a special G protein (or proteins) that is insensitive to cholera or pertussis toxins (Ryan et al., 1990; Flavahan and Vanhoutte, 1990). PLC catalyzes phosphotidylinositol 4,5-bisphosphate (PIP2) in the membrane to produce inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG) (Dorian and Moskowitz, 1986; Lambert et al., 1986; Bartha et al., 1989; Niesen and Wood, 1989; Voyno-Yasenetskaya et al., 1989). IP3 triggers the release or mobilization of Ca2+ from undefined intracellular stores (Lambert et al., 1986; Ryan, 1989; Lückhoff and Clampham, 1992; Graier et al., 1991), most likely from the endoplasmic reticulum. Then, extracellular Ca2+ enters the cell via either receptor-operated cation channels or Ca2+ leak channels and extracellular Ca2+ influx is thought to be driven by the membrane hyperpolarization induced by the activation of Ca2+-dependent K+ channels as I have
described before. DAG, the other product after PIP2 cleavage, activates protein kinase C (PKC) which phosphorylates the PLC-coupled G-protein thereby inhibiting a production of IP3 and consequently attenuating the IP3-activated intracellular Ca2+ release in a negative feed-back fashion (Brock and Capasso 1988; Ryan, 1989). An increase in cytosolic Ca2+ has been implied to induce the synthesis and release of EDRF which may further act within EC's to elicit cell deformation. Another endothelial autacoid, prostacyclin (PGI2), could also be produced by bradykinin but via a different pathway (Bartha et al., 1989; Förstermann et al., 1990). The binding of a B2 receptor signals G-protein-linked phospholipase A2 (PLA2) which gives rise to the liberation of arachidonic acid (AA) from membrane phospholipids and thereafter, stimulate the generation of PGI2 (Busse et al., 1989). Again, Ca2+ is required for PGI2 synthesis and release (Hallam et al., 1988). However, the production of PGI2 appears sensitive to cytosolic Ca2+; whereas, EDRF release depends on extracellular Ca2+ influx. These two endothelial autacoids are vasodilators. EDRF acts on smooth muscle cells via a cGMP pathway and PGI2 via a cAMP pathway. Unfortunately, their action on permeability of microvessels remains unclear. Some investigators have proposed that these endothelial autacoids may be involved in the process of an increase in microvascular permeability because a number of vasoactive agents including bradykinin that stimulate the release of these factors have been demonstrated to produce the increase in microvascular permeability. In addition, the increase in intracellular Ca2+ and the membrane hyperpolarization may work synergistically or separately on EC's themselves and probably also transduce signals to pericytes (Tilton, 1991) to form intercellular gaps (Majno and Palade, 1961; Majno et al., 1961; Hulström and Svensjö, 1980) by contractile (Schnittler et al., 1990; Adelstein, 1980) or noncontractile mechanisms (Boswell et al., 1992).
Fig. 24. Signal transduction pathways of the bradykinin action on the microvascular permeability.
Specific Aims and Significance of This Study

As I have described so far, bradykinin induces an increase in cytosolic Ca\textsuperscript{2+} concentration of vascular EC's either in culture or \textit{in situ}. Additionally, some studies using cultured EC's have demonstrated that the change in cytosolic Ca\textsuperscript{2+} is characterized by a biphasic profile, \textit{i.e.} an initial, transient peak followed by a lowered but sustained elevation in the continuous presence of bradykinin. The initial phase is thought to be due to an IP\textsubscript{3}-dependent release of Ca\textsuperscript{2+} from intracellular stores and the sustained phase involves Ca\textsuperscript{2+} influx from the extracellular medium which is driven by the membrane hyperpolarization upon activation of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels. Interestingly, the biphasic increase in cytosolic Ca\textsuperscript{2+} was detected in microvascular EC's, \textit{in situ}, and it was similar in the time-course to the biphasic increase in hydraulic conductivity (L_p) of the microvessel, although this observation was achieved by using a Ca\textsuperscript{2+} ionophore, inomycin, rather than bradykinin. In some other studies, the bradykinin-induced increase in microvascular permeability has been shown to exhibit a transient peak and a sustained plateau. If the bradykinin-provoked cellular event of Ca\textsuperscript{2+} is correlated directly with the bradykinin-induced microvascular event of permeability, then a bradykinin-induced hyperpolarization via affecting Ca\textsuperscript{2+} influx could be the only determinant for a sustained increase in microvascular permeability.

Therefore, to test the involvement of V_m of microvascular EC's in the process of a bradykinin-induced increase in permeability, the following hypothesis is evaluated: \textbf{Bradykinin induces a hyperpolarization of microvascular EC's that may be due to the activation of Ca\textsuperscript{2+}-dependent K\textsuperscript{+} channels and the membrane hyperpolarization determines the occurrence of increased permeability of the microvessel.}

My hypothesis is based primarily on the consistent findings from cultured EC's as
connected into a proposed pathway for the action of bradykinin (Figure 25). Although a bulk of evidence strongly suggests that the \( V_m \) of EC's is involved in the regulation of vascular functions, the role of \( V_m \) in mediating permeability has never been investigated either on an intact microvessel or on a monolayer of cultured EC's most likely due to the hardness in conducting the two measurements, particularly the measurement for \( V_m \), at the same time. In my previous study (Miao and Joyner, 1994), I have developed a technique for detecting \( V_m \) of EC’s, \textit{in situ}, using the fluorescent probe, bisoxonol, and this technique makes it possible to observe a bradykinin-induced change in microvascular permeability while monitoring the variation in \( V_m \) from EC’s, \textit{in situ}, in perfused hamster mesenteric microvessels.

Hence, this study is designed:

1) to monitor changes in \( V_m \) using bisoxonol and on the same microvessel, to measure changes in the albumin flux across the vessel wall, using another fluorescent dye, TRITC-albumin, before and after application of bradykinin to the microvessel;

2) to inhibit the bradykinin-induced increase in the albumin flux by using high \( K^+ \) solution to attenuate the effect of the bradykinin-induced hyperpolarization that is presumably due to the activation of \( \text{Ca}^{2+} \)-dependent \( K^+ \) channels.
METHODS

Animals and Solutions

Young male Syrian golden hamster, weighing 80-150 g, were used in this study. All solutions used directly for experiments were prepared daily. Stock solutions were diluted with corresponding solutions to application concentrations on the date of an experiment. The solutions used for this study are:

1) Ringer's solution: 120 mM NaCl, 4.6 mM KCl, 2 mM CaCl₂-2H₂O, 1.45 mM MgSO₄-7H₂O, 25 mM NaHCO₃, 5.5 mM D-glucose, and 5 mM HEPES-NaOH;

2) 50 mM K⁺ solution: 74.6 mM NaCl, 50 mM KCl, 2 mM CaCl₂-2H₂O, 1.45 mM MgSO₄-7H₂O, 25 mM NaHCO₃, 5.5 mM D-glucose, and 5 mM HEPES-NaOH;

3) bradykinin (10⁻⁴ M) stock solution: Bradykinin (M.W. 1060.2 and 85% peptide content) was dissolved in saline by the ratio of 1 mg to 8 ml and stored in a freezer;

4) bisoxonol (2x10⁻³ M) stock solution: Bisoxonol (M.W. 517) was dissolved in absolute ethanol and stored in a refrigerator;

5) TRITC-albumin/BSA (1 g%/1 g%) stock solution: TRITC-albumin conjugate and BSA were dissolved in Ringer's solution and stored in a freezer.

Except that bisoxonol and TRITC-albumin were from Molecular Probes, Inc., all chemicals were purchased from either Sigma Chemical Co. or Fisher's Scientific Inc.

Preparation of Hamster Mesenteric Microvessels for Intravital Epifluorescence Microscopy

The preparation of hamster mesentery microvessels was conducted following the routine procedure as I have described in Chapter III. Under the condition of subcutaneous
anesthetization with sodium pentobarbital (60 mg/Kg body weight), the animal was placed on a warmly heated cushion and the left side of the abdomen was subjected to a small (1 cm in length) incision to expose the abdominal cavity. Then the mesentery was found and gently pulled out. After the animal was moved from the cushion to a special tray designed for this study and was properly stabilized, the mesentery was draped over onto a cover slip pedestal in the animal tray. A piece of cotton strip was soaked with Ringer’s solution and placed around the exposed intestine to keep it moisturized. Thereafter, the animal was moved with the tray onto the stage of the microscope system (Figure 9). From now on, the mesentery was suffused constantly with a solution (initially Ringer’s) at 37 °C from the gravity reservoir which was bubbled continuously with oxygenated atmosphere of 95% O₂ and 5% CO₂ (pH 7.4). The suffusion solution after superfusing over the mesentery was then collected and aspirated from the bottom of the tray into a container. Upon confirmation of tissue stabilization, a convergent postcapillary venule (diameter=16~42 μm) with brisk blood flow was chosen for cannulation. Two minutin pins mounted separately on micromanipulators (Prior, England) were positioned alongside the selected vessel branches to restrain the tissue from movement. At this point of time, the mesenteric microvessel was ready for resulting cannulation and subsequent manipulations.

**Cannulation of the Microvessel with Perfusion Micropipettes**

Two beveled glass pipettes (tip diameter=6~12 μm), one serving as the washout pipette was filled with 2 μM bisoxonol / 0.1% BSA in Ringer’s solution and the other serving as the test pipette was filled with 2 μM bisoxonol / 0.05%BSA / 0.05% TRITC-albumin in Ringer’s solution, were mounted on two hydraulic micromanipulators (Prior, England) and connected to two hydraulic manometers that were used to adjust hydraulic
pressure (cm H$_2$O) applied to the Micropipettes. Initially, the washout pipette was advanced into the lumen of a branch of the convergent microvessel visualized under the microscope. The perfusion pressure (20–30 cmH$_2$O) of the pipette was set a little higher than the original pressure in the microvessel so that the vessel was now perfused with Ringer's solution from the pipette. Then, the other branch of the microvessel was cannulated, in the same manner, with the test pipette. These cannulations and following observations throughout the experiment are accomplished on the stage of a Leitz (Diavert) inverted epifluorescence microscope with a x40 (NA 0.7) objective and a x10 eyepiece under a dim red light (>650 nm) in the room. The microscope was equipped with a HBO 100-W mercury lamp (Leitz), a heat filter (KG-2, Zeiss), two dichroic mirrors with one for bisoxonol fluorescence (BP 390-490, RKP 510, LP 515 nm, Leitz) and the other for TRITC fluorescence (BP 530-560, RKP 580, LP 580 nm, Leitz) that were housed in a manually-operated cube wheel, a photomultiplier tube compacted with a photometer system (Leitz MVP) and a chart recorder (Linear).

**Measurement of Bisoxonol I, from EC's lining the Microvessel Wall**

When the microvessel was perfused constantly with the solution from the washout pipette, the light source from the lamp was passed first through the heat filter to the appropriate cube for bisoxonol fluorescence. After passing through a light filter in the cube, the excitation light (490 nm) was generated and reflected through the microscope objective to the microvessel. The emission light from the excited vessel was passed through the cube with a band-pass filter (515 nm) to a measuring window (Leitz 4-leaf diaphragm) in the photomultiplier tube. The window was placed over the vessel about 50-100 µm downstream from the convergent point by positioning this portion of the vessel on the optical axis of the
microscope. The dimension of the window was set to 50x70 \( \mu m \) with its width slightly larger than the diameter of the microvessel but the length generally fixed at 70 \( \mu m \). After bisoxonol was equilibrated across the EC's membrane (after 15-minute loading), bisoxonol \( I_f \) from the loaded cells was measured in arbitrary units by the photometer and displayed on the chart recorder for 2 seconds at 30-second intervals continuously until it reaches a stable value. The fluorescent image of the vessel was simultaneously detected by a SIT camera (MTI) projecting to a TV and videorecorder set. At specific time points, bright-field and fluorescent images of the microvessel were digitized (512x512, 16 bit deep) by an Image-1 processing system (Universal Imaging) as a 16-frame average and stored on an optical disk for subsequent image analysis.

**Measurement of Albumin Flux from the Microvessel**

The measuring system was set exactly the same as the one for bisoxonol detection except manually turning the cube wheel to the dichroic mirror specifically for generating TRITC excitation (546 nm) and emission (580 nm) lights. Initially, the same microvessel was perfused with the washout pipette; then, the perfusion of the vessel was rapidly switched to the test pipette that contains TRITC-albumin and the pressure in the washout pipette is adjusted until the dye from the test pipette was balanced in the branch on the washout side. This adjusted pressure is named balance pressure \( (P_b) \). Therefore, the microvessel was now perfused under \( P_b \) rather than under the original pressure set for the test pipette (Figure 26). To measure permeability of the microvessel was to measure the TRITC-albumin flux \( (J_a) \) across the vessel wall. When starting the perfusion with TRITC-albumin from the test pipette, at the first moment, the microvessel was rapidly filled with the dye; this initial filling caused a swift increase in \( I_f \) in a step function manner and was recorded as \( I_f^* \). While the
Fig. 25. Schematic representation of the methodology for measuring the albumin flux ($J_A$) across the microvessel wall. A: the washout pipette in the left branch of the microvessel; B: the test pipette containing TRITC-albumin in the right branch of the vessel; C: the measuring window in the photomultiplier tube over a segment of the vessel; $2r$: the diameter of the vessel; $P_B$: the balance pressure indicated by an arrow pointing to a place where the test solution is balanced in the left branch; $I_f$: the fluorescence intensity of TRITC-albumin measured via the window.

\[
J_A = \frac{1}{\Delta I_p} \left( \frac{dI_f}{dt} \right) \frac{r}{2}
\]
vessel was perfused continuously with the dye for 10-20 seconds, TRITC-albumin \( I_1 \) showed a slow but steady increase with a slope of \( \frac{dI_1}{dt} \). After this detection, the perfusion was switched back to the washout pipette thereby clearing out the TRITC-albumin from the microvessel and returning \( I_1 \) gradually to \( I_0 \). After these parameters was obtained, the flux of TRITC-albumin across the microvessel wall could be calculated out according to the equation (Huxley, et al., 1987):

\[
J_A = \frac{1}{I_0} \left( \frac{dI_1}{dt} \right) \left( \frac{r}{2} \right)
\]

where \( J_A \) is the TRITC-albumin flux (cm/s), and \( r \) is the radius (μm) of the microvessel that is assumed to be a cylinder.

**Experimental Protocol**

1) Effects of bradykinin on \( V_m \) and albumin flux

The microvessel was initially perfused with Ringer's solution from the washout pipette and suffused with Ringer's solution from the reservoir. After turning the specific filter cube for TRITC-albumin and opening the pathway for the light source, the test pipette was rapidly switched on to perfuse (\( P_c = 19-31 \) cmH₂O) the microvessel with TRITC-albumin for 10-20 seconds as I described in the above paragraph. After the TRITC-albumin fluorescence curve had been recorded for the resting level of the albumin flux (\( J_A \)), bradykinin (1 μM) in Ringer's was applied for 10 minutes to the microvessel via suffusion by switching a solenoid valve to a corresponding reservoir. During this period, the response of \( J_A \) was repeatedly measured at 0.5, 1, 2, 3, 4, 5, 7, and 10 minute. Then, the suffusate was changed back to Ringer's solution. During this off-period of bradykinin, \( J_A \) was repeatedly measured at 1, 3, 5, and 10 minute. Usually, \( J_A \) could return to the control level with 10 minutes.
Then, fluorescence filters were switched for bisoxonol. At this time, the microvessel was still perfused and suffused with Ringer's solution. The perfusion pressure was maintained equal to $P_b$ used for $J_A$ measurement. After the bisoxonol $I_r$ baseline ($I_{r0}$) had been stabilized and registered for 2-5 minutes, the same bradykinin (1 $\mu$M) Ringer's solution was applied to the microvessel. The response of bisoxonol $I_r$ was recorded continuously at every 30 seconds for a period of 2 seconds in the same manner as described above. Ten minutes after application of bradykinin, the suffusate was changed back to Ringer's. The variation in bisoxonol $I_r$ was continuously measured for 10 minutes. During and after administration of bradykinin, changes in bisoxonol $I_r$ from EC's was compared to the baseline, i.e. $I_{r0}$, and calculated by:

$$\Delta I_r\% = \frac{(I_r - I_{r0})}{I_{r0}} \times 100\%.$$ 

The responses of the albumin flux and $V_m$ of microvascular EC's were compared by correlating the change in bisoxonol $I_r$ to the change in $J_A$. Their responding time-courses were compared as well.

Before, during, and after the application of bradykinin, fluorescent images of the microvessel for bisoxonol and TRITC-albumin fluorescence were acquired and processed at their steady states.

2) Effects of high $K^+$ on the bradykinin-induced changes in $V_m$ and $J_A$

After the bradykinin-induced responses of bisoxonol $I_r$ and $J_A$ had been detected according to the above procedures, the same microvessel was subjected to recannulation with another two pipettes. One worked as the washout pipette and was filled with 50 mM $K^+$ solution containing 0.1% BSA and 2 $\mu$M bisoxonol, the other worked as the test pipette and was filled with 50 mM $K^+$ solution containing 0.05% TRITC-albumin/0.05% BSA and 2 $\mu$M
bisoxonol. Following similar procedures with the same periods of time, the changes of bisoxonol $I_r$ and $J_A$ were measured before, during, and after applying the suffusate of bradykinin (1 μM) in 50 mM K$^+$ solution to the microvessel. Then, the changes in bradykinin-induced responses of $V_m$ and $J_A$ under the high K$^+$ condition were compared to those under the normal Ringer's condition in a paired manner. Fluorescent images were taken routinely as well.

**Data Analysis**

Except for the curves that represent continuous recordings for changes in bisoxonol $I_r$ and $J_A$, all data points were summarized and presented as means ± SE. The difference between means of paired two-group and of above two-group samples were tested using the paired t-test and the two-way analysis of variance followed by multiple comparison, respectively. P-levels less than 0.05 were considered statistically significant. Analysis of linear correlation and regression was conducted according to the method of least-squares computation.
RESULTS

Measurement of $J_A$ with TRITC-Albumin in the Presence of Bisoxonol

Albumin flux ($J_A$) across the microvessel wall was measured using a fluorescent tracer, TRITC-albumin, while another fluorescent dye, bisoxonol, was present. Under the fluorescence field for TRITC, bisoxonol fluorescence did not appear. As represented in Figure 27, a mesenteric postcapillary venule was under perfusion from the washout pipette in the bright field (panel 1). When the excitation light was turned on for bisoxonol, images of bisoxonol-loaded EC's showed the brightest regions, indicating the lining of the microvessel (Panel 3). After the excitation light was switched for TRITC and the vessel was perfused with the TRITC-albumin solution from the test pipette, the lumen of the vessel was filled and illuminated with the TRITC chromophore whereas the vessel wall formed by EC's was disappeared although bisoxonol was still in the perfusate (Panel 2).

Effect of Bradykinin on Albumin Flux and Bisoxonol $I_f$

The application of bradykinin (1 μM) in the suffusate elicited responses of both albumin flux ($J_A$) and bisoxonol $I_f$ in the microvessel. In Figure 28, fluorescent images of the same vessel are compared for bradykinin-induced variations in the TRITC and bisoxonol fluorescence. Panel 1, 2, 3, and 4 show the profiles of the microvessel with TRITC fluorescence at the control, the bradykinin-induced peak, the sustained, and the returned level. Correspondingly, panel 5 through 8 exhibit the changes in bisoxonol fluorescence of the microvessel at these four stages. As depicted by the continuous recording curves in Figure 29, $J_A$ was about $1 \times 10^{-6}$ cm/s at the basal level; it started to rise at 1 minute after using bradykinin and reached the maximum (about 9 folds) at 3 minutes. Then, it began to
Fig. 26. Images of a microvessel under the intravital microscope. Panel 1: the perfused microvessel in the bright field; panel 2: the vessel in the TRITC fluorescent field; and panel 3: the vessel in the bisoxonol fluorescent field.
dramatically drop to about $4 \times 10^{-4}$ cm/s at 4 minute and this sustained level was maintained
till 3 minutes after removing bradykinin from the suffusate. After this point of time, $J_A$
gradually decreased and at 10 minute, returned to the control value. In comparison with $J_A$,
bisoxonol $I_f$ started to decrease within 0.5 minute after bradykinin was applied to the
microvessel, and at 1.5 minute it reached the lowest level (-42%) that was maintained for
about 2.5 minutes. Thereafter, bisoxonol $I_f$ was fluctuated towards the side of its control
level and at 10 minute, it was about -33%. After bradykinin was washed off, bisoxonol $I_f$
gradually moved back and about 4 minutes later, it returned to the original value even though
fluctuations still persisted afterwards.

The responses of both bisoxonol $I_f$ and $J_A$ to bradykinin showed to be biphasic with
$J_A$ being more topical. Both the initiation and the peak of bisoxonol $I_f$ appeared to occur
earlier than $J_A$. From summarized time data for this set of experiments ($n=10$), the initial and
the maximal time points for bisoxonol $I_f$ were $0.82 \pm 0.15$ and $3.59 \pm 0.44$ minutes,
respectively. Whereas for $J_A$ responses, they were $2.14 \pm 0.19$ and $4.32 \pm 0.44$ minutes,
which were significantly slower than those for bisoxonol $I_f$ ($p<0.05$).

Concerning statistical differences of either bisoxonol $I_f$ or $J_A$ at different time points,
their basal, bradykinin-induced maximal, sustained values at 10 minute after applying
bradykinin, and returned values at 10 minutes after removing bradykinin were summarized
and compared as exhibited in Figure 30. For either bisoxonol $I_f$ or $J_A$, significant differences
were found among the basal, maximal, and sustained levels; whereas, the difference between
the means of basal and returned levels was not significant ($p>0.05$).

The analysis of linear regression and correlation was conducted to correlate the
change in bisoxonol $I_f$ ($\% \Delta I_f$) to the net increases in $J_A$ at the peak level. Figure 31 describes
Fig. 27. Continuous recordings of changes in bisoxonol $I_r$ (%$\Delta I_r$) from microvascular EC's and the albumin flux ($J_A$) across the microvessel wall before, during, and after applying bradykinin (BK) in the suffusate.
Fig. 28. Fluorescent images of a microvessel during measurements of TRITC-albumin flux (panel 1 through 4) and $V_m$ (panel 5 through 8). Panels 1, 2, 3, and 4 show the images of the microvessel in the TRITC fluorescent field acquired in the basal, the transient, the sustained, and the returned phases during an action of bradykinin; panels 5, 6, 7, and 8 are bisoxonol fluorescent images of the vessel in corresponding phases.
Fig. 29. Comparisons between different levels of changes in bisoxonol $I_f$ and the albumin flux ($J_A$) in microvessels in response to bradykinin (1 µM) in the suffusate. Note: *p<0.05, compared to other levels in the same group.
Fig. 30. Correlation of a net increase in the maximal albumin flux ($\Delta J_A^{\text{max}}$) to the maximal response of bisoxonol $I_r$ to bradykinin (1 $\mu$M). The linear correlation was significant ($p<0.05$). The dashed lines indicate the 95% confidence interval.

$y = -0.08x + 4.96$

$r = 0.722$
Fig. 31. Correlation between the net increase in the sustained albumin flux at 10 minute \( (\Delta J_A^{10}) \) after using bradykinin (1 \( \mu \)M) and the maximal change in bisoxonol \( I_r \). The correlation line (solid) is linear and significant (p<0.05), and the dashed lines indicate the 95% confidence interval.
\( \Delta J_A^{\text{max}} \) as a function of \( \%\Delta I_t \) in response to bradykinin. In a range of 0—80% as bisoxonol \( I_t \) decreases, \( J_A^{\text{max}} \) increases from about 5 to 11 \((x10^{-6} \text{ cm/s})\), showing a significant linear correlation \((r=0.722, p<0.05)\). The relationship between \( \%\Delta I_t \) and \( \Delta J_A^{10'} \) is exhibited in Figure 32. Within the same range of the change in bisoxonol \( I_t \), \( \Delta J_A^{10'} \) varies between about 0 to 4 \((x10^{-6} \text{ cm/s})\) linearly along with \( \%\Delta I_t \) \((r=0.808, p<0.05)\).

Effects of Bradykinin on Albumin Flux and Bisoxonol \( I_t \) under High K+ Condition

Effects of applying bradykinin (1 \( \mu \text{M} \)) in the suffusate to the microvessel on \( J_A \) and bisoxonol \( I_t \) were investigated under normal (4.6 mM) and high K+ (50 mM) conditions in this series of experiments \((n=6)\). Figure 33 exhibits the fluorescent images of a microvessel taken under TRITC and bisoxonol excitation light. Panel 1, 2, and 3 show the TRITC fluorescent images of the microvessel under 50 mM K+ before, during, and after bradykinin application. TRITC fluorescence of the image in the presence of bradykinin appears very little enhancement as compared to that either before using or after removing bradykinin. Bisoxonol fluorescence of the microvessel under 50 mM K+ are correspondingly displayed in panel 4, 5, and 6 in which the application of bradykinin decreased fluorescence emission from bisoxonol-loaded EC's (panel 5) as compared to those before using bradykinin (panel 4) and after removing bradykinin from the vessel (panel 6). The typical curves of continuous recording from an experiment are presented in Figure 34. The changes in bisoxonol \( I_t \) and \( J_A \) in response to bradykinin in Ringer's solution (panel A) were similar to those I have described above. The differences were the slower peak-reaching time and the greater peak value for both \( \Delta I_t \) and \( J_A \) in this case. As compared to the normal K+ condition, high K+ brought bisoxonol \( I_t \) up to 120% initially (panel B), indicating a strong depolarization of the
cell membrane. Upon the exposure to bradykinin, microvascular EC's underwent a membrane repolarization within 1 minute and gradually reached a peak level (ΔI_p=40%) at 3.5 minute. Then, this repolarization appeared to be maintained until bradykinin was removed from the suffusate. The returning speed of bisoxonol I_p to the original level was faster (about 2 minutes) than that (about 3 minutes) under the normal K⁺ condition (panel A). The rate of albumin flux at the basal level (0.4x10⁻⁶ cm/s) did not appear to be affected by high K⁺ when panel B was compared to panel A. After bradykinin was administered on the microvessel, J_A began to rise in 1 minute, and it reached the peak of 9 (x10⁻⁶ cm/s) at 3 minute followed by its decreased and sustained phase with a value of about 1.5 (x10⁻⁶ cm/s). After bradykinin was eliminated, J_A returned to the control level. Both the maximal and the sustained value of J_A showed to be lowered by high K⁺.

The averaged time points of the initial and the maximal responses to bradykinin were 0.5 ± 0 and 3.83 ± 0.40 for bisoxonol I_p and 1.42 ± 0.35 and 3.25 ± 0.17 for J_A, respectively. A significant difference was found for the initial time between bisoxonol I_p and J_A whereas the maximal time between the two groups was not significant.

To summarize the data for this set of experiments, the bradykinin-induced changes in J_A and bisoxonol I_p under normal and high K⁺ conditions were expressed (Figure 35) as the means of their basal, maximal, sustained values at 10 minute after using bradykinin, and returned values at 10 minute after removing bradykinin from the suffusate. Within each group, the maximal change showed a significant difference from the sustained level (p<0.05) except for ΔI_p under 50 mM K⁺; and likewise, the sustained value was significantly different from its basal value (p<0.05) except for the J_A group under high K⁺ condition. However, the difference between a basal and a returned level was not statistically different (p>0.05).
When each pair of corresponding values between the Ringer's and the 50 mM K⁺ groups were subjected to a comparison, significant differences (p<0.05) were found for both the maximal and the sustained levels of $I_A$ but not for either the basal or the returned levels between the two groups. The difference in $\Delta I_f$ of bisoxonol between these two groups was obviously significant.

The change in bisoxonol $I_f (\Delta I_f)$ at the maximal repolarization induced by bradykinin under high K⁺ condition was analyzed in a paired manner along with the net decrease in the maximal $I_A (\Delta I_A^{\text{max}(R-K)})$ in comparison to the maximal $I_A$ under normal K⁺ condition. A linear correlation of $\Delta I_A^{\text{max}(R-K)}$ to $\% \Delta I_f (r=0.761)$ was yielded as shown in Figure 36. Within the range of 0–90% for the change in bisoxonol $I_f$, the decrease in $I_A^{\text{max}}$ was predicted to be $0-4.5 \times 10^{-6}$ cm/s as referred to the correlation line.
Fig. 32. Fluorescent images of a microvessel under the high K⁺ (50 mM) condition.
Panels 1, 2, and 3 show changes in TRITC fluorescence of the microvessel before (basal),
during (maximal), and after (returned) using bradykinin; correspondingly, panels 4, 5,
and 6 are the images of bisoxonol-loaded EC’s in response to bradykinin.
Fig. 33. Continuous recordings of responses of bisoxonol $I_r$ and the albumin flux ($J_A$) to bradykinin (BK, 1 μM) under normal Ringer’s (R) and 50 mM $K^+$ (K) condition.
Fig. 34. Comparisons of changes in bisoxonol $I_f$ and the albumin flux ($J_A$) in response to bradykinin (1 μM) between different phases and between effects of normal and high $K^+$ at the same phases. Note: *p<0.05 compared to either sustained, the basal or the returned level in the same group; #p<0.05 compared to the other levels in the same group and the corresponding levels in Ringer's groups.
Fig. 35. Correlation of the difference in the maximal $J_A$ between normal and high $K^+$ effects in response to bradykinin to the bradykinin-induced maximal change in bisoxonol $I_I$ under high $K^+$ condition. $\Delta J_A^{\text{max}(R-K)} = J_A^{\text{max}}$ under Ringer's - $J_A^{\text{max}}$ under 50 mM $K^+$ condition. The linear correlation is significant ($p<0.05$) and the dashed lines indicate 95% confidence intervals.
DISCUSSION

Application of Double Fluorescent Probes Permitted Observations of Both $V_m$ and $J_A$ on the Same Microvessel

In the present study using *in situ* perfused microvessels, the application of two fluorescent probes, TRITC-albumin and bisoxonol, made it possible to investigate both the albumin flux ($J_A$) across the microvessel wall and the $V_m$ in microvascular EC's on the same vessel. As my previous observation (see Chapter III) indicated, there is a crossover of TRITC fluorescence on bisoxonol $I_r$ whereas the presence of bisoxonol does not interfere with TRITC fluorescence. Therefore, in this study, TRITC-albumin was used in the test pipette for measuring the albumin flux while bisoxonol was present. When bisoxonol $I_r$ was measured, the microvessel was perfused with the solution from the washout pipette that contained bisoxonol but no TRITC-albumin. In this way, bisoxonol was present constantly in the perfusate so that EC's could be equilibrated always with bisoxonol for continuously tracing a change in $V_m$ and meanwhile the recorded bisoxonol $I_r$ contaminated by the crossover of TRITC fluorescence could be avoided. As shown in Figure 27, the fluorescent images of the microvessel can be distinguished by the TRITC fluorescence in the lumen and the bisoxonol fluorescence in the cells. This manipulation allowed bradykinin-induced changes in $V_m$ to be compared and correlated with the corresponding changes in microvascular permeability.

**Bradykinin-Induced Hyperpolarization Was Correlated with the Increase in Albumin Flux in Microvessels**

*Bradykinin Induced a Hyperpolarization of Microvascular EC's, in situ*
The hyperpolarization of vascular EC's induced by bradykinin has been observed in cultured EC's's from the bovine aorta (Schilling, 1989; Sauve et al., 1990; Mehrke et al., 1991), the porcine coronary artery (Brunet et al., 1989; Cowan et al., 1992; Sharma and Davis, 1994), and the guinea-pig coronary artery (Mehrke et al., 1990; Mehrke et al., 1991), and intact EC's from porcine coronary artery (Bény, 1989; Bény, 1990) detected by either patch-clamping or intracellular recording. The hyperpolarized amplitude has been reported to be from 6 to 40 mV apparently depending on a variation in the cell origin and preparation, the resting $V_m$, the concentration of bradykinin, and the recording technique. In the present study, bradykinin elicited a membrane hyperpolarization of microvascular EC's indicated by the decrease in bisoxonol $I_P$. On 10 different microvessels, bradykinin (1 μM) in the suffusate produced the decrease in bisoxonol $I_P$ by a range of 18–73% with the mean of 39.2%. Referring to my calibrated rate of 5.2%/mV, the average amplitude of the hyperpolarization was about 8 mV. The resting $V_m$ calibrated in my study was -76 mV and thus, the $V_m$ after the bradykinin-induced hyperpolarization could become -84 mV which is close to the K+ equilibrium potential ($E_K$=−90 mV). As compared to most reported values for the bradykinin-induced hyperpolarization, my result showed a relatively small hyperpolarization for microvascular EC's, in situ, and it could be due to a relatively higher resting $V_m$ for these cells.

The bradykinin-induced hyperpolarization in most of reported observations was showed to be a transient response. In the patch-clamp study by Mehrke and Daut (1990) using cultured guinea-pig coronary artery EC's, the membrane hyperpolarization occurred instantly after the application of bradykinin (0.1–20 nM) and it reached a peak (33 mV on the average) at about 1.5 minutes; then, the hyperpolarization started to decrease until it
returned to the resting $V_m$ (-35 mV on the average) at about 5 minutes followed by voltage oscillations of low amplitude. However, in the present study, the initial time of a bradykinin-induced hyperpolarization was about 0.8 minute and the peak-reaching time was about 3.6 minutes. Both these time points showed a slower response of the $V_m$ to bradykinin in microvascular EC's, *in situ*, than that in cultured EC's. Furthermore, a gradual decrease in $V_m$ after the peak of a hyperpolarization along with $V_m$ oscillations was observed but the $V_m$ did not returned to the resting level within 10 minutes after applying bradykinin. The slower occurrence of the bradykinin-induced hyperpolarization in this study may be due to a longer time for bradykinin to penetrate the mesenteric connective tissue and the basement membrane before it acts on the EC's membrane. Additionally, bisoxonol belongs to a slow-response group of voltage-sensitive probes whose reaction to the change in $V_m$ could be initiated within seconds but it would take longer to reach the maximal level. This characteristic of bisoxonol was noticed by Brueuer and colleagues (1984). In L-homokaryon cells, after changing the $V_m$ from its resting level (-30 mV) to about -90 mV, bisoxonol $I_r$ started to reduce with several seconds but it reached the lowest level at about 20 minutes and when the $V_m$ was altered back from -90 to -65 mV, it took about 11 minutes for bisoxonol $I_r$ to reach a plateau value. This result strongly suggest that bisoxonol is a slow-response dye. The time required by bisoxonol for equilibrating across the membrane in response to a change in $V_m$ also depends on the size of the cell and the amplitude of a $V_m$ variation. However, since the response of bisoxonol $I_r$ could be induced within seconds after a change in $V_m$ and the bradykinin-hyperpolarization in cultured EC's showed to be transient, this hyperpolarization in microvascular EC's appeared to last longer, indicating that these *in situ* cells may synchronize the signal generated by the stimulation of bradykinin via intercellular
coupling and maintain this cell activation for completing a functional response of the microvessel.

*Bradykinin Increased the Albumin Flux Across the Microvessel Wall*

In association with the observation of a Vm response, the change in the albumin flux (Ja) across the microvessel wall was measured in this study. As I described earlier, the flux of a given substance across the endothelial barrier consists of a diffusive movement and a hydraulic coupling. Since I kept the concentration of TRITC-albumin and the balance pressure constant during the whole period of measuring Ja before and after using bradykinin, the contribution from a concentration gradient of TRITC-albumin and the difference of both the hydrostatic and the osmotic pressure across the vessel wall should be constant. Thus, a change in Ja would be due to a change in the number and/or the size of those pores in the endothelial barrier which have been suggested to result from the gap formation or gap augment between EC’s.

The results from this series of experiments showed that in response to bradykinin, Ja exhibited a dramatic increase in a biphasic manner (Figure 29). The basal level of Ja was measured to be 1.001 (x10^6 cm/s). After applying bradykinin (1 μM) in the suffusate, Ja began to increase at about 2 minutes and it reached a transient peak of 9.168 (x10^6 cm/s) at about 4.5 minutes. Then, Ja gradually reduced but relatively sustained at the level of 3.070 (x10^6 cm/s) detected at 10 minute after using bradykinin. After removing bradykinin from the microvessel, Ja started to return to the basal level and at 10 minute, it reached 1.047 (x10^6 cm/s) that was not significantly different from the control value (p>0.05). These results indicate that bradykinin induces an increase in microvascular permeability to the macromolecule, albumin, and the response of microvessels to bradykinin is reversible. This
demonstration is consistent with the studies using Ca$^{2+}$ ionophore A23187, ionomycin, and serotonin in hamster and frog microvessels (Curry and Joyner, 1988; Curry et al., 1990; Joyner and Curry, 1986; Olesen, 1985). In these studies, microvascular permeability to albumin increased to a transient peak within 1-3 minutes followed by a sustained lower value after 4-8 minutes that was maintained as long as the stimulant was in the perfusate. The transient peak of $J_A$ in this study appeared to occur later than the time points reported by the above studies. The reason of this difference could be that the application of bradykinin in my experiments was via suffusion rather than via perfusion.

The Increase in Albumin Flux Was Correlated with the Change in $V_m$ of Microvascular EC's

When the time course of the bradykinin-induced hyperpolarization was compared with that of the bradykinin-induced increase in albumin transport, both the initial and the maximal response time points of bisoxonol $I_f$ were significantly earlier than those of $J_A$. Taken the longer time for bisoxonol equilibration into account, the response of $V_m$ to bradykinin could be even earlier.

The linear correlation of the increase in $J_A$ to the decrease in bisoxonol $I_f$ yielded significant results. Concerning that the basal level of $J_A$ may affect the overall increased $J_A$, the net increases in $J_A$ at the transient peak ($\Delta J_{A_{\text{max}}}$) and at the sustained level ($\Delta J_{A_{10^6}}$) were used to correlate with the change in bisoxonol $I_f$ ($%\Delta I_f$) at the peak level (Figure 31 and 32). Significant correlations of $%\Delta I_f$ to $\Delta J_{A_{\text{max}}}$ and $%\Delta I_f$ to $\Delta J_{A_{10^6}}$ were achieved and the intercepts on the $\Delta J_{A_{\text{max}}}$ and the $\Delta J_{A_{10^6}}$ were found to be about 5 and 0 ($\times10^{-6}$ cm/s), respectively. In association with to the characteristic of the time courses for the two events, these results could suggest that the bradykinin-induced hyperpolarization determines the
increase in albumin transport across the microvessel wall, the transient peak in part and the sustained level in all. The bradykinin-induced hyperpolarization of vascular EC's has been demonstrated to linearly correlate with the increase in cytosolic Ca\(^{2+}\) (Sharma and Davis, 1994) and the increase in cytosolic Ca\(^{2+}\) has been evidenced to determine frog microvascular permeability to albumin (He et al., 1990). Although different stimulants, ionomycin and substance P, and different cell and animal models were used in these studies, their results are agreeable with my findings. However, as I described earlier, most investigations support the hypothesis that in the biphasic increase in cytosolic Ca\(^{2+}\) in response to inflammatory agonists including bradykinin, the initial transient peak results from the release of intracellularly stored Ca\(^{2+}\) and the sustained phase is due to the extracellular Ca\(^{2+}\) influx which is driven by the membrane hyperpolarization (Adams et al., 1989). If the change in cytosolic Ca\(^{2+}\) is directly correlated to the increase in microvascular permeability, then the hyperpolarization could mediate only the sustained level of increased permeability. Whereas, my results indicate that the bradykinin-induced hyperpolarization not only determines the sustained phase but also partially affect the transient peak of the albumin flux. The rest of a peak value may be related to other regulating factors, such as endothelial autacoids and the protein kinase C (Hock, 1992).

**Bradykinin-Induced Increase in the Albumin Flux Was Reduced by The Membrane Depolarization**

*Activation of Ca\(^{2+}\)-dependent K* Channels May Not Be the Only Reason for a Bradykinin-Induced Variation in *V*:

The membrane hyperpolarization induced by bradykinin was further investigated by using a high K* (50 mM) solution in the absence and presence of bradykinin (1 μM). As
shown in Figure 35, when the microvessel was exposed to the high K⁺ solution, the average increase in bisoxonol Iᵣ was about 115% indicating a membrane depolarization of EC's by 22 mV that was comparable with the amplitude found in my previous study (see Chapter III). After applying bradykinin in the suffusate, bisoxonol Iᵣ from EC's began to decrease within 1 minute and at about 3.5 minute reached a lowest level of 50% that indicates a membrane repolarization from the original depolarized $V_m$ of -54 mV to a level of -66 mV. This repolarization peak was then followed by a small and sustained depolarization that was detected to be 3 mV by a 15% increase in bisoxonol Iᵣ from the peak level ($p>0.05$). After removing bradykinin from the suffusate, bisoxonol Iᵣ returned to a level of 130% that was not significantly different from the original value ($p>0.05$).

The bradykinin-induced hyperpolarization of the EC's membrane under the normal condition has been demonstrated to be due to the activation of Ca³⁺-dependent K⁺ channels. This hyperpolarization could be inhibited by extracellular high K⁺ (Mehrke and Daut, 1990; Cowan et al., 1992). My results did show that bradykinin could induce a membrane hyperpolarization in microvascular EC's, in situ. However, at a depolarized level of $V_m$ under 50 mM K⁺, bradykinin still produced a strong repolarization of the cells. This considerable negative shift in $V_m$ under high K⁺ could not be explained by the activation of Ca³⁺-dependent K⁺ channels. The reason is that a decreased electrochemical gradient of K⁺ across the EC's membrane by raising extracellular K⁺ to 50 mM would tend to further depolarize the membrane from the original -54 mV towards its equilibrium potential (now $E_K=-11$ mV, assuming intracellular K⁺ is still 130 mM) if the K⁺ conductance is increased by the activation of these channels. In the study by Mehrke and Daut (1990), cultured guinea-pig coronary EC's showed a strong hyperpolarization of 40 mV in response to
bradykinin from the resting $V_m$ of -35 mV. When the $K^+$ concentration was raised to 50 mM in the medium, the bradykinin-induced hyperpolarization was abolished, and the hyperpolarization turned into a depolarization when extracellular $K^+$ was further increased to 100 mM. The bradykinin-induced repolarization in this study is unique. Electrical mechanism(s) for this action is not clear and might be implicate the activation of $Cl^-$ currents or the $Na^+-K^+-ATPase$. In the study by Brock and colleagues (1986), bradykinin was observed to stimulate $Na^+-K^+-Cl^-$ cotransport in cultured bovine aortic and human umbilical vein EC's. If the stoichiometry of these ion fluxes holds true about 1:2:2 under high $K^+$ condition, the low extracellular $Na^+$ condition due to the substitution by $K^+$ could possibly favor ion effluxes activated by bradykinin thereby inducing the membrane repolarization. Nevertheless, this study suggests that bradykinin-induced variations in $V_m$ are complicated in terms of ionic activities under the in situ condition and the activation of $Ca^{2+}$-dependent $K^+$ channels may not be the only reason for the action of bradykinin on $V_m$. Bradykinin may differentially regulate the extracellular $Ca^{2+}$ influx at different $V_m$ levels which may in turn mediate vascular responses under varied extracellular ionic conditions.

*Bradykinin-Induced Increase in Albumin Flux Was Inhibited by High $K^+$*

When microvessels were exposed to high $K^+$ (50 mM), the basal level of $J_A$ did not change significantly as compared to that under the normal $K^+$ (4.6 mM) condition ($p>0.05$). After bradykinin was applied to the vessel, a similar but smaller increase in $J_A$ occurred in a biphasic manner (Figure 7). This response was initiated within 1.5 minutes and maximized at about 3.5 minute. Then, it rapidly reduced to a sustained level and at 10 minute it was only about $1 \times 10^{-6}$ cm/s that was not significantly different from either the original or the returned value ($p>0.05$). The initial time for the response of $J_A$ was significantly greater than
that for the response of bisoxonol $I_t$ ($p<0.05$) but the peak-reaching time was similar ($p>0.05$). As compared to the maximal increase in $J_A$ under Ringer's solution, the net decrease in the maximal $J_A$ ($\Delta J_A^{max(K+)}$) was linearly correlated to the value of bisoxonol $I_t$ after the bradykinin-induced repolarization reached a maximal level ($\%\Delta I_t$) as shown in Figure 7. This correlation indicates that under the depolarized condition established by high $K^+$, the bradykinin-induced repolarization still contribute to the increase in $J_A$ for the transient peak. The intercept of on the axis of $\Delta J_A^{max(K+)}$ was exhibited to be about 0 (x10⁻⁶ cm/s). This result could not be extrapolated to the correlation obtained from the earlier study under the normal $K^+$ condition. The possible reason is that the mechanism with which the bradykinin-induced variation in $V_m$ mediates the increase in vascular permeability may be different under high $K^+$ condition as I have pointed out before.

Summary

Using double fluorescent probes, this study made it possible to observe the change in $V_m$ of microvascular EC's while the response of microvascular permeability is measured on the same microvessel. It is the first time that variations in $V_m$ of EC's have been correlated to the vascular function, i.e., the regulation of microvascular permeability. A inflammatory agonist, bradykinin, induced the increase in $V_m$ either at the resting or at the depolarized level, which suggest that the activation of $Ca^{2+}$-dependent $K^+$ channels may not be the only electrical event of the EC's membrane elicited by this inflammatory agonist. The bradykinin-induced variations in $V_m$ are associated with the occurrence of the increase in microvascular permeability. But, what is involved in terms of ionic activity for the membrane repolarization in response to bradykinin and what is the intracellular mechanism for this repolarization to mediate an increase in microvascular permeability? These
questions merit further investigations.
Chapter V

SUMMARY, CONCLUSIONS, AND PERSPECTIVES

Since the last decade when the techniques for patch-clamping (Hamill et al., 1981) and maintaining vascular EC's in culture (Ryan, 1984) became available, electrophysiological studies have accumulated a great deal of evidence for the involvement of electrical activities of the EC's membrane in cellular functions. Particularly, variations in $V_m$ of vascular EC's induced by a variety of inflammatory agonists including bradykinin have been hypothesized to control the concentration of cytosolic $Ca^{2+}$ which, in turn as an intracellular messenger, affects the synthesis and the release of endothelial autacoids and determines the kinetics of various intracellular $Ca^{2+}$-dependent events, such as the activation of $Ca^{2+}$-dependent ion channels, the regulation of $Ca^{2+}$-dependent protein kinases and $Ca^{2+}$-binding proteins, and the participation in rearranging the cytoskeleton system. Additionally, the variation in $V_m$ as an electrical signal has been proposed to be transmitted to neighboring endothelial, smooth muscle cells and perhaps pericytes thereby influencing their functional status. Hence, the membrane electrical activities of vascular EC's become associated with not only cellular but vascular functions.

However, most electrophysiological studies to date have been conducted on cultured vascular EC's and few on intact EC's under nearly physiological conditions due to technical difficulties. Besides, observations on cultured single cells or even cell monolayers cannot correlate cellular events directly to vascular responses. Thus, a series of my studies have been conducted to investigate electrical properties of intact or in situ vascular EC's in both
large vessels and microvessels. In addition, the variations in $V_m$ of microvascular EC's in response to an inflammatory mediator, bradykinin, have been studied and correlated to the bradykinin-induced increase in microvascular permeability. To summarize and conclude the results in these studies, I have found that the resting $V_m$ of intact EC's from the aorta and the vena cava in hamsters appears similar in value (about -40 mV). The contribution of the overall $K^+$ conductance to the resting $V_m$ is rather small (about 20%), and $Na^+$ and $Cl^-$ play a significant role in determining the resting $V_m$. This result is inconsistent with the observations using cultured vascular EC's but comparable to those for intact EC's, which indicates that electrical properties of the EC's membrane may be altered during the process of cell culture. In comparison with these intact EC's from large vessels, microvascular EC's have a higher value of the resting $V_m$ (-76 mV) and the membrane permeability to $K^+$ is stronger, consistent with the high resting $V_m$. The electrical polarity of microvascular EC's, 

*in situ*, is suggested by the differential depolarizing action of $K^+$ on the luminal side versus the abluminal side of the cells. The bradykinin-induced hyperpolarization has been proposed to be due to the activation of $Ca^{2+}$-dependent $K^+$ channels. Whereas, in my study, the variation in $V_m$ in response to bradykinin was not inhibited by high $K^+$, strongly suggesting that some other electrical activities of the cell membrane could be involved as well. The bradykinin-induced variations in $V_m$ of EC's were found to mediate microvascular permeability to albumin. This result, for the first time, provides direct evidence for an important role of $V_m$ in regulating vascular functions and furthers our understanding of inflammatory reactions with regard to the mechanisms underlying changes in microvascular permeability.

Having addressed some important issues, my study also raises new questions which
are characteristic of membrane electrical properties of in situ EC's. For instance, 1) what determines the asymmetrical distribution of the K* membrane conductance in microvascular EC's? 2) do intact EC's from large vessels have polarized membrane electrical properties as well so that the K* contribution to the resting $V_m$ has a smaller fraction on the luminal side of the EC’s membrane? 3) what are other electrical events controlling bradykinin-induced variations in $V_m$, besides the proposed activation of Ca$^{2+}$-dependent K* channels? These questions are important in thoroughly elucidating the ionic basis of electrical activities of the EC’s membrane and their significant implications in regulating vascular functions. Therefore, further investigations remain to be conducted.

The fluorescence microscope and image processing system have permitted us to employ double fluorescent probes for observing changes in $V_m$ as a modulator in the process of the increase in microvascular permeability. This manipulation provides a feasible technique for correlating cellular mechanisms directly to vascular functions. Thus, besides using bisoxonol to monitor changes in $V_m$, a variety of other fluorescent probes are available which can be introduced into this working system for appropriate cellular events, such as Fura-2 or Fura Red for intracellular Ca$^{2+}$, SNAFL or SNARF for intracellular pH, SBFI and PBFI for intracellular Na* and K*, respectively, and derivative dyes of phallacidin and phaloidin for actin microfilaments, etc. For mechanisms of bradykinin-induced changes in microvascular permeability, I can further my investigation the implications of endothelial autacoids (EDRF or PGL2), intracellular signals (G-proteins, phospholipases, Ca$^{2+}$, protein kinases, cyclic nucleotides, inositol polyphosphates and diacylglycerols), cytoskeleton, intercellular junction proteins, cell-cell interactions between EC's and pericytes, neutrophils, mast cells, or macrophages. Effects of other inflammatory mediators, cytokines, oxygen free
radicals, and mechanical stimulations (perfusion pressure and shear stress) on $V_m$ of EC's can also be investigated in relation to the regulation of microvascular permeability.
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ABSTRACTS:


