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Electrical method for detection of endothelial cell shape change in real time: Assessment of endothelial barrier function

(electrical measurements/endothelial cell shape/α-thrombin)

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ABSTRACT **We have developed an electrical method to study endothelial cell shape changes in real time in order to examine the mechanisms of alterations in the endothelial barrier function. Endothelial shape changes were quantified by using a monolayer of endothelial cells grown on a small (10^{-3} cm^2) evaporated gold electrode and measuring the changes in electrical impedance. Bovine pulmonary microvessel endothelial cells and bovine pulmonary artery endothelial cells were used to study the effects of α-thrombin on cell-shape dynamics by the impedance measurement. α-Thrombin produced a dose-dependent decrease in impedance that occurred within 0.5 min in both cell types, indicative of retraction of endothelial cells and widening of interendothelial junctions because of "rounding up" of the cells. The α-thrombin-induced decrease in impedance persisted for ≈2 hr, after which the value recovered to basal levels. Pretreatment of endothelial cells with the protein kinase C inhibitor, calphostin C, or with 8-bromoadenosine 3′,5′-cyclic monophosphate prevented the decreased impedance, suggesting that the endothelial cell change is modulated by activation of second-messenger pathways. The α-thrombin-induced decrease in impedance was in agreement with the previously observed increases in transendothelial albumin permeability and evidence of formation of intercellular gaps after α-thrombin challenge. The impedance measurement may be a valuable *in vitro* method for the assessment of mechanisms of decreased endothelial barrier function occurring with inflammatory mediators. Since the rapidly occurring changes in endothelial cell shape in response to mediators such as thrombin are mediated activation of second-messenger pathways, the ability to monitor endothelial cell dynamics in real time may provide insights into the signal-transduction events mediating the increased endothelial permeability.**

The vascular endothelium plays a central role in the maintenance of vascular homeostasis. Vascular endothelial cell monolayer functions as a barrier between the blood and interstitial compartments (1). A decrease in the barrier properties of vascular endothelium leads to tissue edema. Increased endothelial permeability to plasma proteins is the characteristic feature of many inflammatory conditions. Proinflammatory mediators such as α-thrombin, histamine, platelet-activating factor, and oxygen radicals have been shown to increase vascular endothelial permeability to macromolecules (2–8). However, the intracellular mechanisms by which these agents mediate the response remain unclear.

The cultured endothelial cell monolayer grown on a porous filter has been used extensively to study the barrier function of endothelial cells (9, 10). We have examined in a series of studies the permeability-increasing properties of α-thrombin, a potent proinflammatory mediator (2–5, 11). Morphological studies indicated that α-thrombin causes endothelial cell retraction, which is believed to lead to intercellular gap formation and to the observed increases of the paracellular transport of macromolecule solutes such as albumin (12, 13). To date, there have been no methods available for assessment of endothelial-cell shape changes in real time. In this paper, we describe a new method to study the shape change of endothelial cells involving the determination of the electrical impedance characteristics of endothelial cell monolayers grown on small gold-film electrodes. The results indicate that α-thrombin causes a rapid dose-dependent decrease in impedance of the endothelial cell monolayer, indicating widening of the paracellular pathways. This response was inhibited by pretreatment of the cells with a protein kinase C (PKC) inhibitor and 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), suggesting that the secondary messengers, PKC and cAMP, modulate endothelial barrier function by preventing the thrombin-induced alterations in endothelial cell shape.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from GIBCO. Human α-thrombin and catalytically inactive diisopropylphosphoryl α-thrombin (DIP-α-thrombin) were gifts from John W. Fenton II, Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY. Calphostin C was purchased from Kamiya Biomedical (Thousand Oaks, CA). 8-Br-cAMP was purchased from Sigma.

Electrode Preparation. Electrodes were prepared in a manner similar to that described by Giaever and Keese (14, 15) with some modifications. Gold was evaporated at 10^{-6} torr through a mask onto a polycarbonate plastic substrate to provide two strips of gold (≈0.5 cm wide). A narrow (100 μm) line of gold projected a few millimeters beyond the end of one of the strips. A piece of Parafilm was cut to size and applied to the gold/polycarbonate surface by using heat and mechanical pressure such that it insulated the central region of the gold strips. Two electrodes extended beyond one side of the Parafilm: a small active electrode (≈10^{-3} cm^2) and a large counter electrode (≈0.25 cm^2). Copper leads were soldered with indium to the gold at the other side of the Parafilm. The solder junctions and exposed gold around the junctions were covered with a layer of insulating wax. The completed two-electrode device was mounted with wax to the bottom of

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Abbreviations: BPMVEC, bovine pulmonary microvessel endothelial cells; BPAEC, bovine pulmonary artery endothelial cells; PKC, protein kinase C; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphate; ECIS, electric cell-substrate impedance sensor; DIP-α-thrombin, diisopropylphosphoryl α-thrombin.

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a 35-mm tissue culture dish, and the entire assembly was sterilized with UV irradiation.

Cell Cultures. Bovine pulmonary microvessel endothelial cells (BPMVEC) and bovine pulmonary artery endothelial cells (BPAEC) were cultured by procedures of Del Vecchio *et al.* (16, 17). The cell passage number used for the present study was between 20 and 25. Both BPMVEC and BPAEC were seeded (4×10^5 cells per electrode dish) and were allowed to grow to confluency. In all cases, confluent endothelial monolayers grown on the electrode were used for the impedance measurement. The electrode-containing dishes were run with 1.5 ml of culture medium [DMEM with 10% (vol/vol) fetal bovine serum]. α-Thrombin, calphostin C, and 8-Br-cAMP were diluted in the culture medium and then added to the endothelial cell monolayer at the indicated concentrations.

Impedance Measurement. *Background.* The measurement of cell shape of endothelial cells grown in a monolayer was based on a technique developed to study the dynamic cell behavior in culture (14, 15, 18–20). Because the method is unique, it will be described in some detail. In this system, referred to as electric cell-substrate impedance sensor (ECIS), the cells are cultured on a small gold electrode, and culture medium is used as the electrolyte. An approximately constant current source applies an ac signal of 1 μA between this small electrode and a large counter electrode, while the voltage is monitored with a lock-in amplifier. Voltage and phase data are stored and processed with a personal computer. The same computer controls the output of the amplifier and switches the measurement to different electrodes in the course of an experiment.

The critical feature of the setup is the relatively small size of the active electrode (10^{-3} cm^2). With larger electrodes, the cell-related signals become difficult to detect. This is the consequence of solution resistances in the system that predominate over the electrode's impedance and mask any changes caused by the cells. When electrodes are reduced to $\approx 10^{-3}$ cm^2 or smaller, the impedance of the electrode–electrolyte interface at 4000 Hz predominates. In this situation, the presence of the cells and morphology changes are clearly revealed. The size of the electrode restricts the maximum cell populations that can be observed at one time to about 100 cells, but it should be noted that the shape change of even a single cell can be easily measured.

Present Setup. Fig. 1 shows the schematic of the electrodes and the basic setup of electrodes used in these measurements. The small electrode and the larger counter electrode were connected to a phase-sensitive lock-in amplifier, and a 1-V, 4000-Hz ac signal was supplied through a 1-MΩ resistor to approximate a constant-current source. This has the advantage, if the sample is regarded as a resistor and capacitor in series, of having the measured in-phase voltage proportional to the resistance and the out-of-phase voltage proportional to the reactance. Because the larger changes occur in the resistance, we have chosen to concentrate on those changes in the present study.

RESULTS AND DISCUSSION

In the present study, we used ECIS to assess whether thrombin produced a shape change of endothelial cells, since retraction and "rounding up" of endothelial cells have been proposed to occur with various permeability-increasing mediators. Previous morphological studies suggest that α-thrombin causes endothelial-cell retraction. Laposata *et al.* (13), using a grid assay method to study α-thrombin-induced effects on human umbilical vein endothelial cells, observed gap formation between cells within 2 min after the addition of α-thrombin and recovery to normal shape after 2 hr. They also showed that the cells did not detach from the substratum. Suidan *et al.* (21) have investigated the α-thrombin-induced cell retraction in neuroblastoma cells with time-lapse videomicroscopy. Upon monitoring cell retraction every 20 sec, they observed the maximum cell retraction within 1 min.

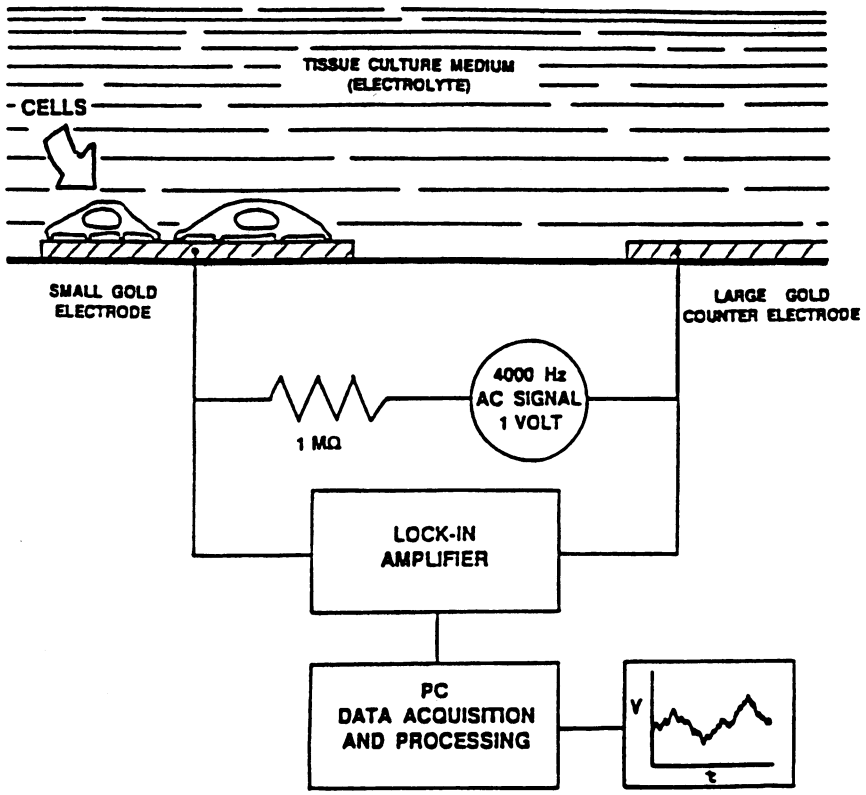

FIG. 1. Basic setup used to measure endothelial cell monolayer impedance.

We measured in-phase voltage changes to monitor the cell shape change under physiological conditions using the culture medium as the electrolyte solution. Fig. 2 shows the effects of different concentrations of α-thrombin on BPMVEC. The addition of medium alone did not change the in-phase voltage. However, the voltage decreased within 30 sec after the addition of α-thrombin to the electrode system containing the BPMVEC monolayer. The decrease observed in BPMVEC showed a characteristic biphasic pattern. The first part of the response occurred within 2 min and then there was partial recovery which was followed by a longer decrease in the voltage. The response of α-thrombin was always dose-dependent. The α-thrombin-induced in-phase voltage changes with BPAEC (Fig. 3) showed a slightly different profile. In this case, the voltage decreased immediately after the addition of α-thrombin. At low concentrations of α-thrombin (25 nM and 50 nM), the in-phase voltage recovered to normal values in a short period of time. However, at the high concentration studied (100 nM), the decrease in the voltage persisted for a longer period. The sustained decrease in voltage observed with the addition of α-thrombin (100 nM) is in close agreement with the α-thrombin-induced increase in albumin permeability observed at this concentration (3–5). Moreover, the increase in endothelial permeability to albumin in response to α-thrombin occurred within 2 min and was associated with alterations in the actin cytoskeleton (22), findings consistent with the present observation.

Morphological studies (13) and albumin permeability measurements in endothelial monolayer (3–5) have shown recovery of endothelial cells from α-thrombin-induced effects after 1–2 hr. When we continuously measured the in-phase voltage after the addition of 25 nM α-thrombin to the electrode system containing BPMVEC monolayer, the decrease in the voltage persisted for at least 2 hr, and then the cells recovered their normal voltage (Fig. 4), in accord with previous observation of recovery of the permeability-increase (3–5).

α-Thrombin binds to endothelial-cell surface receptor and activates this receptor by a unique mechanism described recently by the cloning of this receptor from Dami (megakaryocyte-like cell line) and Chinese hamster lung fibroblasts

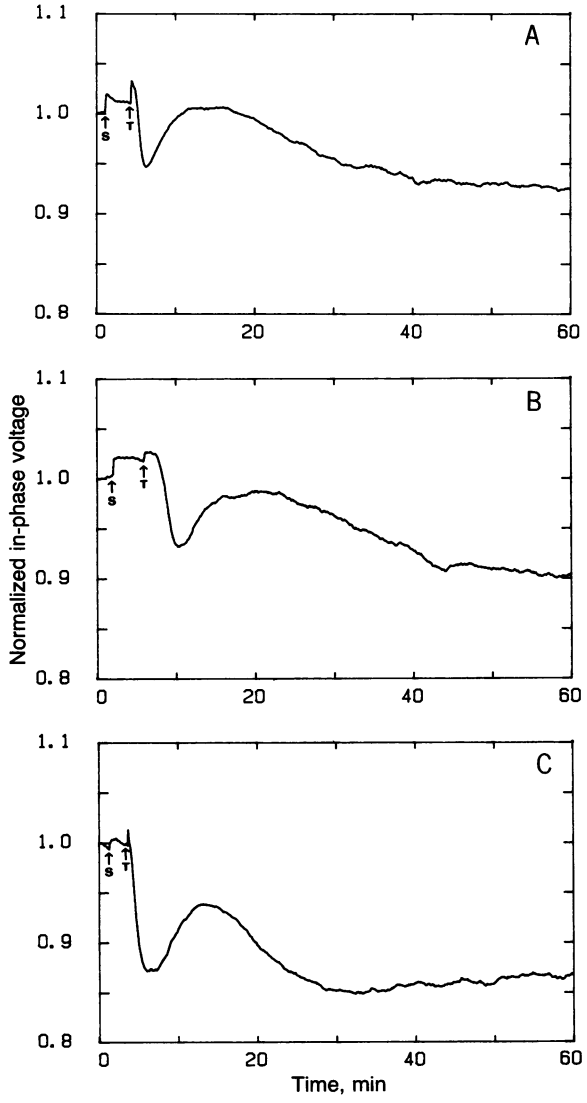

FIG. 2. Dose-dependent decreases in in-phase voltage of BPMVEC induced by 25 nM α-thrombin (*A*), 50 nM α-thrombin (*B*), and 100 nM α-thrombin (*C*). The arrow "S" indicates the addition of culture medium alone. The arrow "T" indicates the addition of α-thrombin. The addition of culture medium alone did not change the impedance of the monolayer. Other details are described in *Materials and Methods*. In-phase voltages measured at time zero were 5.194 mV, 5.433 mV, and 5.331 mV for *A*, *B*, and *C*, respectively.

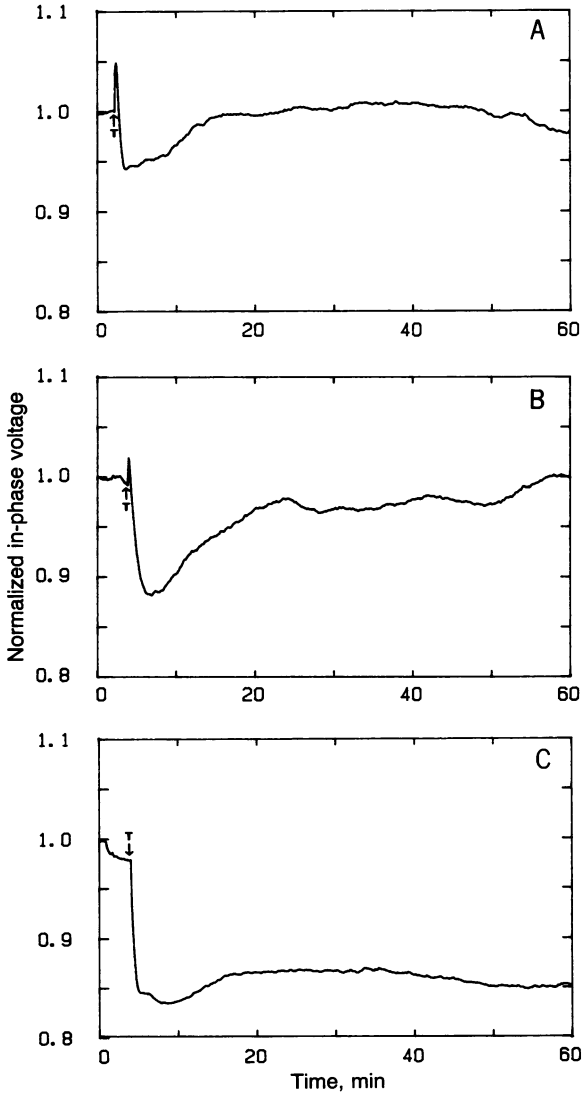

FIG. 3. Concentration-dependent decrease in in-phase voltage of BPAEC induced by 25 nM α-thrombin (*A*), 50 nM α-thrombin (*B*), and 100 nM α-thrombin (*C*). The arrow "T" indicates the time at which α-thrombin was added to the electrode containing the BPAEC monolayer. In-phase voltages measured at time zero were 3.895 mV, 4.575 mV, and 4.056 mV for *A*, *B*, and *C*, respectively.

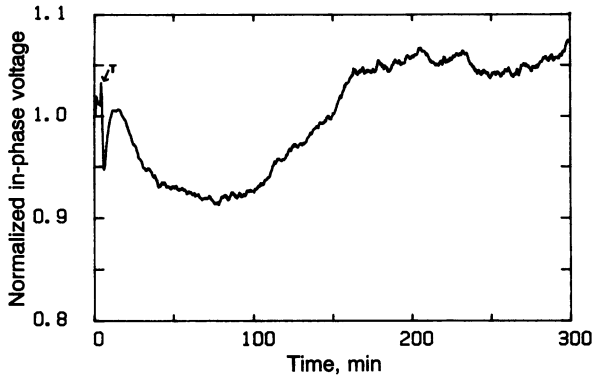

FIG. 4. Reversibility of α-thrombin-induced decrease in in-phase voltage in BPMVEC. α-Thrombin (25 nM) was added to the electrode system containing BPMVEC monolayer, and the in-phase voltage was monitored for a longer period of time. In-phase voltage measured at time zero was 5.194 mV.

(23, 24). These studies indicated that thrombin-receptor activation occurs by cleavage of the receptor at a point 41 amino acids from the receptor's start methionine. The newly formed NH_2-terminal region acts as a "tethered ligand" and binds to a domain on the receptor, which thereby activates the thrombin signal (23). For this process to occur, proteolytically active-form α-thrombin is essential. To study whether α-thrombin-induced endothelial cell shape change is a receptor-mediated event, we measured in-phase voltage changes using 50 nM catalytically inactive DIP-α-thrombin in BPMVEC (Fig. 5). DIP-α-thrombin alone did not alter the voltage of BPMVEC, an observation similar to the absence of morphological (13) and permeability-increase in endothelial cells challenged with the catalytically inactive thrombin (5). The present observations support the hypothesis that the involvement of a proteolytically activated thrombin receptor is required for mediation of endothelial-cell shape change.

It is well known that the cytoskeletal actin plays an important role in maintaining the cell structure (25). Studies have shown that PKC-mediated phosphorylation of actin-binding proteins leads to alterations in cell shape and widening of interendothelial junctions (26–31). Previous studies from our laboratory indicate that the activation of PKC by phorbol 12-myristate 13-acetate resulted in increase in endothelial albumin permeability presumably through increase in the diameter of the paracellular pathways (11). Furthermore, the α-thrombin-induced increase in endothelial albumin permeability was inhibited by PKC inhibition (11). Recent morphological evidence also shows that PKC inhibitors prevented α-thrombin-induced cell shape changes (21). In the present study, we examined whether a specific PKC inhibitor [calphostin C (32)] altered the α-thrombin-induced decrease in the in-phase voltage. Calphostin C interacts in a light-dependent manner with or near the diacylglycerol binding domain of PKC (33). The results of pretreatment of endothelial cells with calphostin C for 20 min are shown in Fig. 6 *A* and *B*. Pretreatment of BPMVEC with 20 nM calphostin C in the absence of light did not prevent the α-thrombin-induced decrease in voltage (Fig. 6*A*). However, calphostin C (10 nM) pretreatment in the presence of light inhibited the second, longer-lasting decrease in the in-phase voltage induced by α-thrombin (Fig. 6*B*). These results support the morphological (13, 21) and albumin permeability studies in endothelial cells on the inhibitory effects of PKC inhibition

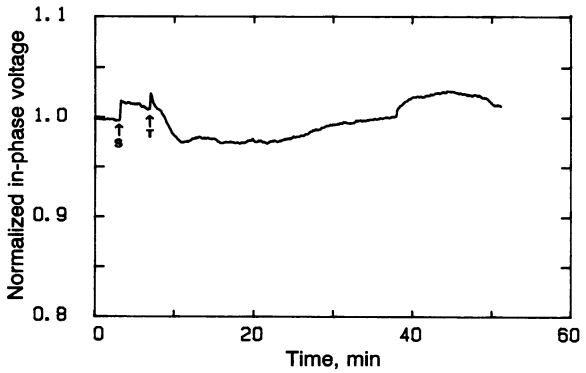

FIG. 5. Effect of catalytically inactive thrombin (DIP-α-thrombin) on the in-phase voltage of the BPMVEC monolayer. DIP-α-thrombin (50 nM) was added to the electrode system containing BPMVEC monolayer, and the in-phase voltage was measured. In-phase voltage measured at time zero was 4.507 mV.

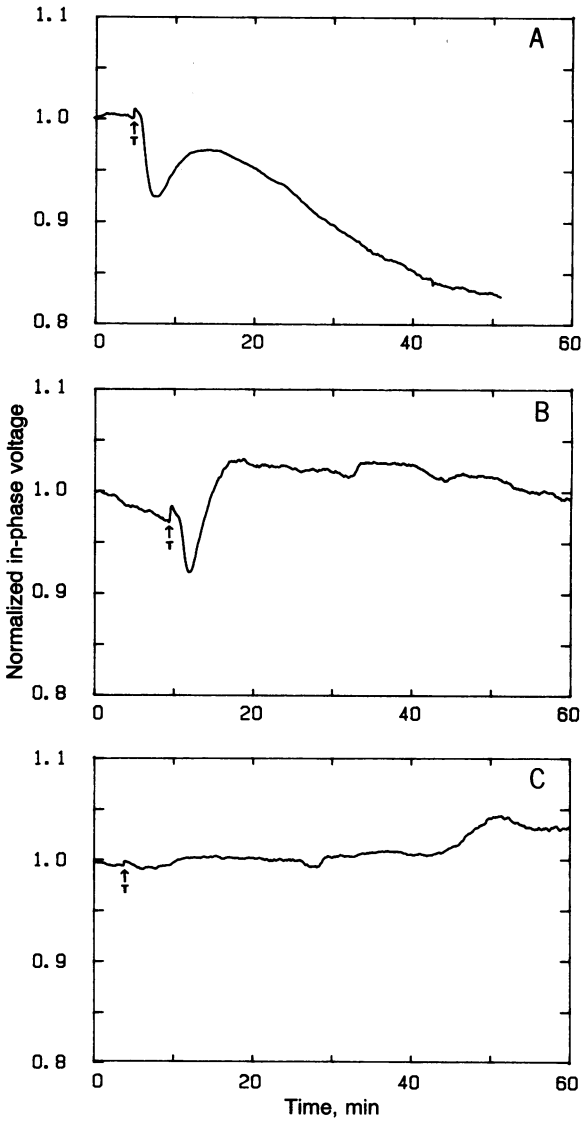

FIG. 6. Inhibition of α-thrombin-induced decrease in in-phase voltage by pretreatment of endothelial cell monolayers with PKC inhibitor, calphostin C, and 8-Br-cAMP in BPMVEC. (*A*) Calphostin C (20 nM) was preincubated with endothelial cell monolayer in the absence of light for 20 min, and then the in-phase voltage was measured by challenging with 50 nM α-thrombin. (*B*) Calphostin C (10 nM) was preincubated with endothelial cell monolayers in the presence of light, and then in-phase voltage was measured by challenging with 50 nM α-thrombin. (*C*) The endothelial cell monolayer was pretreated with 1 mM 8-Br-cAMP for 30 min, and then the effect of 50 nM α-thrombin was measured. In-phase voltages measured at time zero were 3.858 mV, 6.123 mV, and 4.861 mV for *A*, *B*, and *C*, respectively.

on cell shape and barrier function (11). The results suggest that PKC activation mediates the more sustained decrease in voltage observed with BPMVEC, indicating a critical role of PKC in regulation of endothelial barrier function.

A number of studies have demonstrated that increased intracellular cAMP prevents the increase in albumin permeability in endothelial cells produced by a variety of inflammatory mediators (34–37). In the present study, preincubation of BPMVEC with 1 mM 8-Br-cAMP prevented the α-thrombin-induced decrease in the in-phase voltage (Fig. 6*C*), supporting the hypothesis that increased cAMP inhibits "rounding up" of endothelial cells and thereby prevents the increase in endothelial permeability.

In conclusion, we describe a novel method for assessment of changes in cell shape in real time. As the cells attach and spread on the small electrodes, their effective area is partially blocked, and this results in an increase in impedance. We have used the device to detect the response of murine macrophages to biologically active compounds (38). In more recent work, we have used a spectrum of ac frequencies from 22 to 90,000 Hz. The effects of cells on impedance measurements at these different frequencies have been related to parameters of cell morphology using a simple model (18). These results show that the ECIS measurements can be used to measure the height of the spaces that are known to exist between the ventral side of the cell and the substratum (39). In addition, as the present results indicate, it is possible to obtain information regarding the intercellular impedance changes of cell monolayers such as the endothelial and epithelial barriers.

Use of a spectrum of ac frequencies and analysis of both the in- and out-of-phase component of the voltage should make it possible to make more definitive statements (such as relative change in the height of cells and diameter of intercellular junctions) resulting from thrombin and other permeability-increasing mediators.

The results obtained with this method are in close agreement with the morphological and macromolecular permeability data using the same endothelial cell monolayers. The electrical impedance method utilizing ECIS may be useful for assessment of the endothelial barrier function and thus may provide insights into the role of second-messenger pathways in mediating alterations in endothelial permeability.

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1. Crone, C. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45,** 77–83.
2. Del Vecchio, P. J., Siflinger-Birnboim, A., Shepard, J. M., Bizios, R., Cooper, J. A. & Malik, A. B. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **46,** 2511–2515.
3. Garcia, J. G. N., Siflinger-Birnboim, A., Bizios, R., Del Vecchio, P. J., Fenton, J. W., II, & Malik, A. B. (1986) *J. Cell. Physiol.* **128,** 96–104.
4. Siflinger-Birnboim, A., Cooper, J. A., Del Vecchio, P. J., Lum, H. & Malik, A. B. (1988) *Circ. Res.* **36,** 216–227.
5. Aschner, J. L., Lennon, J. M., Fenton, J. W., II, Aschner, M. & Malik, A. B. (1990) *Am. J. Physiol.* **259,** L270–L275.
6. Rotrosen, D. & Gallin, J. I. (1986) *J. Cell. Biol.* **103,** 2379–2387.
7. Bussolino, F., Camussi, G., Aglietta, M., Braquet, P., Bosia, A., Pescarmona, G., Sanavio, F., D'urso, N. & Marchisio, P. C. (1987) *J. Immunol.* **139,** 2439–2446.
8. Shasby, D. M., Lind, S. E., Shasby, S. S., Goldsmith, J. C. & Hunninghake, G. W. (1985) *Blood* **65,** 605–614.
9. Malik, A. B., Lynch, J. J. & Cooper, J. A. (1989) *J. Invest. Dermatol.* **93,** 62S–67S.
10. Peterson, B. T. (1992) *Am. J. Physiol.* **262,** L243–L256.
11. Lynch, J. J., Ferro, T. J., Blumenstock, F. A., Brockenauer, A. M. & Malik, A. B. (1990) *J. Clin. Invest.* **85,** 1991–1998.
12. Galdal, K. S., Evensen, S. A. & Nilsen, E. (1983) *Thromb. Res.* **32,** 57–66.
13. Laposata, M., Dovnarsky, D. K. & Shin, H. S. (1983) *Blood* **62,** 549–556.
14. Giaever, I. & Keese, C. R. (1986) *IEEE Trans. Biomed. Eng.* **33,** 242–247.
15. Giaever, I. & Keese, C. R. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 3761–3764.
16. Del Vecchio, P. J. & Lincoln, D. W., II (1983) in *The Endothelial Cell—A Pluripotent Cell of the Vessel Wall*, eds. Thilo-Karner, D. G. S. & Freshney, R. J. (Basel Publishers, Basel), pp. 67–83.
17. Del Vecchio, P. J., Siflinger-Birnboim, A., Belloni, P. N., Holleran, L. A., Lum, H. & Malik, A. B. (1992) *In Vitro*, in press.
18. Giaever, I. & Keese, C. R. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7896–7900.
19. Giaever, I. & Keese, C. R. (1989) *Physica D* **38,** 128–133.
20. Mitra, P., Keese, C. R. & Giaever, I. (1991) *BioTechniques* **11,** 504–510.
21. Suidan, H. S., Stone, S. R., Hemmings, B. A. & Monard, D. (1992) *Neuron* **8,** 363–375.
22. Lum, H., Aschner, J. L., Phillips, P. G., Fletcher, P. W. & Malik, A. B. (1992) *Am. J. Physiol.*, in press.
23. Vu, T. K. H., Hung, D. T., Wheaton, V. I. & Coughlin, S. R. (1991) *Cell* **64,** 1057–1068.
24. Rasmussen, U. B., Vouret-Craviari, V., Jallat, S., Schlesinger, Y., Pages, G., Pavirani, A., Legeq, J. P., Pouyssegur, J. & Van Obberghen-Schilling, E. (1991) *FEBS Lett.* **288,** 123–128.
25. Burridge, K., Fath, K., Kelly, T., Nuekolls, G. & Turner, C. (1988) *Annu. Rev. Cell Biol.* **4,** 487–525.
26. Ojakian, G. K. (1981) *Cell* **23,** 95–98.
27. Mullin, J. M. & O'Brien, T. G. (1986) *Am. J. Physiol.* **251,** C597–C602.
28. Yamasaki, H., Enomoto, T., Martek, N., Shiba, Y. & Kanno, Y. (1983) *Exp. Cell Res.* **146,** 297–308.
29. Werth, D. K., Niedel, J. E. & Pastan, K. (1983) *J. Biol. Chem.* **258,** 11423–11426.
30. Huang, C. H., Devanney, J. F. & Kennedy, S. P. (1988) *Biochem. Biophys. Res. Commun.* **150,** 1006–1011.
31. de Lanerolle, P. & Nishikawa, M. (1988) *J. Biol. Chem.* **263,** 9071–9074.
32. Kobayashi, E., Nakano, H., Morimoto, M. & Tamaoki, T. (1989) *Biochem. Biophys. Res. Commun.* **159,** 548–553.
33. Bruns, R. F., Miller, D. F., Merriman, R. L., Howbert, J. J., Heath, W. F., Kobayashi, E., Takahashi, I., Tamaoki, T. & Nakano, H. (1991) *Biochem. Biophys. Res. Commun.* **176,** 288–293.
34. Stelzner, T. J., Weil, J. V. & O'Brien, R. F. (1989) *J. Cell. Physiol.* **139,** 157–166.
35. Oliver, J. A. (1990) *J. Cell. Physiol.* **145,** 536–542.
36. Langeler, E. G. & Van Hinsbergh, V. W. M. (1991) *Am. J. Physiol.* **260,** C1052–C1059.
37. Casnocha, S. A., Eskin, S. G., Hall, E. R. & McIntire, L. V. (1989) *J. Appl. Physiol.* **67,** 1997–2005.
38. Kowolenko, M., Keese, C. R., Lawrence, D. A. & Giaever, I. (1990) *J. Immunol. Methods* **127,** 71–77.
39. Heaysman, J. E. M. & Pegrum, S. M. (1982) in *Cell Behavior*, eds. Bellairs, R., Curtis, A. & Dunn, G. (Cambridge Univ. Press., London), pp. 49–76.