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Cell Volume Regulation of Cerebrovascular Endothelium In Vitro

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Regulation of cell volume as a fundamental cellular function of high biological priority was studied in cultured cerebrovascular endothelium. The use of a multiparameter flow cytometric system allowed simultaneous measurements of cell volume, viability, and membrane potential or intracellular pH. Endothelium, the cellular constituent of the blood-brain barrier (BBB), swells immediately on exposure to low osmolality. This is associated with membrane depolarization and a fall of intracellular pH. Within 30-60 min, cell volume and membrane potential recover completely, although the extracellular osmolality is kept low. Intracellular pH does not normalize fully. Measurements of intracellular K^+ and Na^+ concentrations reveal their involvement in the regulatory process. The findings strongly suggest that the cerebrovascular endothelium has a highly effective built-in capacity for homeostatic control essential for normal BBB function.

The mechanisms of cell volume control have become a matter of increasing interest in general physiology. Furthermore, many pathological conditions including those of the brain involve disturbances of cell volume regulation and maintenance; cytotoxic brain edema may serve as an example. A better understanding of cell volume control mechanisms may provide a basis for influencing the respective disorders more specifically (Kempski et al., 1983, 1984).

A prerequisite for the normal function of nerve and glial cells is a homeostatically controlled environment. This requires strict control over the exchange of substances between blood and brain. The cerebral microvessels are actively participating in this function, generally described as the blood-brain barrier (BBB) (Bradbury, 1979); cerebrovascular endothelium represents its cellular elements. Thus the ability of the capillary endothelium to regulate its cell volume should be a prerequisite for the preservation of structural integrity and, therefore, barrier function.

The mechanisms involved in the intricate regulation of cell volume can be studied by exposing viable cells to either hypo- or hyperosmotic medium. Various cell types from different species have been described to normalize cell volume under these conditions (reviewed in Macknight and Leaf, 1977; Kregenow, 1981; Spring and Ericson, 1982; Cala, 1983; Gilles, 1983). Previous studies suggest that changes of membrane potential (Grinstein et al., 1982) and intracellular pH (Kregenow, 1981) might be involved in the regulatory process.

So far, studies concerned with volume regulation have been restricted to a single parameter, e.g., cell volume or membrane potential only. The recent availability of a multiparameter flow cytometric system (Kachel et al., 1977) now allows the simultaneous analysis of cell volume, viability, and membrane potential or intracellular pH in identical cells (Valet et al., 1981, Nerl and Valet, 1982).

In the present study, these parameters were examined in cerebrovascular endothelial cells exposed to a hypotonic environment in vitro. The results demonstrate that these cells have a full capacity to normalize their volume and membrane potential but a limited ability to restore the intracellular pH during exposure to hyposmotic medium.

MATERIALS AND METHODS

Cell culture

Pure endothelial cells were cultivated as previously described by Spatz et al. (1980). In brief, brains of 2-day-old Osborne-Mendel rats are freed from leptomeninges under sterile conditions. The fraction of microvessels is obtained by homogenization and centrifugation using a discontinuous sucrose gradient (1.0–1.5 M).

After several washes, cells are dissociated from the microvessels by a 5 min incubation in 0.01% trypsin-collagenase in Hank's solution without Ca^{2+} and Mg^{2+}. The cells obtained by that procedure are seeded in a plastic flask and kept at 37°C in an atmosphere of 5% CO_2/95% air. For subcultivation, the cells are dissociated with 0.1% trypsin in Ca^{2+}- and Mg^{2+}-free

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Hank's. Propagated cells of the generations 2–9 were used for the experiments. The feeding medium consists of 65% medium 199, 1% BME amino acids, 1% BME vitamins, 20% fetal calf serum, 1% antibiotic antimycotic mixture (all from Gibco, Grand Island, N.Y.), 2% of 50% glucose solution , and 10% of 5 mg/ml peptone (powder, type 1, Sigma, St. Louis, MO) at pH 7.2.

The purity of cultures was assessed by histochemical demonstration of enzymes characteristic for cerebrovascular endothelium (Spatz et al., 1980). These cells contain β_2- and α_2 - adrenergic receptors coupled to adenylate cyclase, which is also responsive to the prostaglandin E series and other vasoactive substances (Karnushina et al., 1982, 1983).

Hypotonic exposure

For the experiments, cells were harvested from confluent cultures as described above. A dense cell suspension in serum-free medium was prepared and stored at 4°C. Aliquots of 5 μl of this suspension were added to 500 μl of isotonic (i.e., 340 mOsm/liter: 150 mM NaCl, 10 mM Tris-HCl, 5.4 mM KCl, 1 mM $MgCl_2$, 2.4 mM $CaCl_2$) or hyposmotic (i.e, 170 mOsm/liter: 75mM NaCl, 5 mM Tris-HCl, 5.4 mM KCl, 1 mM $MgCl_2$, 2.4 mM $CaCl_2$) medium at 37°C and a pH of 7.35 for up to 60 min.

Flow cytometry

Measurements of cell volume, viability, membrane potential, and intracellular pH were performed with a "Fluvo-Metricell" flow cytometer as described by Kachel et al. (1977). The system allows the measurement of cell volume and two fluorescences simultaneously. Cell volume is measured by an advanced Coulter method employing a hydrodynamic focussing technique (Kachel, 1976). Intracellular pH is obtained in a one-step measurement by use of the fluorochrome ADB (1.4-diacetoxy-2.3-dicyano-benzol).

Fluorescence is excited between 300 and 400 nm with an HBO-100 high-pressure mercury arc lamp. Intracellular pH can be calculated from the ratio of two emission fluorescences measured between 420 and 440 nm and 500 and 580 nm according to the technique described by Valet et al. (1981). In contrast to vital cells, dead cells stain with 20 μg/ml of propidium iodide for exclusion from the pH determination. Transmembrane potential is measured by use of the lipophilic cationic dye 3,3′-dihexyl-oxocarbocyanine-iodide (DiOC6(3)). The emitted fluorescence is registered between 500 and 580 nm after excitation between 450 and 500 nm. The transmembraneous potential is proportional to the intracellular DiOC6(3) concentration (Nerl and Valet, 1982). The cellular DiOC6(3) concentration is calculated in relative units as ratio of DiOC6(3) fluorescence per cell and cell volume.

The dyes were added to the cell suspension 2–5 min prior to each measurement for equilibration between extra- and intracellular compartment. Stock solutions contained 1 mg/ml ADB in dimethyl-formamide and 15μg/ml DiOC6(3) in ethanol. Final concentrations were 20μg/ml ADB and 0.3 μg/ml DiOC6(3). Further details of the flow cytometric techniques employed will be described in a separate publication by Valet (in preparation).

Intracellular electrolytes

Intracellular electrolyte concentrations were measured in separate experiments in a set-up that allows control of pH, pO_2, and temperature (Kempski et al., 1983). Samples of suspended cells collected during the initial control period and 60 min after hyposmotic exposure were centrifuged through phthalate oil to reduce extracellular contamination (Bui and Wiley, 1981, Kempski et al., 1984). Contamination was further lowered by a short wash in $CaCl_2$ solutions of corresponding osmolality. The extracellular fluid contaminant remaining in the sample was determined by tritiated inulin. Electrolytes were then mesured by atomic absorption spectrophotometry. Concentrations were calculated on the basis of cells counted per sample and cell volume data obtained from parallel volume measurements with a Metricell flow cytometer calibrated electrically and with latex beads of defined diameters.

RESULTS

The exposure of brain capillary endothelium to hypotonic solution (170 mOsm/liter resulted in rapid swelling of these cells (Fig. 1a). Cell swelling occurred within a few seconds. Therefore, the increase in cell volume was too fast for detecting early changes since the flow cytometric measurements require at least 1 min. Volume regulation of the endothelium began immediately after cell volume had reached maximal swelling. Even though a secondary swelling of the endothelium occurred 10 min after the initial phase of cellular volume reduction, a complete recovery of cell volume was found within 30 min of hypotonic exposure (Fig. 1a). Both the initial swelling and the early phase of volume regulation of the cells coincided with a lowering of the membrane potential to 60% of normal and a decrease of intracellular pH from 7.33 to 7.10 (Fig. 1a–c). The transmembraneous potential recovered together with cell volume, whereas intracellular pH did not fully normalize within the experimental period of 1 hr (Fig. 1c). At this time (60 min after hyposmotic exposure) the intracellular Na^+ and K^+ concentrations were markedly reduced in the endothelial cells as compared to controls (Table 1). The decrease of intracellular Na^+ and K^+ concentrations together accounts for a fall of cellular osmolality of about 63 mOsm/liter discharged from the cells during the adjustment of cell volume to the hypotonic solution.

DISCUSSION

This study clearly demonstrates that endothelial cells are capable of regulating their volume after osmotic swelling. Membrane potential, which depolarized during swelling, normalized together with cell volume, whereas intracellular pH did not fully recover the control level.

Volume-regulating systems leading to more or less complete normalization of cell volume after osmotic swelling have been observed in different cell types. The phenomenon is generally referred to as regulatory volume decrease (RVD) (for reviews see Macknight and Leaf, 1977; Kregenow, 1980; Spring and Ericson, 1982; Cala, 1983; Gilles, 1983). RVD represents an excellent model for functional studies of cell volume control. Although many data on this subject have accumulated,

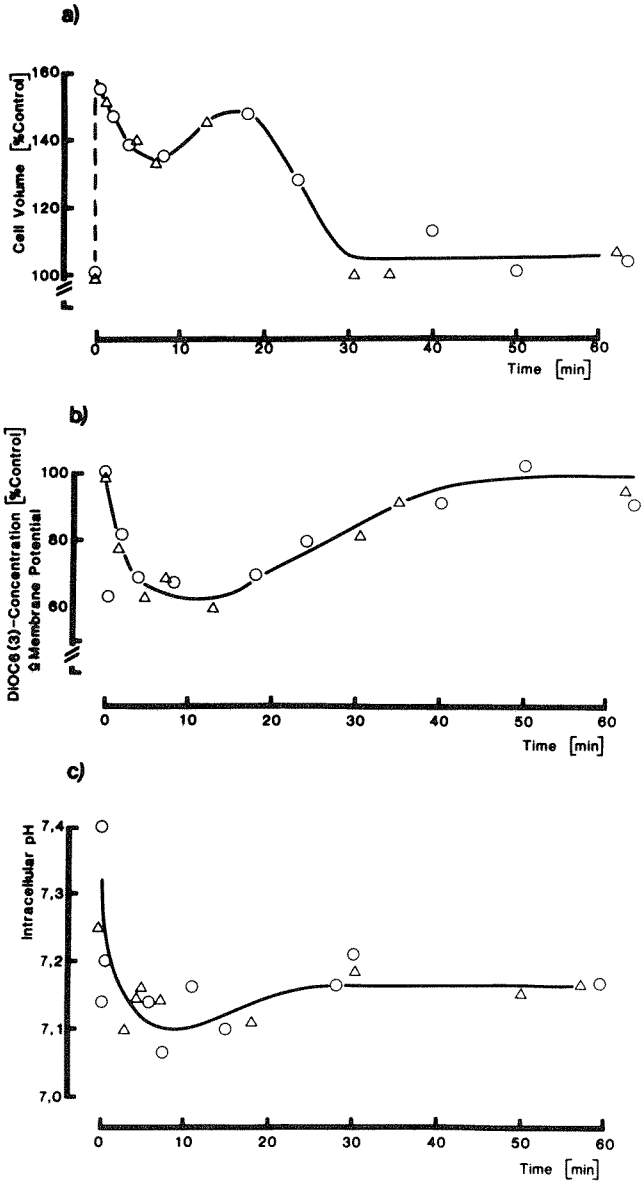

Fig.1 Cell volume (a), membrane potential (b), and intracellular pH (c) of cerebrovascular endothelium after hypoosmotic exposure (160 mOsm/liter). The two different symbols represent results obtained from two sets of endothelial cultures.

important questions remain unresolved. There are several inconsistent findings obtained from different cell types and/or species, probably resulting from their various functional capacities. However, similar experiments have never been done on cerebrovascular endothelium, which is of particular interest because of its homeostatic role in the brain.

A detailed analysis of the processes occurring during hypotonic exposure may help explain the current findings in endothelium. Hypotonicity was brought about by decreasing the osmolality in the presence of physiological K^+, and Ca^{2+} and Mg^{2+} concentrations in the medium. Immediate cell swelling resulted from a suddenly generated osmotic gradient between the extra- and intracellular compartment. The rapid net influx of water into the endothelial cells, facilitated by the high

TABLE 1. Intracellular K^+ and Na^+ concentrations (duplicate measurements) of endothelial cells suspended in isotonic and in hypoosmotic solution for 60 min

	Control (340 mOsm/liter)	60-min hypoosmotic exposure (170 mOsm/liter)
Intracellular Na^+ concentration	49.4 ± 12.3[1]	21.6 ± 2.7
Intracellular K^+ concentration	107.1 ± 12.9	72.8 ± 11.2

[1]Concentrations are given as mM/liter cell mass on the basis of cell volume and the cell count.

membrane permeability of the solvent, came to an end when the intracellular osmolality was equal to that of the external environment. This time point is likely to coincide with maximal swelling. Assuming intracellular solute concentrations then to be half normal due to the osmotic inflow of water, the intracellular K^+ concentration would have fallen from originally 107 to 53 mMol/liter cell mass at the time of maximal swelling. The decrease of the intra-extracellular K^+ gradient should have reduced the transmembrane potential, which was indeed found (Fig. 1b).

The exact nature of the volume-regulating response, RVD, is not completely understood in either cerebrovascular endothelium or in other cells. Regulatory extrusion of fluid clearly requires the discharge of osmotically active solutes, such as e.g., K^+ ions from the cellular compartment. Enhanced efflux of K^+ down the electrochemical gradient together with an increased membrane permeability could be the underlying mechanism. In fact, membrane permeability for $^{42}K^+$ is increased during the initial phase of RVD in glioma cells (Kempski et al., in preparation), lymphocytes (Bui and Wiley, 1981; Deutsch et al., 1982; Grinstein et al., 1982),and other cell types. In addition, the extracellular medium becomes alkaline during RVD of duck red blood cells (Kregenow, 1981), and it has been suggested that RVD results from a passive exchange of Cl^- against OH^- or, more probably, against HCO_3^-. Similar mechanisms may be active in endothelial cells, as indicated by the low intracellular pH in the early phase of volume regulation.

Taken together, it is suggested that RVD is accomplished by enhanced efflux of K^+ ions accompanied by intracellular anions and water. Uncertainty exists, however, concerning the types of anions involved in RVD. In addition to fluxes of HCO_3^-, Cl^-, an efflux of amino acids has been reported (Hoffmann and Hendil, 1976; Hoffmann and Lambert, 1983).

On the other hand, potential simultaneous normalization of volume and membrane may appear to exclude each other. If one assumes that K^+ is the only cation responsible for the osmotic water efflux, then normalization of the membrane potential should not have been possible, since this would have required intracellular reaccumulation of positively charged solutes like K^+.

The discrepancy would be resolved if an exhange of intracellular Na^+ versus K^+ is considered as an additional mechanism. As seen in Table 1, hypotonic dilution and subsequent RVD led to a marked loss of intracellular Na^+ against an extra- to intracellular gradient. The final Na^+ concentration was 28 mM below the concentration found under control conditions, thus contributing to the depletion of intracellular solutes. Moreover,

the intracellular K^+ concentration of 73 mM was 20 mM higher than expected if K^+ was the only cation accompanying water during RVD. Similar observations were made in glioma cells (Kempski et al., 1984).

The sum of positively charged particles amounts to 94 mM when calculated on the basis of the final Na^+ and K^+ concentration. This is in agreement with the expected intraendothelial concentration of cations after hypotonic exposure.

Activation of cellular Na^+/K^+ carrier systems during hypotonic suspension together with an increased membrane permeability for K^+ may be involved in both RVD and recovery of membrane potential. However, it remains to be clarified whether an ouabain-dependent Na^+/K^+ pump takes part in endothelial volume regulation, for ouabain- resistant RVD has been reported in other cell types (Kregenow, 1981). RVD might be possible despite inhibition of the Na^+/K^+ pump, provided the intra/extracellular K^+ gradient is steep enough. Such a process would be consistent with the cited observations that RVD in other cell types is not prevented by ouabain and that RVD is an electrically silent phenomenon in cells of some nonmammals (Cala, 1980). On the other hand, ouabain blocked RVD completely in lymphoma (Rosenberg et al., 1972) and kidney cells (Gagnon et al., 1982) and in human lymphocytes, which regulate volume very quickly, ion fluxes during RVD are electrogenic (Grinstein et al., 1982).

In conclusion, cerebrovascular endothelium has the capacity for full normalization of cell volume and membrane potential but an incomplete ability to restore intracellular pH after exposure to hypotonic medium. The rapid recovery of both cellular volume and membrane potential despite the persistence of an abnormal osmotic environment suggests that cerebral endothelium possesses powerful homeostatic control mechanisms. Further study of these mechanisms may shed new light on how endothelial cells fulfil their function as key elements of the blood-brain barrier.

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