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Hypotonicity-induced changes in anion permeability of cultured rat brain endothelial cells

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Abstract

Iodide efflux, an index of anion permeability, has been monitored in cultured rat brain endothelial cells. Following hypotonicity-induced swelling, large, rapid increases in permeability occur, the extent of these increases depending on the degree of hypotonicity. Such large responses are not observed with rat aortic endothelial cells. Results of anion substitution experiments suggest that iodide efflux is via a chloride channel rather than an exchanger. The efflux increase is blocked by NPPB (100 μM) but not by DIDS or DPC at 100 μM. It is dependent on intracellular ATP but unaffected by removal of external calcium. Increasing internal calcium using A23187 does not produce a change in efflux, but depletion of calcium reduces or eliminates the response to hypotonicity. The response is reduced by pimozide (2–50 μM) that inhibits the actions of calmodulin and by pBPB (10 μM) that affects phospholipase A_2 activity. It is eliminated by 5-lipoxygenase inhibitors (L-656,224 and ETH615, 10 μM) but is unaffected by cyclo-oxygenase inhibitors (indomethacin and piroxicam, 1–100 μM). It is blocked by some modulators of *P*-glycoprotein activity, e.g., verapamil (100 μM), tamoxifen (50 μM), and progesterone (100 μM) but not by others, e,g., forskolin (40 μM), dideoxyforskolin (40 μM), quinidine (100 μM) and cyclosporin A (10 μM).

Keywords: Cell volume regulation; Brain endothelium; Chloride channel; Iodide efflux

Abbreviations: A23187, calcimycin, viz. 6*S*-[6α(2S^*,3S^*),8β(R^*),9β,11α]-5-(methylamino)-2-[[3,9,11-trimethyl-8-[1-methyl-2-oxo-2-(1H-pyrrol-2-yl)ethyl]-1,7-dioxaspiro[5.5]undec-2-yl]methyl]-4-benzoxazolecarboxylic acid; CsA, cyclosporin A; dFSK, 1,9 dideoxyforskolin; DIDS, diisothiocyanostilbene-2,2″-disulfonic acid; DPC, diphenylamine-2-carboxylate; dtBHQ, 2,5-di-*tert*-butylhydroquinone; EGTA, ethylene glycol bis-(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; ETH615, (4-[2-quinolylmethoxy]-*N*-[3-fluorobenzyl]-phenylaminomethyl-4-benzoic acid; HEPES, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]; L-656,224, 7-chloro-2-[(4-methoxyphenyl)methyl]-3-methyl-5-propyl-4-benzofuranol; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoate; pBPB, 4-bromophenacyl bromide

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1. Introduction

The ability of capillary endothelial cells to regulate their volume is a prerequisite for the preservation of structural integrity of the blood-brain barrier. To survive, all cells must avoid excessive alterations of cell volume. The cellular content of osmolytes is continuously changing in cells as a result of various metabolic events including oxidative and detoxification pathways [1], and this is particularly so where there is a constant through traffic of osmotically active species such as amino acids and glucose. Endothelial cells in most areas of the vasculature form a permeable lining between blood and tissues and so allow passage of materials between cells. Brain endothelial cells are, however, different in that they are attached to each other by tight junctions thus forming a high resistance layer. This means that materials crossing between blood and brain cannot pass between cells but must pass through them. These large fluxes of solutes expose brain endothelial cells to the risk of large changes in their cellular contents and hence volume. Efficient volume regulation is therefore likely to be vital to the integrity of the blood–brain barrier.

Cells can conveniently be caused to swell by a rapid entry of water if they are shifted to a medium of lower tonicity. The cells respond to this swelling by a regulatory volume decrease, involving loss of water secondary to loss of certain intracellular osmolytes. In many cell types, this is achieved by exit of K^+ and Cl^- ions through swelling activated channels. There is no single common mechanism by which this volume decrease occurs and different signalling pathways are involved in different cell types, some being dependent on changes in intracellular calcium while others are calcium independent [2,3].

Chloride channels involved in regulatory volume decrease have been identified in various endothelial cell types including those derived from bovine pulmonary artery, human aorta and human umbilical vein [4], but rather few studies have involved endothelial cells derived from brain microvasculature [5]. In the present study, we investigate the properties of volume-sensitive chloride channels in cultured rat brain endothelial cells and the possible intracellular signalling pathways involved in their activation using the iodide efflux technique as a measure of anion permeability. Iodide is a suitable marker for chloride movement since it shows similar conductive permeability to chloride but is a relatively poor substrate for cotransport pathways [6]. We demonstrate here that the increased iodide efflux in response to hypotonicity is relatively insensitive to DIDS and to manoeuvres designed to perturb intracellular calcium. The iodide efflux is also insensitive to blockade of cyclooxygenase but is markedly reduced by inhibitors of phospholipase A_2 and 5-lipoxygenase.

Brain microvascular endothelial cells unlike those of the aorta or other large vessels express *P*-glycoprotein [7], a protein that in some studies has been associated with volume regulatory pathways [8]. We show here that the hypotonicity-induced anion permeability changes in the brain endothelial cells are sensitive to some but not all agents that inhibit the function of *P*-glycoprotein. Whether *P*-glycoprotein is involved in modulating the permeability changes in brain endothelial cells is not clear but aortic endothelial cells not expressing *P*-glycoprotein show smaller increases in iodide permeability in response to hypotonicity.

Preliminary results of this work have already been published in abstract form [9]. Further characterisation of the channels involved in this response has been undertaken using whole cell patch clamp recordings and preliminary results of this work have already been reported in brief [10].

2. Materials and methods

2.1. Cell culture

Brain microvessels were isolated from cortical grey matter of rats as previously described [7] and either used immediately for cell culture or stored frozen in liquid nitrogen in medium containing 10% DMSO for later culture. Brain endothelial cells (BECs) were grown from these microvessels in collagen-coated culture flasks in Hams F10 medium containing 20% plasma-derived serum, 75 μg/ml endothelial cell growth supplement (First Link, Brierley Hill, West Midlands, UK), 80 μg/ml heparin, 0.5 μg/ml vitamin C, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were used for the iodide efflux experiments after 1–5 passages. At the

later passages, fetal calf serum was often used in place of plasma-derived serum in the medium to encourage growth of the endothelial cells without fear of overgrowth by contaminating cells that may be present in the earliest passages. Aortic endothelial cells (AECs) (kindly supplied by Ms. Heather Lauder in the Department of Pharmacology, University of Cambridge, UK) were cultured from aortic rings taken from the rats. These were maintained in the same medium as for the BECs but did not require collagen-coated surfaces on which to grow.

2.2. Iodide efflux experiments

Cells were plated into 12-well plates at a density of 10^5 cells/well 3 days before the experiments. All stages of the experiments were performed at 37°C. Efflux measurements were performed as previously described [11]. Briefly cells were loaded with 92.5 kBq ml^{-1} $^{125}I^-$ in efflux solution for 90 min and subsequently washed three times with 1 ml of efflux solution to remove extracellular tracer. Samples for determination of $^{125}I^-$ efflux were then obtained each 40 s by completely removing the efflux solution and replacing it with 1 ml of fresh efflux solution. At the end of the collections, the $^{125}I^-$ remaining in the cells was extracted by digesting the cells with 1 ml of 0.1 M HNO_3 for 30 min. The samples were transferred to a γ-counter for measurement of $^{125}I^-$.

When given, the hypotonic stimulus was applied by substituting the isotonic efflux solution for the hypotonic solution at the 4 min time-point. This hypotonic solution was then used throughout the rest of the experiment. Where drug effects were being examined, the drug was added to the $^{125}I^-$ solution during the last 30 min of loading and was present in the isotonic and hypotonic efflux solutions used thereafter. When chloride was replaced with gluconate, the cells were loaded with $^{125}I^-$ in chloride containing solutions and the gluconate solution was used for the washes and efflux periods. In an individual experiment each condition was examined in triplicate (sometimes sextuplicate) and three or more wells were used for controls. Values of *n* refer to the number of wells counted for that condition. Data for each substance were obtained on at least two separate occasions unless indicated. Whenever a stimulated increase in rate constant is expressed as a percentage

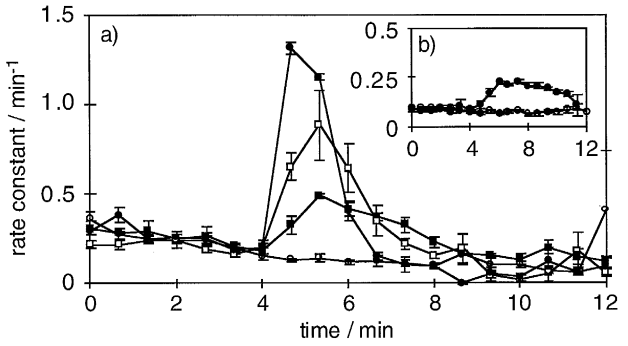

Fig. 1. Effects of different levels of hypotonic challenge on increases in rate constant for iodide efflux from (a) rat brain endothelial cells and from (b) rat aortic endothelial cells. Solutions were changed at the 4 min time-point from isotonic containing 90 mM mannitol to hypotonic in which mannitol concentrations were decreased by 90 mM (●), 60 mM (□), or 30 mM (■). In the controls the replacement solution was isotonic (○). Values shown at each time point are the mean ± S.E. of samples from three separate wells.

of control, the control value is the mean of the stimulated increase in the control wells from the same experiment. Numerical values are stated as mean ± standard deviation. The values plotted in Fig. 1 and Fig. 2 are mean ± S.E.

2.3. Data processing and analysis

The rate constant for efflux was calculated separately from the efflux data for each well as follows.

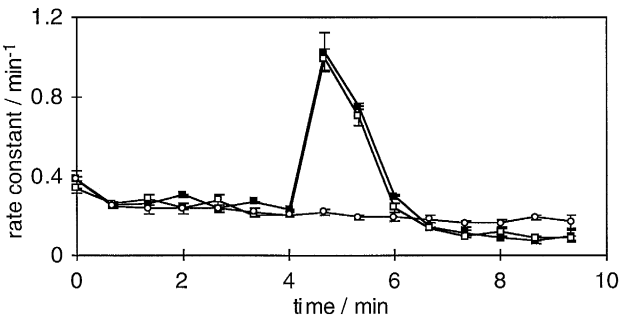

Fig. 2. Effects of replacement of chloride by gluconate and of NPPB on the increases in rate constant for iodide efflux from rat brain endothelial cells. Solutions were changed at the 4 min time point from isotonic containing 90 mM mannitol to hypotonic in which the mannitol was omitted. (■) Control response; (□) response using iso- and hypotonic solutions containing gluconate instead of chloride; (○) response (chloride solutions) with 100 μM NPPB present throughout. Values shown at each time point are the mean ± S.E. of samples from three separate wells.

The total counts in a batch of cells before the sample at time 0 was first calculated by adding together the counts above background in all the samples including the counts removed from the cells using nitric acid. The counts remaining in the cells after each sample were then found by successively subtracting the counts above background collected in each sample. The rate constant at the time of the sample was then calculated as

$$\text{rate constant} = (1/\text{time interval}) \times \ln\left(\frac{\text{counts remaining after previous sample}}{\text{counts remaining after sample}}\right)$$

Loading of the cells was characterized by the number of counts in the cells 160 s after the sample at time 0 (i.e., the sum of the counts in all samples starting with the fifth and including the counts removed using nitric acid).

2.4. Solutions and chemicals

Compositions of the solutions were as follows: the isotonic solution (measured osmolality of 288 mOsmol) contained (mM): NaCl 87, KCl 4, $MgSO_4$ 0.6, $CaCl_2$ 0.3, KH_2PO_4 0.6, Na_2HPO_4 1.1, Hepes 10, mannitol 90 and glucose 6 (pH adjusted to 7.4 with NaOH). The hypotonic solutions were the same but with mannitol reduced or absent. i.e., 60, 30, and 0 mM respectively (producing solutions of measured osmolality of 259, 228 and 197 mOsmol). In some early experiments, instead of 10 mM Hepes the solutions were buffered with 14 mM $NaHCO_3$ and bubbled with 95% O_2/5% CO_2. No differences were observed between the responses obtained with the two buffers. Unless otherwise stated, all compounds and media were either standard analytical grade reagents or were obtained from Sigma (Poole, UK). NPPB was obtained from Research Biochemicals International, Natick, MA, dtBHQ from Aldrich, Poole, UK, and A23187 from Calbiochem Novabiochem, Nottingham, UK. Cyclosporin A was a gift of Ms. K. Wright, MRC Centre, Cambridge, UK, ETH615 was a gift of Dr. E. Petersen, Leo Pharmaceuticals, Ballerup, Denmark, and L-656,224 was a gift of Dr. A.W. Ford-Hutchinson, Merck Frosst Canada, Pointe-Claire-Dorval, Quebec. Sodium gluconate was prepared from gluconic acid (Aldrich, Poole, UK) and sodium hydroxide.

3. Results

The amount of $^{125}I^-$ loaded into the rat brain endothelial cells varied with the number of cells but was typically 2000 counts min^{-1} $well^{-1}$. At the tracer levels used in these experiments, iodide is always at a very low concentration (< 10 nM) and the rate constant for efflux should be independent of the loading. The basal rate constant for efflux was typically 0.15 to 0.3 min^{-1}.

Following the maximum hypotonic challenge, iodide efflux from brain endothelial cells increased rapidly from basal values to reach a peak (0.98 ± 0.37 min^{-1}, mean $\pm$ S.D., $n = 243$) within 40–80 s. As shown in Fig. 1, the magnitude of the peak increase in iodide efflux was dependent on the level of hypotonicity and clear responses were seen even for reductions in osmolality as small as by reduction of the mannitol concentration by 30 mM. By contrast (see inset, Fig. 1b) even with a reduction in mannitol concentration of 90 mM only small peak responses (0.37 ± 0.29 min^{-1} at peak, mean $\pm$ S.D., $n = 19$) were seen with endothelial cells from rat aorta.

A peak rate constant of 1.3 min^{-1} corresponds to a loss within 40 s of 60% of the iodide present at the start of the sample period. Given this rapid loss, tests were undertaken to determine whether the hypotonicity challenge might lead to a general increase in cell membrane permeability. For this purpose, cells were loaded with [^{3}H]vincristine instead of $^{125}I^-$ (30 nM solution for 90 min) and then exposed for 10-min periods firstly to isotonic buffer, then to hypotonic buffer (without mannitol) and then to water alone. After each exposure the buffer surrounding the cells was collected and assessed for its radioactive content. There was no difference in the rate constant for [^{3}H]vincristine efflux into isotonic or hypotonic buffer but there was a large and rapid efflux following exposure to water alone (data not shown). Likewise, when the basal release of ATP was monitored, no difference was found between isotonic and hypotonic buffer exposure. Only when the cells were exposed to water a large release of ATP was observed (Barrand and Kennedy, unpublished observations).

Table 1
Effect of inhibition of enzymes in the arachidonic acid metabolic pathway, phospholipase A_2, lipoxygenase and cyclooxygenase, on hypotonicity-induced iodide efflux from rat brain endothelial cells

	Concentration	Response remaining as a percentage of control mean ± S.D. (n)
Pimozide	2 μM	38 ± 8 (3)
	10 μM	18 ± 12 (3)
	50 μM	9 ± 18 (6)
pBPB	10 μM	15 ± 20 (9)
ETH615	1 μM	100 ± 9 (3)
	10 μM	1 ± 5 (9)
L-656,224	10 μM	1 ± 11 (9)
Indomethacin	1 μM	98 ± 6 (3)
	10 μM	101 ± 6 (3)
	100 μM	90 ± 16 (3)
Piroxicam	1 μM	125 ± 17 (3)
	10 μM	105 ± 20 (3)
	100 μM	87 ± 38 (6)

Inhibitors were present during the last 30 min of iodide loading and throughout the efflux period. Efflux was stimulated by changing from isotonic solution containing 90 mM mannitol to hypotonic solution in which the mannitol had been omitted. Responses with and without the inhibitory agents are compared at the peak increase in rate constant for efflux. None means that no increase in iodide efflux could be seen in the presence of the inhibitor.

The decrease in the efflux rate constant after the peak may be a consequence of regulatory volume decrease which would remove the stimulus to increased anion permeability. However, further evidence is required before reaching this conclusion [11]. A similar decrease in the rate constant would be seen if the iodide loaded into two distinct kinetic compartments: a large compartment, perhaps cytoplasm, in which the iodide is available for rapid stimulated efflux and a small compartment, perhaps intracellular organelles, in which it is not. The calculated rate constant would then decrease as the tracer was depleted from the large compartment.

Evidence that the iodide efflux occurs through ion channels rather than by exchange for extracellular chloride was obtained by replacing the chloride in the external medium with gluconate. Gluconate is a poor substrate for exchange. Thus if efflux were to occur via this mechanism the substitution should substantially reduce the rate. As shown in Fig. 2, this did not happen which suggests that the efflux occurs via ion channels. The driving force for efflux by this mechanism is expected to be maintained as the intracellular potential will be kept negative by efflux of potassium. In three experiments with gluconate the increase in the rate constant as a percentage of the increase seen in the chloride controls was 128 ± 44%, mean ± S.D., $n = 9$. Further evidence for efflux via channels rather than exchange was obtained using whole-cell patch clamp experiments in which a marked increase in chloride conductance occurs when these cells are exposed to hypotonic solutions [10]. The efflux was however relatively insensitive to chloride channel inhibitors. While a high concentration of NPPB did reduce the response the same concentration of DIDS and DPC produced little if any inhibition. As a percentage of the increase seen in the controls the increase in the rate constants for efflux in the presence of the inhibitors were: NPPB 100 μM

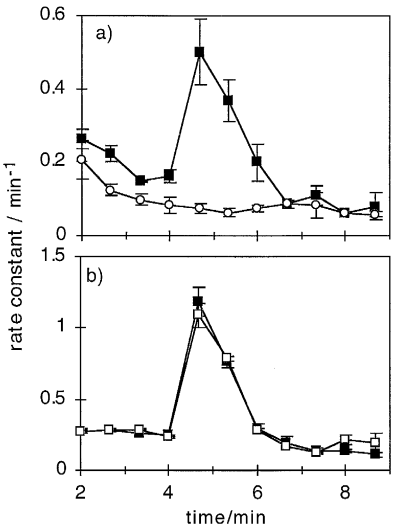

Fig. 3. Effects of (a) the lipoxygenase inhibitor, ETH615 at 10 μM, and (b) the cyclooxygenase inhibitor, indomethacin at 100 μM on the rate constant for iodide efflux from rat brain endothelial cells. Solutions were changed at the 4 min time-point from isotonic, i.e., containing 90 mM mannitol, to hypotonic, i.e., with 90 mM mannitol omitted. Control responses in the absence of inhibitors are shown as closed symbols, test responses in the presence of the inhibitors as open symbols. In the test experiments, inhibitors were present during the last 30 min of loading with iodide and throughout the subsequent efflux period. Values shown at each time point are the mean ± S.E. of samples from three separate wells.

$14 \pm 17\%$ ($n = 6$), DIDS 100 μM $91 \pm 12\%$ ($n = 12$); and DPC $104 \pm 27\%$ ($n = 6$) (one experiment).

Depletion of intracellular ATP by replacement of glucose by 6 mM deoxyglucose and addition of 10 mM sodium azide to both loading and efflux solutions always completely blocked the response ($n = 9$). When depletion was started only at the time of the washes (i.e., 5 min before the change to hypotonic solution) the response was completely eliminated in two experiments ($n = 6$) and reduced ($77 \pm 60\%$, $n = 3$) in a third. These results are consistent with other reports that the increased anion permeability requires internal ATP.

The possible involvement of calcium in the iodide efflux response was investigated. Firstly removal of extracellular calcium by using calcium free solution containing 1 mM EGTA during the washes and efflux period did not block the increase in iodide permeability ($108 \pm 54\%$, mean $\pm$ S.D., $n = 12$). Secondly to determine the importance of intracellular calcium, cells were preincubated with thapsigargin at a concentration (1 μM) which is usually sufficient to block uptake of calcium into intracellular stores. This together with calcium-free solution containing 1 mM EGTA produced variable effects on the hypotonic response, with apparently no effect (one experiment), a delay of the response by about 1.5 min (one experiment), reduction to 73% (one experiment), and complete abolition of response (one experiment). A higher concentration of thapsigargin, 10 μM, abolished the response (one experiment). Other methods used to deplete intracellular calcium caused incomplete inhibition of the response: 100 μM dtBHQ, an alternative to thapsigargin for blocking the calcium pump of intracellular stores, reduced the response to $24 \pm 6\%$ ($n = 3$) and the ionophore, A23187, used at 1 μM in calcium free solution containing 1mM EGTA reduced it to $43 \pm 6\%$ ($n = 3$). Finally to test whether an increase in internal calcium can trigger a response, cells suspended in the normal calcium-containing isotonic solution were exposed to 1 μM A23187 instead of hypotonic solution at the 4 min time-point during the efflux period. This produced no significant increase in iodide efflux compared to controls exposed to hypotonic solution ($< 5 \pm 10\%$, $n = 6$).

The involvement of arachidonic acid or its metabolites in the control of the stimulated anion efflux from brain endothelial cells was investigated using inhibitors of calmodulin, phospholipase A_2, cyclooxygenase and 5-lipoxygenase (see Table 1 and Fig. 3). Pimozide, a calmodulin inhibitor, produced greater than 50% inhibition even at 2 μM ($n = 3$) and completely eliminated responses at 50 μM ($n = 6$). Similarly the phospholipase A_2 inhibitor pBPB (10 μM) eliminated or reduced the efflux. Neither indo-

Table 2
Effect of resistance modifiers, i.e., agents that inhibit P-glycoprotein mediated drug efflux from rat brain endothelial cells

Agent		Concentration applied	Response remaining as a percentage of control mean $\pm$ S.D. (n)
Verapamil	(3–10 μM)	50 μM	74 $\pm$ 8 (6)
		100 μM	11 $\pm$ 16 (18)
Dideoxyforskolin	(40 μM)	40 μM	97 $\pm$ 12 (9)
Cyclosporin A	(2 μM)	10 μM	98 $\pm$ 20 (3, 1 exp.)
Forskolin	(40 μM)	40 μM	120 $\pm$ 13 (6, 1 exp.)
Quinidine	(10 μM)	100 μM	91 $\pm$ 4 (6, 1 exp.)
Tamoxifen	(10–50 μM)	50 μM	25 $\pm$ 26 (12)
Progesterone	(25 μM)	50 μM	37 $\pm$ 9 (6)
		100 μM	< 9 (6)

The modifiers were present during the last 30 min of iodide loading and throughout the subsequent efflux period. Efflux was stimulated by changing from isotonic solution containing 90 mM mannitol to hypotonic solution in which the mannitol had been omitted. Responses with and without the agents are compared at the peak increase in rate constant for efflux. None means that no increase in efflux could be seen the presence of the modifier. The concentrations given in brackets for each drug have previously been shown to inhibit *P*-glycoprotein-mediated efflux activity and to produce a threefold increase in intracellular vincristine accumulation [7].

methacin nor piroxicam at 100 μM altered the rate of efflux which indicates that products of cyclooxygenase are not essential for the hypotonicity-induced efflux. By contrast the 5-lipoxygenase inhibitors ETH615 and L-656,224 at 10 μM produced nearly total block, strongly suggesting a role for leukotrienes.

The effects of several different substrates and inhibitors of *P*-glycoprotein on the iodide efflux from brain endothelial cells were investigated (see Table 2). Verapamil, which blocks drug efflux by *P*-glycoprotein at 10 μM [7], did not produce clear inhibition of iodide efflux at concentrations below 50 μM. At 100 μM, verapamil reduced the initial amount loaded into the cells in each well about fivefold and eliminated stimulated release. Quinidine (100 μM), forskolin (40 μM), 1,9-dideoxyforskolin (40 μM), and cyclosporin A (10 μM) at concentrations that are known to block drug efflux [7], produced no reduction in iodide efflux. Treatment with both tamoxifen (50 μM) and progesterone (50 and 100 μM) produced substantial block.

4. Discussion

Mechanisms for regulatory volume increase generally involve uptake of ions by cotransporters [12–14] followed more slowly, at least in some cell types, by uptake of organic osmolytes [3]. Regulatory volume decreases in epithelial and endothelial cells occur primarily by loss of K and Cl ions via channels though in some cell types loss of organic solutes is also significant [3,15]. Many of the studies investigating ionic movements have used whole cell patch clamp recordings. In the present work, we have used the iodide efflux technique to monitor anion permeability in populations of cells, thus obtaining population averages and eliminating any problems of interindividual cell variations.

From these measurements we have been able to show the presence of anion permeability increases following hypotonic challenge in endothelial cells derived from rat brain microvessels. The increases could be detected with osmolality changes as small as 30 mOsmol and depend on the level of hypotonicity. In endothelial cells grown from the rat aorta, the permeability increases were much smaller, suggesting that this type of endothelial cell may not respond so readily to volume changes.

Volume regulatory responses have been investigated in a number of endothelial cell types [4]. In vascular endothelium, regulation of cell volume is thought to be important in maintaining barrier function [12]. This may be particularly relevant in the case of the blood–brain barrier as large fluxes of polar solutes must pass through these cells which exposes them to the risk of large changes in cell solute content and in cell volume. Thus endothelial cells derived from brain microvessels may require more highly developed systems for regulation of their cell volume than endothelial cells from other parts of the vasculature. Hence their ready response following relatively small changes in osmolality.

We have also investigated the properties of this anion efflux pathway in the rat brain endothelial cells. It shows similarities with but also differences from volume-activated chloride channels described in other types of cell. Specifically there are differences in the effects of the chloride channel blockers, DIDS and NPPB. These agents have been shown to block swelling-activated chloride currents in various cell types including primary cultures of human astrocytoma cells [16], and some endothelial cell types, e.g., those derived from human umbilical vein [17,18], in this case, DIDS showing half maximal inhibition at 120 μM. With rat brain endothelial cells NPPB at 100 μM blocked the permeability increase, but DIDS had little if any effect. DPC at 100 μM was likewise without effect.

The effect of DIDS on swelling-induced chloride ion movements in brain endothelial cells have already been studied using whole cell patch clamp recordings [10]. In these experiments, it was shown that DIDS at 100 μM could suppress outward current representing inward movement of chloride. However, it was not able to reduce the inward current representing outward movement of chloride ions. These earlier observations are thus consistent with a lack of effect of DIDS in the iodide efflux studies, since the resting potential of these cells is expected to be negative relative to the reversal potential for the chloride channels.

In line with what has been observed in human umbilical vein endothelial cells [19], the response in the rat brain endothelial cells is dependent on ATP

levels, reduction of intracellular ATP abolishing the response.

A number of different intracellular pathways may mediate these hypotonicity induced changes in anion permeability and different ones have been implicated in different cell types. In some cells such as human fibroblasts [20], the responses depend on extracellular and intracellular calcium. By contrast, in the present study, removal of external calcium had no noticeable effect on the response seen in the rat brain endothelium and a deliberate increase in intracellular calcium induced by A23187 did not provoke a permeability increase. Nevertheless, the response does seem to require the presence of calcium. In some experiments, depletion of internal stores using thapsigargin (1 μM) and removal of external calcium together brought about a reduction in response. This thapsigargin effect was somewhat variable, a phenomenon possibly accounted for by the presence of *P*-glycoprotein in the cells [7]. It has been shown previously that expression of *P*-glycoprotein is one of the factors leading to resistance to thapsigargin [21]. Further evidence that calcium is important in the responses was obtained by using a higher concentration of thapsigargin, by employing an alternative inhibitor, dtBHQ, and by depletion of internal calcium using A23187. The higher concentration of thapsigargin abolished the response while dtBHQ and A23187 with external EGTA both substantially reduced it. These observations can be explained if a low concentration of calcium within the cell is required for a response to occur, but an increase in calcium is not the trigger for the response, an idea proposed by Szücs et al [22]. Quite possibly, enzymes involved in the response pathway may require calcium, but only at or somewhat below the levels normally present within the cell.

In support of this idea, it was observed that the calmodulin inhibitor, pimozide, and inhibitors of the calcium-requiring enzymes, phospholipase A_2 and 5-lipoxygenase, reduced or blocked the response. Such observations suggest an important role for leukotrienes generated in response to activation of phospholipase A_2. These effects are reminiscent of the responses observed in fibroblasts [20] and Ehrlich ascites tumour cells [23]. By contrast, cyclooxygenase inhibitors failed to produce any significant effect. These effects differ from those observed in endothelial cells derived from human umbilical vein [17], where phospholipase blockade but not lipooxygenase inhibition reduced the responses.

Other differences between different endothelial cell types concern the effects of agents that modulate *P*-glycoprotein function. Certain *P*-glycoprotein modulators, e.g., tamoxifen, progesterone and verapamil were found to have effects on the anion permeability changes observed in the rat brain endothelial cells, though in the case of verapamil, this was at a concentration substantially higher than that shown to inhibit *P*-glycoprotein activity [7]. However, other agents, i.e., dideoxyforskolin, cyclosporin A used at concentrations known to block drug efflux activity in these cells [7] were without effect on the hypotonicity-induced increases in anion efflux. This is in contrast to the results seen by others in other endothelial cell types where both dideoxyforskolin [17] and cyclosporin A [18] were able to inhibit the responses, albeit at concentrations (60 μM and 10 μM, respectively) slightly higher than those used in the present study (40 μM and 2 μM, respectively).

Opinion is still very much divided on the influence that *P*-glycoprotein may have on swelling-activated chloride channels. The basis of this dispute may lie in the existence of different types of these chloride channels in the different cells that have been studied. Whether or not *P*-glycoprotein is involved in regulating or modifying the activity of the channels present in the rat brain endothelial cells is not known. We have already demonstrated from RT-PCR and Western blot analysis that both mdr1a and to a greater extent mdr1b *P*-glycoprotein is expressed in these cells in primary culture and that this *P*-glycoprotein is functionally active [7]. It remains to determined therefore if the function of the swelling-activated channels is in any way impaired when *P*-glycoprotein expression is transiently ablated by antisense oligonucleotide treatment. These studies are currently in progress.

It seems therefore that in the rat brain endothelial cells, the characteristics of the chloride channels activated by swelling and the pathways involved in regulating these channels may be different from those observed by others in other endothelial cell types [4,17,18]. This is not surprising, considering the different roles played by the endothelium in different situations. It has been suggested that different chan-

nel proteins may be responsible for anion movements in different cell types and that more than one type of anion-selective channel could play a role in volume regulation [24,25]. Whether therefore brain endothelial cells contain channels different from those in other endothelial cell types or whether they contain the same channels that are expressed in a different regulatory environment is not yet clear. Answers to this question will require further identification of the channels expressed in these cells both at the pharmacological and the molecular level. Such studies are already being undertaken by others to identify the chloride channels in endothelial cells of bovine pulmonary artery [26]. Since it is likely that factors within the brain may influence events in endothelial cells derived from the blood–brain barrier, further studies of volume regulation in brain endothelial cells following co-culture with astrocytes could prove very informative.

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