Changes in anion permeability following hypotonic challenge in rat brain endothelial cells: different responses in primary cultures and in immortalised RBE4 cells

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Abstract Hypotonicity-induced anion permeability changes were investigated but not detected in immortalised (RBE4) rat brain endothelial cells using iodide efflux measurements. Large, rapid increases were however observed in primary cultured cells. Both cell types were reinvestigated following culture in a common growth factor-depleted medium. Responses were still undetectable in the immortalised RBE4 cells. Reduced responses were observed in the primary cultured cells that also showed altered morphology and decreased activity of another transporter, P-glycoprotein. Thus both immortalisation and different culture conditions may alter functional expression in these cells of transporters involved in hypotonicity-induced anion permeability changes.

Key words: Iodide efflux; Anion permeability; Primary culture; Immortalised rat brain endothelial cells

1. Introduction

The endothelial layer of the cerebral microvessels provides a tight barrier to the exchange of materials between blood and brain parenchyma so all solutes and water entering or leaving the brain must pass through, rather than between, the endothelial cells. This exposes the cells to large fluxes of osmotically active species. Brain endothelial cells therefore require an efficient means of regulating their volume so that structural integrity of the blood-brain barrier is maintained. Regulatory mechanisms include activation of channels that allow chloride ions to move out of cells leading to decreased cellular volume. Chloride channels involved in regulatory volume decreases have been identified in endothelial cells from a number of different sources [1]. We have shown already that endothelial cells derived from brain microvessels are capable of marked increases in anion permeability in response to relatively modest changes in osmolality [2] though the pathways by which these channels become activated and the factors that modulate their activity have still to be clearly elucidated.

In vitro models, using cultured brain endothelial cells [3], provide a useful means of examining in detail molecular events under defined and controlled conditions and so have many advantages over in vivo systems. Primary cultures have been used successfully to investigate function and activity of a number of different receptors, transporters and ion channels in brain endothelial cells [4–8]. However, such models have disadvantages in that microvessel isolation and separation and selective culture of the brain endothelial cells have to be undertaken on a regular basis; senescence occurs after several passages and other cell types may be present, albeit in small numbers, amongst the endothelial cells.

To avoid these problems, immortalised cell lines of brain endothelial cells have been produced [9,10] including one, RBE4, developed from rat brain endothelial cells by transfection with plasmid containing the E1A adenovirus [11]. This has a non-transformed endothelial phenotype, retains sensitivity to astroglial factors [11,12] and displays many blood-brain barrier characteristics [13] including expression of various transporters such as GLUT1 [14] and P-glycoprotein [15,16]. There may however be differences between such immortalised cells and primary cultured cells. In the present paper, we investigate anion permeability changes in the immortalised rat brain endothelial (RBE4) cells following hypotonic challenge and compare the responses with those of primary cultured rat brain endothelial cells. As described previously, we use radiolabelled iodide efflux as a measure of anion permeability [2].

2. Materials and methods

2.1. Cell culture

Primary cultures were obtained from brain microvessels isolated from cortical grey matter of rats as previously described [7]. Endothelial cells were grown from these microvessels in collagen-coated culture flasks in Ham's F10 medium containing 20% plasma-derived serum (PDS), 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 (medium A). The cells used for the iodide efflux experiments and uptake experiments were of passages 1–4. RBE4 cells were from subclone B of those originally derived from second-passage rat brain endothelial cell primary cultures by transfection with the plasmid pE1A neo, which confers immortalisation without oncogenic transformation [11]. They were maintained in F-10 Ham's/α-minimum essential medium 1:1 (v/v Sigma), supplemented with 10% (v/v) FCS, 1 ng/ml bFGF (Sigma), penicillin/streptomycin/glutamine and 300 μg/ml G418 (medium B) in collagen-coated flasks. In some experiments, each of the cell types was also cultured for 1–2 weeks in a medium (medium C) containing Ham's F10 with only 10% (v/v) FCS and the penicillin/streptomycin/glutamine supplement as above. G418 was still included with the immortalised RBE4 cells.

2.2. Iodide efflux experiments

Cells were plated into 12-well plates 3 days before the experiments in sufficient numbers to produce a density of around 2×10^5 cells/well on the day of the experiment. All stages of the experiments were performed at 37°C. Efflux measurements were performed as previously described [2]. 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. For determination of $^{125}I^-$ efflux, samples were obtained by collecting

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the efflux solution each 40 s and replacing it with 1 ml of fresh efflux solution. The $^{125}I^-$ remaining in the cells at the end of the experiment 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 their $^{125}I^-$ content.

The composition of the efflux solution was as follows (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) (measured osmolality of 288 mOsm). The hypotonic solution was similar in composition except for absence of mannitol thus producing an osmolality of 197 mOsm. The hypotonic stimulus was applied by replacing the isotonic efflux solution with hypotonic solution at the 4 min time point. This hypotonic solution was then used throughout the rest of the experiment.

As explained previously [2], the rate constants for efflux were calculated as:

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

Loading of the cells with $^{125}I^-$ was calculated from the sum of the counts in all samples excluding the first four but including the counts remaining in the cells at the end of the experiment.

2.3. Drug accumulation studies

Cells were plated as for iodide efflux experiments. Intracellular vincristine accumulation in the absence or presence of inhibitors of P-glycoprotein activity, i.e. verapamil (10 μM) and cyclosporin A (2.5 μM), was determined as described previously [7]. Following exposure for 90 min at 37°C to 30 nM [^{3}H]vincristine (5–10 Ci/mmol) (Amersham Int., Bucks, UK) in Ham's F10 medium containing 5 mg/ml BSA, cells in each well were washed three times in ice cold PBS, lysed in 0.1% SDS and the [^{3}H]vincristine content assessed by liquid scintillation counting.

3. Results and discussion

3.1. Basal and hypotonicity-induced anion permeability

Following a hypotonic stimulus, i.e. reduction of osmolality by 90 mOsm, iodide efflux from primary cultures increased rapidly from basal values to reach a peak within 40–80 s (Fig. 1 and Table 1, experiment 1). These data are consistent with results described previously in these cells [2]. By contrast, the immortalised RBE4 cells plated out in wells on the same plate as the primary cultured cells and treated with the same protocol on the same day with the same solutions did not produce any increase in anion permeability after hypotonic challenge. The basal rate constants for efflux from the two cell types were estimated from the mean of values obtained

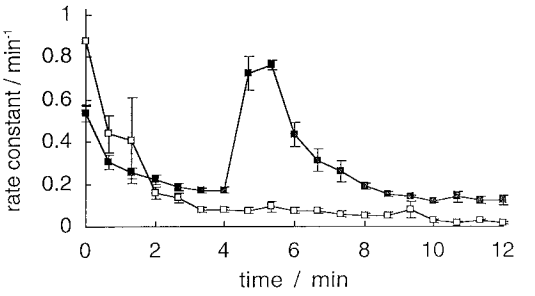

Fig. 1. Effects of hypotonic challenge on increases in rate constant for iodide efflux from rat brain endothelial cells grown as primary cultures (■) in medium A and grown as the immortalised cell line, RBE4 (□) in medium B. Solutions were changed at the 4 min time point from isotonic containing 90 mM mannitol to hypotonic in which mannitol was absent. Values shown are the means ± S.E.M. from three separate experiments with determinations from three separate wells being undertaken in each case (i.e. $n = 9$).

for each well at the two time points just before hypotonic challenge. These were slightly but significantly different between the two cell types (Table 1, experiment 1).

The amount of $^{125}I^-$ loaded into the cells also differed between the primary cultures and the immortalised cells with around 5-fold more $^{125}I^-$ entering the primary cultured cells. The total amount of $^{125}I^-$ loaded was typically around 14 000 counts min^{-1} $well^{-1}$ in the primary cultures and 2500 counts min^{-1} $well^{-1}$ in the RBE4 cells although the cell numbers per well were similar. The route or routes by which iodide enters the cells during the loading period has not been identified. However, a lower level of loading should not affect anion permeability measurements since only tracer amounts of iodide were used (concentrations < 10 nM) and the efflux rate constant should be independent of the amount in the cells.

3.2. Altering culture medium

We investigated the possibility that the difference in response might be due to differences in composition of the media used to culture the primary cultures and the RBE4 cells. It was difficult to make direct comparisons by looking at responses with each cell type grown in the medium of the other since preliminary experiments established that neither cell type

Table 1
Anion efflux rate constants in primary cultured and immortalised RBE4 rat brain endothelial cells

Experiment		Primary culture		Immortalised cells	
		Basal efflux	Peak efflux	Basal efflux	Peak efflux
1	Medium A	0.17 ± 0.01 ($n = 18$)[a]	0.76 ± 0.02 ($n = 9$)		
	Medium B			0.08 ± 0.01 ($n = 18$)[a]	0.1 ± 0.02 ($n = 9$)
2	Medium B			0.07 ± 0.01 ($n = 6$)	0.07 ± 0.01 ($n = 6$)
	Medium C			0.05 ± 0.01 ($n = 6$)[b]	0.08 ± 0.01 ($n = 6$)[b]
3	Medium A			0.1 ± 0.01 ($n = 12$)[c]	
	Medium B			0.04 ± .003 ($n = 12$)[c]	
4	Medium A	0.23 ± 0.02 ($n = 22$)[d]	0.91 ± 0.04 ($n = 11$)[e]		
	Medium C	0.10 ± 0.003 ($n = 22$)[d]	0.35 ± 0.01 ($n = 12$)[e]		

Values shown in min^{-1} are the mean ± S.E.M.
Experiment 1: difference between cell types in basal efflux constants[a] is significant to $P < 0.0001$ by Student's unpaired t-test.
Experiment 2: difference between basal and peak efflux constants[b] in medium C is significant to $P < 0.05$ by paired t-test.
Experiment 3: difference between media in basal efflux constants[c] is significant to $P < 0.0001$ by unpaired t-test.
Experiment 4: differences between media in basal[d] and in peak[e] efflux constants are both significant to $P < 0.0001$ by unpaired t-test.

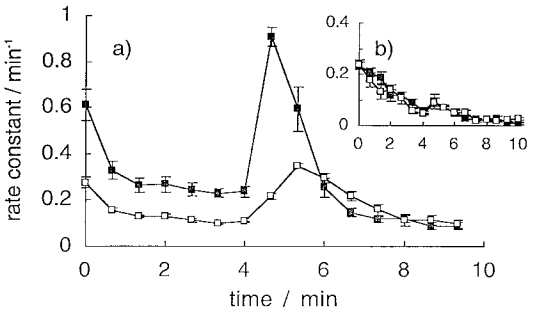

Fig. 2. Effects of hypotonic challenge on increases in rate constant for iodide efflux from (a) rat brain endothelial cells or from (b) immortalised RBE4 cells when grown for 1 week either in their original culture medium (■) (medium A and medium B respectively) or in the common medium C (□). Solutions were changed at the 4 min time point from isotonic containing 90 mM mannitol to hypotonic in which mannitol was absent. Values shown are the means ± S.E.M. from three (for the immortalised cells) or four (for primary cultured cells) separate experiments with determinations from three separate wells being undertaken in each case, i.e. $n=9$ or $n=12$ respectively.

would grow well under these conditions. Indeed, the primary cells failed to produce enough cells for iodide efflux experiments. We therefore devised a common culture medium, medium C, in which both cell types could grow (see Section 2). This medium contained no heparin and no added growth factors apart from those present in the serum. Geneticin was still present in the medium for the RBE4 cells to maintain selection pressure for the transfected cells. It was envisaged that lower levels of growth factors might be advantageous in slowing growth and so possibly encourage a more differentiated phenotype. Though growth factors will stimulate expression of genes involved with proliferation, they may at the same time decrease expression of genes whose products are not required for proliferation. Examples of this have been reported in cultured human umbilical vein endothelial cells [17]. Amongst such genes might be those encoding chloride channels involved in volume regulation.

Growth of the primary cultures and of the immortalised RBE4 cells were compared in their original medium and in the common medium. With the primary cultures, if grown in their original medium, medium A, there was a 6–7-fold increase in cell number over 5 days and, if grown in the common medium, medium C, lacking added growth factors, there was only a 5-fold increase in the same time period. This difference was more marked with the immortalised RBE4 cells that have a high growth rate in their original medium, medium B, with a 20-fold increase in number over 5 days compared with only a 6–7-fold increase in medium C.

3.3. Influence of culture medium on anion permeability in immortalised RBE4 cells

Cells were cultured in the different media for at least 1 week before being plated out for efflux experiments. In the RBE4 cells grown at high rate in their original medium, medium B, hypotonic challenge as previously did not elicit any increase in anion permeability and there was only a very small response in the RBE4 cells grown at the slower rate in the common medium, medium C (Fig. 2b and Table 1, experiment 2). Both the extent of loading and rate constants for basal efflux were similar in the two groups of cells. It seems therefore that the reduced growth rate did not have a very great effect on anion permeability responsiveness in the immortalised RBE4 cells.

In two separate efflux experiments in which sufficient RBE4 cells were obtained after 1 week culture in the primary culture medium, i.e. medium A, no significant response to hypotonic challenge was observed. The basal efflux rate constants were however significantly higher than those observed in the RBE4 cells cultured in their normal medium, i.e. medium B and tested at the same time (Table 1, experiment 3).

3.4. Influence of culture medium on anion permeability in primary cultured cells

A substantial change in responsiveness was however seen with the primary cultured cells when grown at slower rate in the growth factor-depleted common medium but this was a decrease rather than an increase in responsiveness. In experiments performed with cells grown in medium C for at least 1 week, there was a significantly smaller peak response in terms of changes in anion permeability to hypotonicity-induced swelling (Fig. 2a) and the basal efflux rate constants were lower in these cells (Table 1, experiment 4). The initial loading of the cells was also less in the cells grown in medium C (typically around 14000 counts min^{-1} $well^{-1}$ in medium A-cultured cells compared with 5000 counts min^{-1} $well^{-1}$ in medium C-cultured cells). It has been observed in other cell systems that confluence can affect the level of expression of certain chloride channels [18]. It is unlikely in the present study that confluency had any influence since in experiments where cells were plated at different numbers so as to achieve different levels of confluence, there was no significant change in the level of responsiveness of the cells.

3.5. Influence of culture medium on cellular morphology

Changes in cell morphology paralleled these changes in responsiveness in the primary cultures. After 1 week of culture in medium C, the cells had lost their elongate spindle-shaped morphology (Fig. 3a) and become more flattened and spread out (Fig. 3b). Exclusion of either added endothelial cell growth supplement and/or heparin could be responsible for these changes in morphology. Heparin and related molecules

Table 2
Vincristine accumulation in primary cultured rat brain endothelial cells grown in different culture media

	Medium A	Medium C
Accumulation in absence of inhibitors (fmol/10^5 cells)	14.2 ± 2.4 ($n=6$)	27.7 ± 4.7 ($n=6$)*
Increase in accumulation (proportion of control) in presence of:		
10 μM verapamil	2.7 ± 0.3 ($n=8$)	1.8 ± 0.5 ($n=8$)**
2.5 μM cyclosporin A	2.1 ± 0.2 ($n=8$)	1.6 ± 0.1 ($n=8$)**

In each case differences between values for the two media are significant to $*P<0.05$ and $**P<0.01$ by Student's paired t-test.

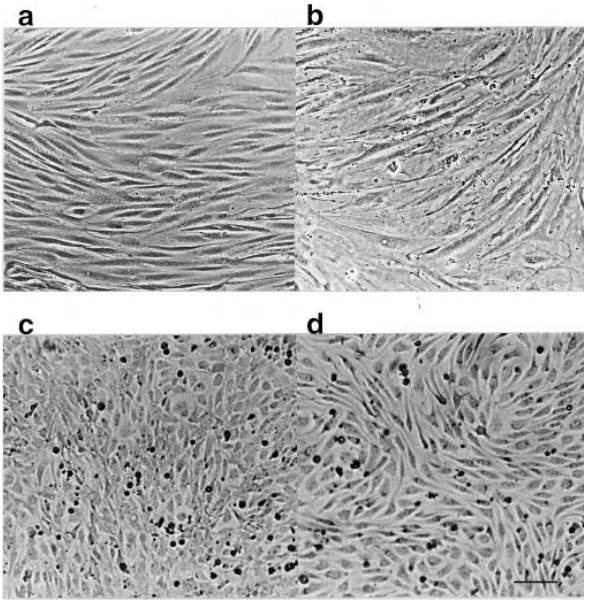

Fig. 3. Appearance under phase contrast of: the rat brain endothelial cells grown as primary cultures (a) in their normal medium A and (b) following growth for 7 days in medium C in which heparin and growth supplements are absent; and immortalised rat brain endothelial cells of the line, RBE4, sub-clone B (c) in their normal culture medium B and (d) following growth for 7 days in medium C. Bar = 1 00 μm.

are known to be important participants in growth factor signalling, being involved with modulation of ligand-receptor interactions [19]. The other alteration in composition, substitution of FCS for PDS has been performed many times previously with these cells without effects on morphology or other properties under investigation. The morphology of the immortalised RBE4 cells was similar in the two different media (Fig. 3c,d).

3.6. *Influence of culture medium on vincristine accumulation in primary cultured cells*

Interestingly, not only anion permeability but also drug efflux activity via the drug transporter, P-glycoprotein, was altered in the primary cultured cells under the different culture conditions. This transporter is known to be present in brain microvasculature [20,21] and has been detected in endothelial cells grown from isolated microvessels [5,7,22,23] and in the immortalised RBE4 cells [15,16]. In the present study, P-glycoprotein activity was examined in the primary rat brain endothelial cells grown in the different media. Activity was assessed from the extent of vincristine accumulation in the presence or absence of inhibitors of P-glycoprotein as previously described [7]. In the absence of inhibitors, there was less vincristine accumulated during 90 min in the primary cultured cells grown in their original medium than in the growth factor-depleted medium (see Table 2). This difference suggests a greater efflux capacity for the cells in their original medium. Indeed, when P-glycoprotein activity was inhibited by verapamil (10 μM) and by cyclosporin A (2.5 μM) the increases in vincristine accumulation in the primary cultured cells were significantly larger when grown in their original medium than when grown in the growth factor-depleted medium (see Table 2).

4. Conclusions

The results reported here show that immortalised RBE4 cells, unlike the primary cultured endothelial cells, have little ability to respond to hypotonic stimulus by increasing their anion permeability. The chloride channel responsible for this response in the primary cultures has yet to be identified though its characteristics have been described already [2]. It is not yet known if this chloride channel is normally expressed in vivo or if it only appears or becomes activated following culture from isolated brain microvessels. Immortalisation itself may not be the prime cause of the differences in response in the two brain endothelial cell types. The conditions of culture may also contribute to these differences since both basal and hypotonicity-induced efflux rate constants are altered in the primary cultured cells in different media. These results provide caveats for the use of in vitro cultures as models of brain endothelial cell behaviour in vivo. But they do indicate the value of these systems for observing events under specified conditions, thereby unravelling some of the potential pathways involved in modulation, both physiological and pathological, of gene expression at the blood-brain barrier.

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