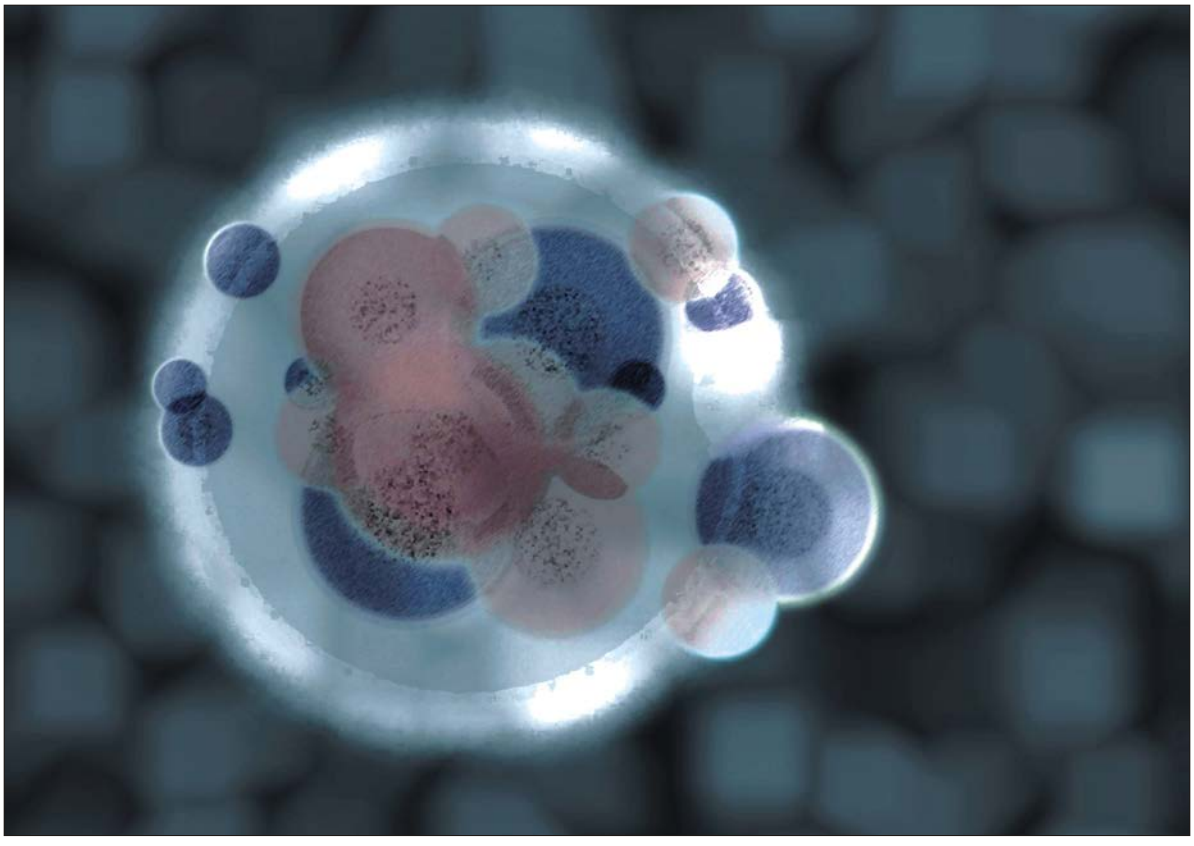

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Bioelectrical and Permeability Properties of Brain Microvasculature Endothelial Cells: Effects of Tight Junction Modulators

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Abstract: In the current investigation, primary porcine brain capillary endothelial (PBCE) cells were isolated and used as an *in vitro* blood-brain barrier model. The bioelectrical and permeability properties were assessed in PBCE cells treated with Astrocytes Conditioned Medium (ACM), hydrocortisone and cAMP elevators. The primary PBCE cells cultured with ACM plus cAMP elevators provided significantly higher trans-endothelial electrical resistance (~700 Ω cm^2) in comparison with those cells cultured with ACM (~400 Ω cm^2) or untreated control (~200 Ω cm^2), while PBCE cells cultured with ACM plus hydrocortisone (550 nM) resulted in ~550 Ω cm^2. The bioelectrical properties were further validated by means of the permeability coefficients of transcellular and paracellular markers with results of ~20 and ~5 ($\times 10^{-6}$ cm sec^{-1}) for propranolol and sucrose, respectively in PBCE cells treated with ACM and cAMP elevators. Upon the obtained bioelectrical and permeability results, PBCE cells cultured with astrocytes conditioned medium and cAMP elevators can be proposed as a high-throughput *in vitro* model for drug screening and brain targeting.

Key words: Blood-brain barrier, bioelectrical properties, cell culture model, cell delivery, cellular permeability, cell and tissue engineering

INTRODUCTION

Brain capillary endothelial cells confer barrier functionality to impede the entrance of toxic agents to the CNS. Because of the barrier confinement, metabolism and existence of transporters at the Blood-Brain Barrier (BBB), studies on the permeation of pharmaceuticals across BBB represent a major challenge in neuropharmaceutical research. Selective transport of endogenous/exogenous compounds through the biological membranes and barriers such as BBB explicitly highlights importance of cellular transport machineries (de Boer and Gaillard, 2007; Omidi and Gumbleton, 2005). To date, for brain drug delivery and targeting, cell based *in vitro* BBB models have been widely exploited. Of these cell-based models, no immortalized cell line has so far been reported to represent restrictive barrier functions of *in vivo* (Gumbleton and Audus, 2001). Basically, cell-based BBB models should primarily display discriminative barrier functionality, thus primary BCE cells isolated from various species such as bovine (Audus and Borchardt, 1987) and porcine (Franke *et al.*, 2000) have been exploited as *in vitro* models.

In vitro cell-based BBB models represent a powerful complement to the *in vivo* system, allowing clarification of cellular, biochemical and molecular features of transport systems mechanistically (Takakura *et al.*, 1991). Nevertheless, none of the immortalized brain endothelial cell lines appear to generate the necessary restrictive paracellular barrier properties that would allow their effective use in transendothelial permeability screening, even though they may be suitable for drug uptake/efflux studies. *In vitro* BBB models that tend to possess the characteristics of discrimination between paracellular and transcellular probes are predominantly primary or low passage model systems requiring the isolation of vessels from brain tissue itself, either bovine or porcine. Various tight junction modulators have been used to provoke imitation of restrictive barrier formation in BCE cells. Among which, the effect of cAMP on BBB function has been studied by Hurst and Fritz (1996), Rubin *et al.* (1991) and Takagi *et al.* (1991). Using combination of astrocyte conditioned-medium and cAMP elevators, Rubin *et al.* (1991) reported a cell culture *in vitro* BBB model that generated high resistance tight junctions and exhibited low rates of paracellular permeability.

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Hurst and Fritz (1996) presented a coculture BBB model of the immortalized human umbilical vein endothelial cells ECV304 with the rat C6 glioma cells, however ECV304 cells were later on reassigned as the T24 bladder epithelial carcinoma cell (Kiessling *et al*., 1999; Suda *et al*., 2001), with rat C6 glioma cells can generate a BBB model with high TEER (~400-600 Ω cm^2). Previously, we showed that b.End3 cells can serve as an appropriate *in vitro* BBB model for carrier-mediated transport studies, but not for drug screening due to its lower barrier discrimination (Omidi *et al*., 2003). To provide a highly discriminative cell-based BBB model, we used the primary PBCE cells cocultured with astrocytes (Omidi *et al*., 2003; Smith *et al*., 2007). In the current study, we produced a simple *in vitro* BBB model using PBCE cells treated with astrocytic factors along with some tight junction modulators.

MATERIALS AND METHODS

Materials: The following items were obtained from Sigma-Aldrich Chemical Co. (Poole, UK): glutaraldehyde, hydrocortisone and alkaline phosphatase (ALP). The M199 medium, DMEM/F12 medium, Foetal Bovine Serum (FBS), heat-inactivated-FBS, penicillin G and streptomycin were obtained from InVitrogen (Paisley, UK). Percoll and the radioisotopes DL-[4-^{3}H]propranolol hydrochloride and [U^{14}C] sucrose, [^{3}H]diazepam and [^{14}C]mannitol were obtained from Amersham Life Science (Little Chalfont, UK). Tissue culture treated multi-well plates and Transwell-clear™ polyesteren membrane (pore size 0.4 μm) inserts (diameter 6.5 and 24 mm) were obtained from Corning Costar (High Wycombe, UK). Neutrally-buffered 2% osmium tetroxide in veranal acetate buffer and Araldite (CY212) resin were obtained from TAAB (Aldermaston, UK). OptiPhase HiSafe3™ liquid scintillation fluid was obtained from Fisher Scientific Chemicals (Loughborough, UK). Dispase II, dispase/collagenase (from Vibrio alginolyticus/Bacillus polymyxa) and rat-tail collagen type I were obtained from Roche (East Sussex, UK). The rat glioma cell line, C6, was obtained from ECACC (Porton, UK).

Isolation of the porcine brain capillary endothelial cells: Isolation of PBCE cells was performed according to the previously reported methodologies (Audus and Borchardt, 1987; Franke *et al*., 2000; Omidi *et al*., 2003) with some modifications. Briefly, the porcine heads were obtained from slaughterhouse and transported to the lab within 0.5 h. Brains were carefully taken out and washed by ice-cold sterile Phosphate Buffered Saline (PBS). Meninges and large surface vessels were removed from the cerebral cortex of the brains under sterile condition. The cortical grey matter of porcine brains minced to small pieces (<1 mm^3) and suspended in preparation media (i.e., M199 supplemented with 100 units mL^{-1} of penicillin G and 100 μg mL^{-1} of streptomycin) using 50 mL per brain. The preparation media was supplemented with dispase II at a final concentration of 0.5%. The mixture was incubated at 37°C for 2 h using Orbital Shaker SO5, Bibby Stenilin Ltd. (Staffordshire, UK). Then, 150 mL of 30% percoll solution (ρ = 1.032 g mL^{-1}) was added per 100 mL of the digested suspension and the mixture centrifuged at 6000×g for 10 min at 4°C using a swing-out centrifuge, Avanti ™ J-25 centrifuge, Beckman Coulter Inc. (California, USA). The pellet containing microvessels were resuspended in the culture media (i.e., preparation media supplemented with 10% heat-inactivated FBS) to a total volume of 10 mL per brain. 1 mg mL^{-1} collagenase-dispase II (0.1% w/v) was added to the latter mixture and incubated at 37°C for 1 h. The suspension was kept at 4°C for 15-20 min to stop enzymatic digestion and passed through the 180 μm nylon mesh to obtain the detached cells. The resulted cells from 1 brain were suspended in 5 mL of culture media and centrifuged at 1300 x g for 10 min at 4°C on a discontinuous percoll-gradient (heavy percoll, 1.07 g cm^{-3} and light percoll, 1.03 g cm^{-3}). The endothelial cells, mainly present in cell clusters, were removed with a pipette from the middle band at a density between 1.052 and 1.05 g cm^{-3}. The obtained cells were either used as fresh or frozen for later use.

For cultivation, cells were plated at a seeding density of 5.0×10^4 cells cm^{-2} onto the T-flask or inserts coated with the rat tail collagen type I (RTCI) that was prepared with sterile acetic acid 0.2% v/v. The T-flasks (25 or 75 cm^2) were coated with 250 and 1000 μL of RTCI, respectively at a final concentration of 3 μg cm^{-2}. The coated flasks were allowed to dry over night (16-18 h). The small (0.3318 cm^2) and large (4.7 cm^2) transwell membrane inserts (either polyesteren or polycarbonate inserts) were also coated with 50 and 500 μL cm^{-2} of RTCI, respectively at a final concentration of 5 μg cm^{-2}. The inserts were first kept at 37°C for 3 h and then let to dry under laminar flow at room temperature over night. To remove the acetic acid residual, the RTCI coated inserts were subjected to further washing (×3) using PBS before plating the cells.

Sub-cultivation and astrocytic modulation: Once the freshly isolated PBCE cells were harvested, they were cultured as the primary alone or with ACM (at a 1:1 ratio with fresh culture medium) from initial cell seeding onto the RTCI coated T-flasks or inserts. For cultivation as astrocytes coculture (ACC), they were cultured onto the RTCI coated T-flasks or inserts while C6 cells were

cultured on the basal of the plates. The cells were allowed to grow for 24-48 h, then washed with PBS (37°C) to remove cell debris and replenished with fresh culture media.

The primary PBCE cells were plated onto the RTCI coated Transwell-Clear™ filter inserts at a seeding density of 5.0×10^4 cells cm^{-2} alone or treated with ACM. To generate tighter monolayers with high TEER, the culture medium was 'switched' to assay medium (i.e., DMEM/Ham's F12, 100 units mL^{-1} penicillin G, 100 μg mL^{-1} streptomycin supplemented with 500 nM hydrocortisone) without serum at 90 h post-seeding according to previously reported method by Franke *et al.* (2000). At 24 h after this 'switch' procedure, transport studies were undertaken.

Rat glioma C6 cells were cultured onto the 6- or 24-well plates at a seeding density of 5.0×10^4 cells cm^{-2} using M199 medium supplemented with 2 mM glutamine, heat inactivated FBS 10%, antibiotics penicillin G (100 units mL^{-1}) and streptomycin sulfate (100 μg mL^{-1}). For the astrocytic modulation as ACC, C6 cells were cultivated 3 days prior to seeding PBCE cells to maximize the biosynthetic secretion of astrocyte derived factors (Rubin *et al.*, 1991). To prepare ACM, C6 cells were cultured to confluence (72 h), at which point ACM was collected every 24 h from the well of the confluent C6 cultures.

The effects of hydrocortisone (550 nM) and cAMP elevators, i.e. phosphodiesterase inhibitors: Ro20-1724 (20 μM) and cyclic AMP analogue 8-(4-chlorophenylthio)-adenosine 3′, 5′-cyclic monophosphate (CPT-cAMP; 250 μM) on the endothelial cells were also studied. These compounds were applied to both apical and basal compartment for 3 days and medium was replenished every 24 h.

All cultured cells were maintained in a humidified atmosphere (5% CO_2/95% air) and medium was replenished every 24 h.

Light and electron microscopy: The morphology of monolayers of PBCE cells grown onto the cell culture flasks and Transwell™ membranes (0.4 μm pore size) in the absence and presence of C6 coculture were examined by the light microscopy (LM) as well as the transmission electron microscopy (TEM). For TEM, cells were fixed and embedded in accordance with the methodology reported previously (Newman *et al.*, 1999).

Bioelectrical properties: The barrier restrictiveness of PBCE cells monolayers was assessed by means of bioelectrical properties and permeability of transcellular and paracellular pathways probes. Trans-endothelial electrical resistance (TEER) was measured from day 3 to day 8 using Evom™ epithelial volt-ohm-meter, World Precision Instrument (Sarasota, USA).

Permeability screening: PBCE cells were cultured as defined previously and permeability experiments were performed at day 5 or 6 post seeding, i.e., 24-48 h after "switch". The transport of paracellular markers (0.5 μCi/well of [^{14}C]sucrose or [^{14}C]mannitol) and transcellular markers (0.5 μCi/well of [^{3}H]propranolol or [^{3}H]diazepam) were examined to assess the tight junction integrity and restrictiveness of PBCE cell monolayers. Sampling was performed at designated times (15, 30, 45, 60, 75 and 90 min) by removing 100 μL medium from either the apical or basal compartments (for experiment in either the A to B or B to A direction) and replenishing with the same amount of fresh medium. Analysis of radiolabeled probes was carried out using a liquid scintillation counter, Wallac 1409, PerkinElmer Life Sciences (Boston, USA). The apparent permeability coefficients were calculated according to our previously reported study (Omidi *et al.*, 2003).

Statistical analyses: All experiments were replicated and data represented as mean value of 4-6 replications ± Standard Deviation (SD). Statistical analyses were performed using either ANOVA followed with a multiple comparison test or the two-tailed Student t-test. A p-value less than 0.05 was assumed to present a significant differences.

RESULTS

Morphological examinations: For morphologically characterization of PBCE cells, we looked at LM and TEM micrographs of PBCE cells cultured onto the cell culture flasks and Transwell™ polycarbonate inserts. Figure 1 shows LM images of PBCE cells at day 6 (Fig. 1A), which clearly demonstrates attenuated and elongated spindle form architecture. Generation of tight junction can be observed as higher density regions (shown with arrows) between two PBCE cells upon coculture with astrocytes (Fig. 1B).

Bioelectrical properties: Figure 2 represents TEER values for PBCE cells at confluency upon "switch" at day 4 (i.e., 24 h post switch). Cultured PBCE cells with no treatment failed to show significant ($p>0.05$) induction in TEER values post-switch, while those cocultured with astrocytes resulted in significant ($p<0.05$) increased in TEER values up to ~900 Ω cm^2 (data not shown). At day 5 (24 h post switch), PBCE cells treated with ACM (1:1) plus cAMP elevators or hydrocortisone significantly

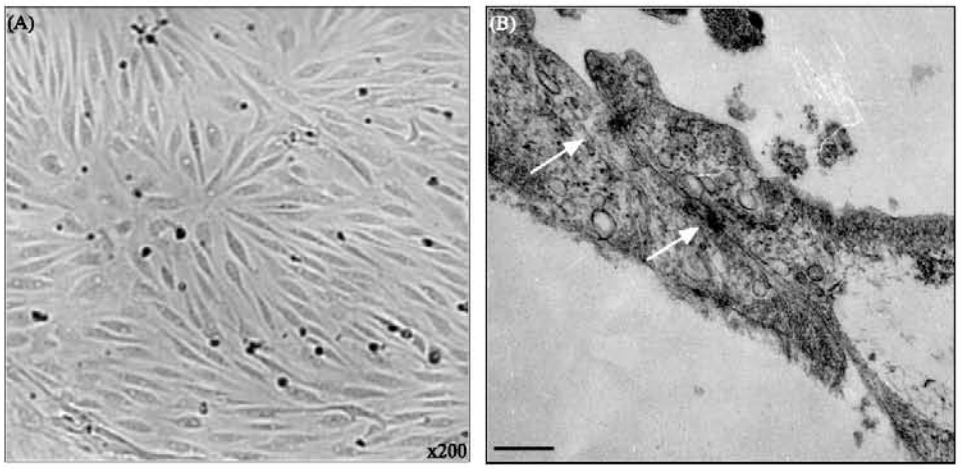

Fig. 1: Light microscopy (A) and transmission electron micrographs (B) of the porcine brain capillary endothelial (PBCE) cells. Squamous morphology of the confluent cells (A) and two elongated primary PBCE cells cultured onto the Transwell™ polycarbonate membranes display typical morphology of the endothelial cells. The white arrows (B) represent the tight junctional elements between two flattened primary PBCE cells. Bar equals to 200 nm

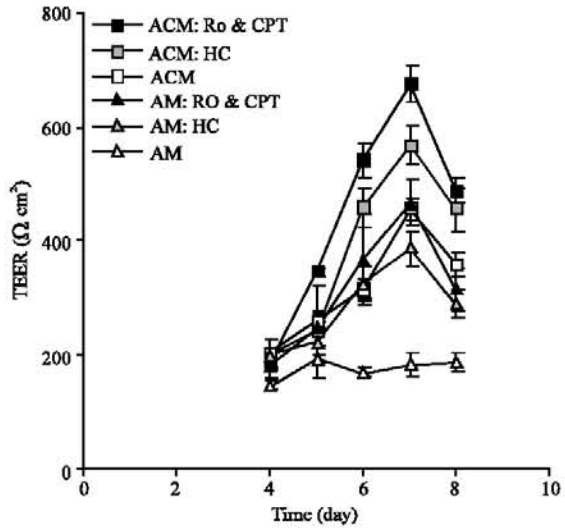

Fig. 2: Trans endothelial electrical resistance (TEER) properties of porcine brain capillary endothelial (PBCE) cells monolayer. Data shows mean ± S.D. 6 replications. * represents significant ($p<0.05$) differences. ACM: Astrocytes conditioned medium; AM: Assay medium; HC: Hydrocortisone; Ro and CPT: cAMP elevators

elevated TEER (Fig. 2). At day 6, increased TEER values were witnessed for PBCE cells treated with ACM plus cAMP elevators (~700 Ω cm^2) and ACM plus hydrocortisone (~600 Ω cm^2).

Permeability analyses: The permeability coefficients of para- and trans-cellular markers in PBCE cell monolayers were measured as shown in Fig. 3. Consistent with the bioelectrical properties, the permeability coefficients ratios of trans-cellular marker (mannitol) over para-cellular marker (propranolol) yielded approximately 3.5 (Fig. 3A). No significant differences were observed in PBCE cells treated with ACM, hydrocortisone and cAMP in comparison with those cells treated with ACM and hydrocortisone. The permeability coefficients ratio for sucrose and diazepam showed similar trends (Fig. 3B). The apical to basal (A-B) and basal to apical (B-A) permeability coefficients of sucrose and diazepam in the primary PBCE cells treated with ACM and hydrocortisone showed no significant differences (Fig. 3B).

DISCUSSION

Drug targeting to the brain is one of the most challenging investigations due to presence of the blood-brain barrier. For drug screening across BBB, it is often essential to use a reliable *in vitro* BBB model which should basically possess high electrical resistance and discriminative barrier properties. Gumbleton and Audus (2001) reviewed potential of the *in vitro* cell cultures models used for permeability screening as a tool and concluded that many of the continuous cell lines derived from brain endothelium lacked the ability to generate a restrictive membrane barrier. In contrast, primary cultures of different species (Siakotos, 1974) have shown the ability to generate a restrictive barrier (Tsuji *et al*., 1992) despite technical complexity in isolation of such primary cultures. To obtain a high throughput BBB *in vitro* model, we aimed to isolated primary PBCE cells according to

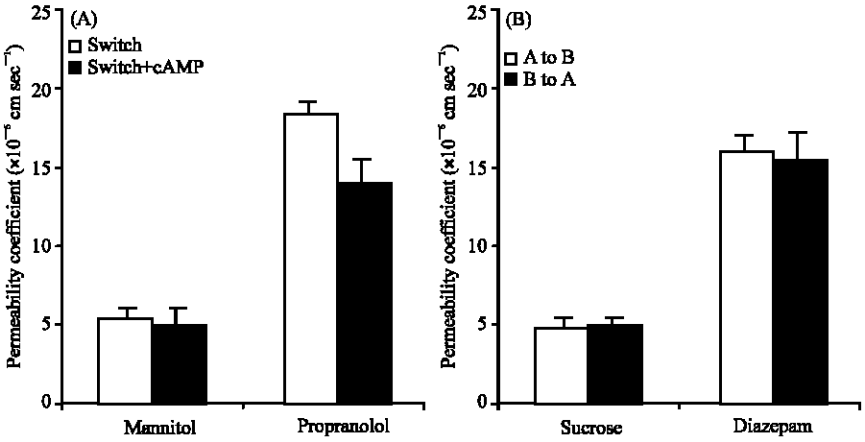

Fig. 3: Permeability coefficients of transcellular and paracellular markers in porcine brain capillary endothelial (PBCE) cells. (A) The effect of switch (removal of serum and addition of 550 nM hydrocortisone) in presence and absence of cAMP elevators on the permeability coefficients of mannitol and propranolol. (B) Permeability coefficients of transcellular and paracellular markers (diazepam and sucrose, respectively) in apical to basal (A to B) and basal to apical (B to A) directions in PBCE cells after switch. Data represents mean±SD of 4-6 replications

previously reported methodologies (Franke *et al*., 2000; Hoheisel *et al*., 1998; Jeliazkova-Mecheva and Bobilya, 2003; Tilling *et al*., 1998) and characterize them towards bioelectrical and permeability properties. Since it is believed astrocytes to be the major modulator of the BBB and regulate the permeability, enzymatic and transport functionalities of the BBB (Allt and Lawrenson, 2000), their impacts in the presence and absence of hydrocortisone and cAMP elevators were assessed.

LM images showed spindle form PBCE cells forming homogenous monolayer at day 6 (Fig. 1A). TEM examination displayed high dense interaction between two elongated PBCE cells implying formation of the tight junction (Fig. 1B) in the presence of ACM and hydrocortisone. It should be prompted that this finding is in agreement with previous results for BBB permeability properties in porcine and murine (Weidenfeller *et al*., 2005).

The bioelectrical examinations resulted in significant responsiveness of the primary PBCE cells to astrocytic factors as well as tight junction modulators. Although the primary PBCE cells cocultured with astrocytes yielded TEER values up to 900 Ω cm^2 as reported previously (Smith *et al*., 2007). PBCE cells treated with ACM reached up to 450 Ω cm^2. It seems the ACM treated cells fail to generate as tight barrier as those cocultured with astrocytes. We speculate that a continued inter-talk between the endothelial cells and astrocytes may confer greater barrier restrictiveness to the endothelial cells cocultured with astrocytes. However, the ACM treated cells showed higher electrical resistance once simultaneously treated with hydrocortisone plus cAMP elevators (700 Ω cm^2).

PBCE cells were further examined using transcellular and paracellular markers. The discrimination ratio obtained from permeability coefficients of diazepam over sucrose was ~3.5 in PBCE cells treated with ACM, hydrocortisone plus cAMP elevators (Fig. 3), however this ratio was approximately 8 in PBCE cells cocultured with astrocytes as previously reported by Smith *et al.* (2007). Intriguingly, similar barrier restrictiveness has been previously reported for PBCE cells showing sucrose permeability values of 1×10^{-6} cm sec^{-1} (Franke *et al*., 1999). It appears that the astrocytic impacts are more profound that the cAMP modulation despite previous reports on usefulness of such modulation (Ishizaki *et al*., 2003; Rubin *et al*., 1991; Wolburg *et al*., 1994). Hamm *et al.* (2004) reported that the permeability of small tracers across BCE cell monolayers was increased upon removal of astrocytes from the coculture. Such changes in permeability were not in association with any detectable alteration in the molecular composition of the tight junction. Perhaps, tighter barrier function occurs on account of the enhancement of phosphorylation states of tight junctional elements PBCE cells upon astrocytes modulation, which is yet to be fully understood. While, Ishizaki *et al.* (2003) showed that cAMP-dependent induction of claudin-5 expression could be involved in promotion of tight-junction function in endothelial cells.

As final conclusion, it can be proposed that PBCE cells treated with astrocytes conditioned medium and cAMP elevators may serve as a high-throughput *in vitro* model for drug screening and brain targeting.

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