similar biosensor chip). These facts suggest that chrysoidine exerts its anti-prion action in a manner that differs from that of Congo red, but this inference demands further evaluation.

In conclusion, we screened chelating chemicals and found that chrysoidine was much more effective in both anti-prion activity and brain endothelial permeability than quinacrine, and it was much less toxic. The mechanism of anti-prion action of this compound did not apparently include alteration of cellular PrP level, direct modification of abnormal PrP, or chelation of metals. Its interaction with PrP121-231 differed greatly from that of Congo red, despite their structural similarity. These findings will contribute to development of therapeutic compounds for prion diseases.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

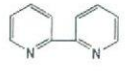
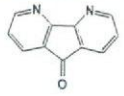
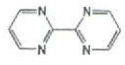
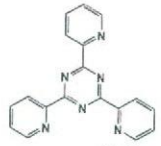
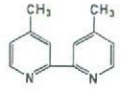
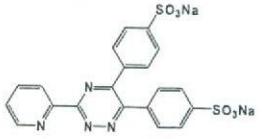
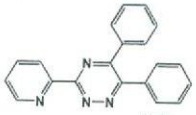
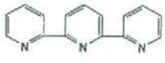
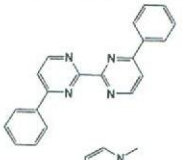
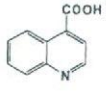
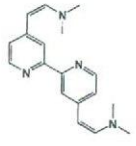
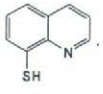
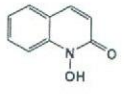
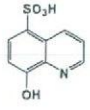
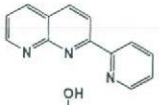
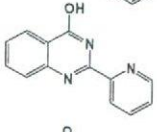
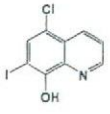
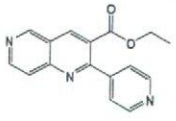
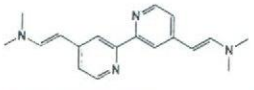
Fig. 1. Anti-prion activity in ScNB cells of chrysoidine pre-incubated with metal ions.
Bars on the left indicate molecular size markers at 37 and 25 kDa.

Fig. 2. Anti-prion activity in ScNB cells of acetylated Yellow AB (A) and azobenzene (B).
Bars on the left indicate molecular size markers at 37 and 25 kDa.

Fig. 3. SPR sensorgrams of chelating compounds interacting with PrP121-231.
Each chemical at 50 μM was analyzed using a *ca.* 3,000 RU PrP-bound biosensor chip.

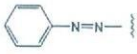
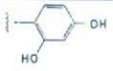
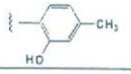
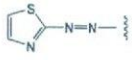
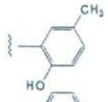
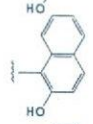
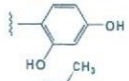
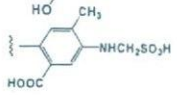
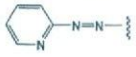
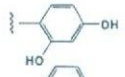
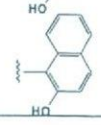
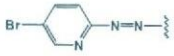
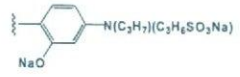
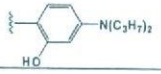
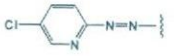
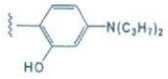
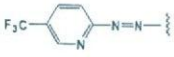
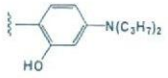
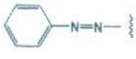
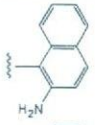
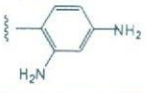
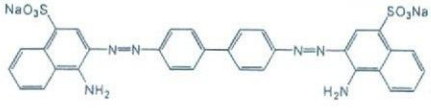
Fig. 4. Permeability coefficients of Na-F, quinacrine and chrysoidine through MBEC4 monolayer.
Each chemical at 100 μM was analyzed. The values are mean ± SEM (n = 3–4 inserts). $^{**}p < 0.01$; significant difference between each group.

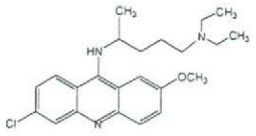
Table 1. Anti-prion activity in ScNB cells of chelating compounds.

Compound	IC_{50} (μM)	CM (μM)	Compound	IC_{50} (μM)	CM (μM)
	-	75		-	>100
	-	>100		-	25
	-	10		-	>100
	-	10		-	10
	-	0.5		5	25
	-	>100		-	5
	-	5		-	25
	-	>100		25	>200
	-	5		-	10
	-	10		-	>100
				-	5

IC_{50}: approximate dose giving 50% inhibition of abnormal PrP formation relative to the control.
CM: approximate maximal dose that does not affect the rate of cell growth to confluence.

Table 2. Anti-prion activity in ScNB cells of chelating azo compounds.

Compound	Terminal 1 - N=N -	Terminal 2	IC_{50} (μM)	CM (μM)
Phenylazoresorcinol			0.3	50
2-Phenylazo-4-methylphenol			0.3	75
4-Methyl-2-(2-thiazolylazo)phenol			-	0.5
1-(2-Thiazolylazo)-2-naphtol			-	0.5
4-(2-Thiazolylazo)resorcinol			3	5
TAMSMB			-	>100
4-(2-Pyridylazo)resorcinol			-	0.25
1-(2-Pyridylazo)-2-naphtol			-	1
5-Br-PAPS			15	20
5-Br-PADAP			4	10
5-Cl-PADAP			2	5
5-CF_3-PADAP			4	10
Yellow AB			0.5	100
Chrysoidine			0.015	>100
Congo red			0.014	not tested

Quinacrine		0.4	2

IC_{50}: approximate dose giving 50% inhibition of abnormal PrP formation relative to the control.
CM: approximate maximal dose that does not affect the rate of cell growth to confluence.

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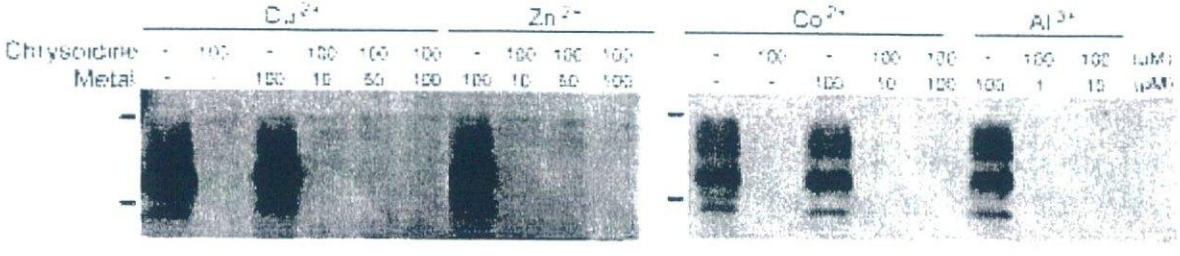

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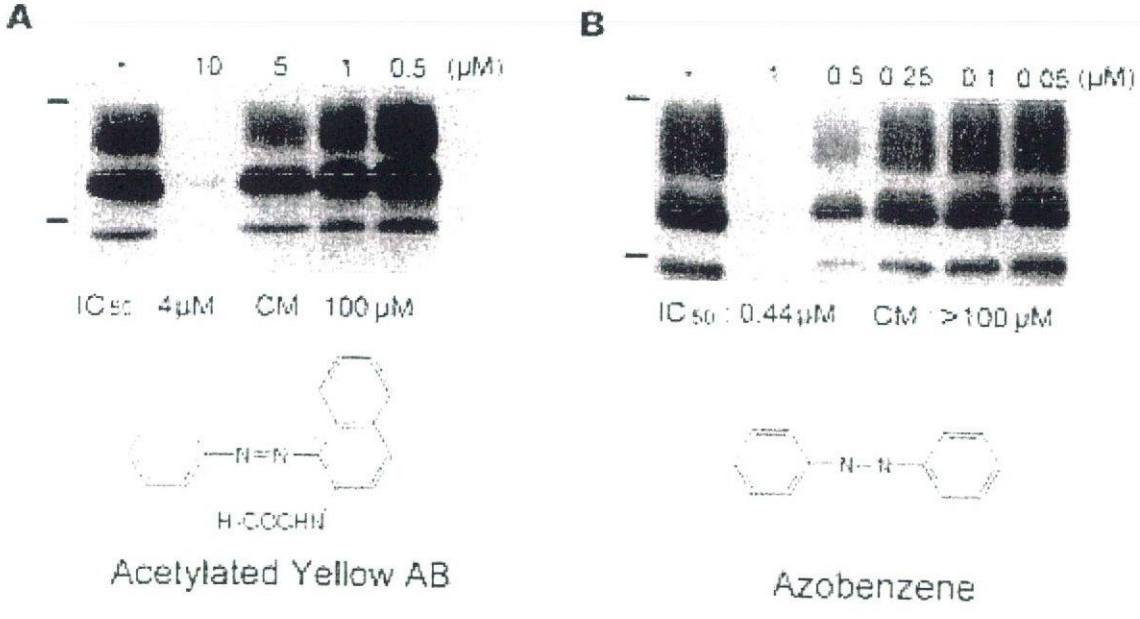

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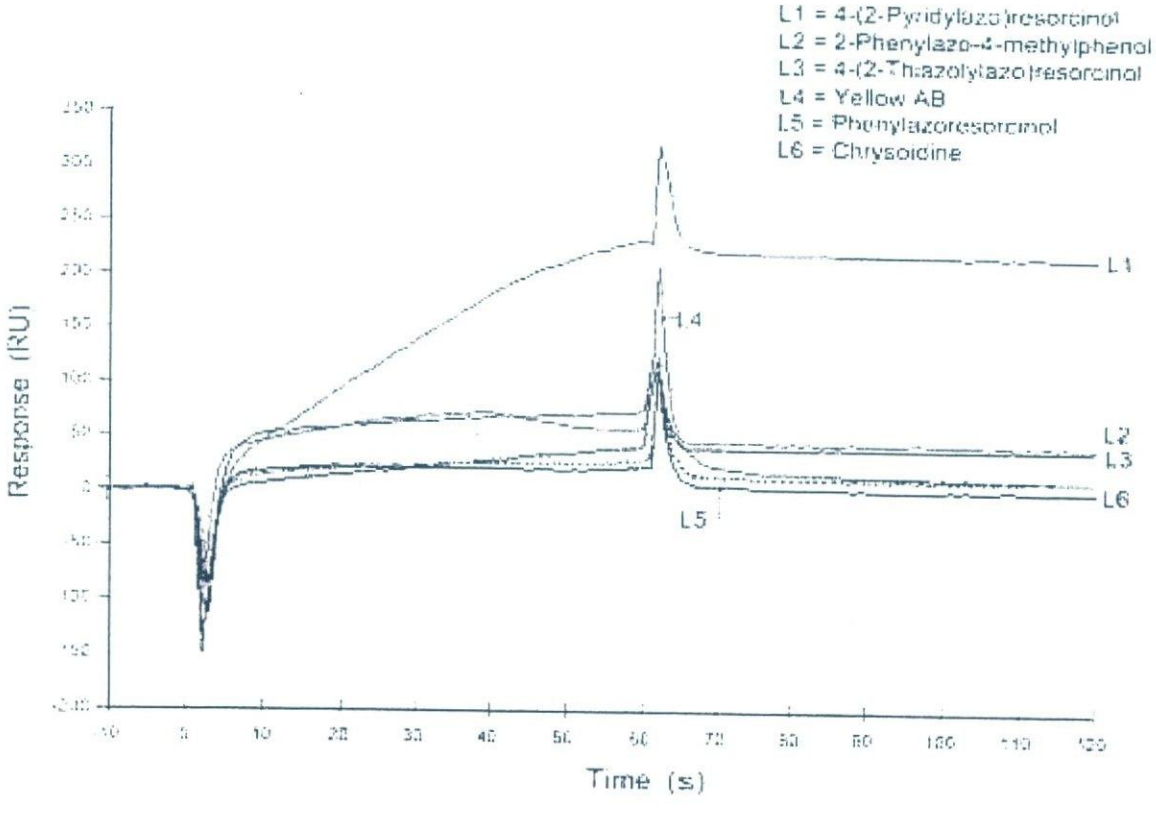

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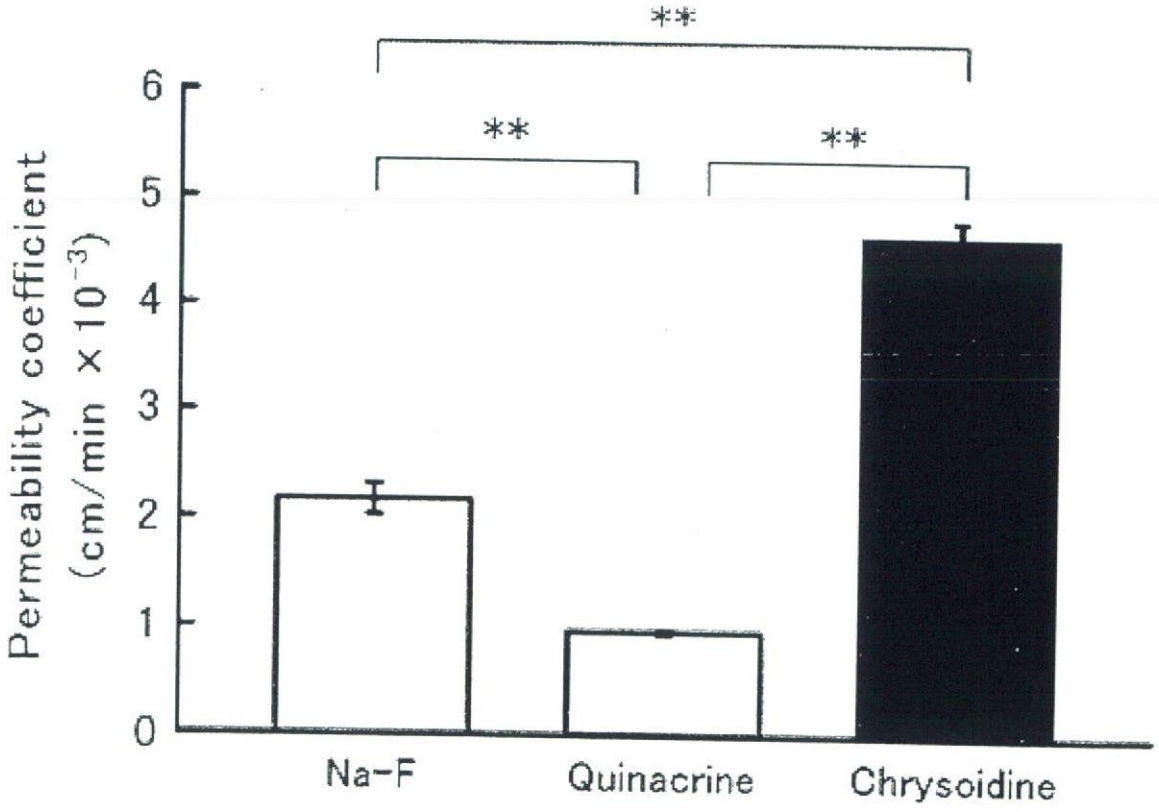

Short Communication

Subject Categories: Central nervous system pharmacology

Protective action of indapamide, a thiazide-like diuretic, on ischemia-induced injury and barrier dysfunction in mouse brain microvascular endothelial cells

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Abstract.

The present study aimed to elucidate the effects of indapamide on ischemic damage to the blood-brain barrier (BBB), in vitro. The ischemia/reperfusion conditions employed here, significantly decreased the viability of mouse brain capillary endothelial (MBEC4) cells, an effect ameliorated by indapamide. Ischemia increased the permeability of MBEC4 cells to two cellular transport markers, sodium fluorescein and Evan's blue-albumin. Indapamide reduced the ischemia-induced hyperpermeability of cells. These results suggest that indapamide may have a protective role in ischemia-induced injury and dysfunction of the BBB.

Keywords: indapamide, ischemia, blood-brain barrier

The blood-brain barrier (BBB) greatly restricts the transport of substances between blood and the central nervous system. The BBB is primarily formed by brain capillary endothelial cells, which are sealed closely with tight junctions (1). Disruption of the BBB can lead to edema and central nervous system pathology in conditions such as stroke. In vitro, hypoxia has been shown to increase the permeability of brain microvascular endothelial cells (2, 3). Targeting the BBB may enhance our understanding of the mechanisms that mediate ischemic brain damage and might aid the development of future treatments for stroke (4, 5).

Indapamide is an indoline derivative of chlorosulfonamide that has both diuretic and antihypertensive properties. Indapamide protects against myocardial ischemia due to its antioxidant action (6). A randomized controlled clinical trial showed that in combination, therapy with perindopril, an angiotensin-converting-enzyme inhibitor, and indapamide reduced the risk of stroke in hypertensive and non-hypertensive patients to a greater degree, compared to single drug therapy with perindopril alone (7). This clinical evidence suggests a possible involvement of indapamide in protecting against recurrent cerebrovascular disease. The present study was, therefore, aimed at evaluating the effects of indapamide on ischemia/reperfusion-induced damage to cerebral microvascular endothelial cells in vitro.

The mouse brain capillary endothelial cells used in the current study (MBEC4) show highly specialized characteristics of brain microvascular endothelial cells (8). MBEC4 cells were isolated from BALB/c mouse brain cortices and immortalized by SV40-transformation (8). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 units/mL of penicillin and 100 μg/mL of streptomycin. Cells were grown in collagen-coated 24-well plates (21,000 cells/cm^2; 1.8 cm^2/well, Corning, Acton, MA, USA) in a humidified atmosphere of 5% CO_2/95% air at 37°C. Ischemia was initiated in vitro by incubating cells with D-glucose-free Krebs-Ringer buffer (143 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM sucrose, pH 7.4) in an anoxic incubator replaced with 5% CO_2/95% N_2 for 10 h at 37°C (ischemia conditions). Subsequently, cells were incubated with serum-free DMEM in 5% CO_2/95% air at 37°C for 1 h (reperfusion conditions). As a control, cells were incubated with normal Krebs-Ringer buffer (143 mM NaCl, 4.7 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM D-glucose, pH 7.4) for 10 h and subsequently incubated with serum-free DMEM for 1 h in 5% CO_2/95% air at 37°C (normal conditions). To test whether indapamide protects against ischemia/reperfusion-induced cell death, cells were exposed to 10-50 μM of indapamide

(Kyoto pharmaceutical industries, Kyoto) or vehicle (0.25% Dimethyl Sulfoxide) during conditions of ischemia and reperfusion or normal conditions. Cell viability was measured by the WST-1 assay (Cell Counting Kit-8, Dojindo, Kumamoto).

Endothelial barrier function was evaluated by measuring permeability of cells to sodium fluorescein (Na-F) and Evan's blue-albumin (EBA) as previously described (9). MBEC4 cells were grown on the collagen-coated membrane (1.1 cm^2, 0.4 μm pore size) of a Transwells™ insert (42,000 cells/cm^2; Corning, Acton, MA, USA) and subsequently exposed to the above-mentioned ischemia conditions for 7 hr. As a control, MBEC4 cells were incubated with normal Krebs-Ringer buffer for 7 h (normal conditions). Cells were exposed to 10-100 μM of indapamide or vehicle during ischemia conditions or normal conditions. To initiate transport experiments, the medium was removed and cells were washed with normal Krebs-Ringer buffer. Krebs-Ringer buffer containing 100 μg/ml of Na-F (MW 376; Sigma, St. Louis, MO, USA) and 670 μg/ml EBA bound to 0.1% BSA (MW 67 kDa) were loaded on to the luminal side of the insert. Samples were removed from the abluminal chamber at 30, 60, 90 and 120 min, and immediately replaced with Krebs-Ringer buffer. The concentrations of Na-F and EBA were determined with a CytoFluor Series 4000 fluorescence multiwell plate reader (Ex(λ) 485 ± 10 nm and Em(λ) 530 ± 12.5 nm, PerSeptive Biosystems, Framingham,

MA, USA) and an Opsys MR microplate reader (630 nm, DYNEX Technologies, Chantilly, VA, USA), respectively. The permeability coefficient and clearance were calculated according to the method of Dehouck et al. (10), as previously described (9).

Data are expressed as the mean ± S.E.M. Statistical analysis was performed using one-way analyses of variance (ANOVA) followed by Tukey-Kramer's post-hoc test. The difference in means was considered to be significant when the p value was less than 0.05.

As shown in Fig. 1, the ischemia (10 h) / reperfusion (1 h) conditions significantly decreased the viability of MBEC4 cells grown on collagen-coated wells, by 26.5% of cells subjected to normal conditions. The effect of ischemia/reperfusion on MBEC4 cells was concentration-dependent: 20-50 μM indapamide improved recovery by 50-86%. These concentrations have no effect on cell viability under normal conditions. As shown in Fig. 2, a 7 h-period of ischemia resulted in increased permeability of MBEC4 cells grown on collagen-coated membranes to Na-F (paracellular transport marker) and EBA (transendothelial transport marker). Following ischemia, the permeability coefficients of Na-F and EBA were significantly increased to 223.7±9.8% and 518.9±42.5% of vehicle, respectively, under normal conditions. The viability of MBEC4 cells grown on membranes after a 7 h-exposure to ischemia conditions and

following termination of the permeability test (2 h) were 72.8 ± 3.6% and 71.3 ± 1.0% of cells subjected to normal conditions, respectively; there was no difference in cell viability before and after the permeability test. Following a 7 h-exposure to indapamide (50-100 μM) under ischemia conditions, hyperpermeability of MBEC4 cells to Na-F and EBA was concentration-dependently reduced by 31.9-47.4% and 41.4-60.6% of vehicle, respectively. This effect was not accompanied by a change in cell viability (vehicle: 72.8 ± 3.6%, 100mM: 75.9 ± 2.3%). Under normal conditions, these concentrations of indapamide had no effect on the permeability coefficients of Na-F and EBA in MBEC4 cells. These findings suggest that indapamide may efficiently inhibit ischemia-induced hyperpermeability rather than inhibit ischemic cell death. MBEC4 cells that were grown on membranes exhibited a higher vulnerability to ischemic cytotoxicity and lower sensitivity to the cytoprotective action of indapamide than those grown on the smooth plastic surface of a well. Further experiments will be required to clarify these issues.

The in vitro ischemia/reperfusion conditions used in the present study significantly reduced the viability of MBEC4 cells and this effect was ameliorated by indapamide. Ischemia conditions increased the permeability of MBEC4 cells to Na-F and EBA, however, this hyperpermeability was reduced by indapamide.

Disruption of the BBB is a critical event during cerebral ischemia as it is followed by the passive diffusion of water, leading to vasogenic edema and secondary brain injury. Cerebral edema is a major and fatal complication of acute ischemic stroke. Free radical overproduction after brain ischemia and reperfusion contributes to brain edema and neuronal damage. Thus, an inhibition of free radical formation is likely to prevent the occurrence of brain edema and neuronal damage. Indapamide has an antioxidant effect and has the potential to scavenge oxygen free radicals and their derivatives. Boucher et al. reported that indapamide treatment has a protective effect on cardiac tissue during the early stages of postischemic reperfusion (6). The present findings suggest that indapamide may protect cerebral endothelial cells from ischemic damage due to antioxidation and/or free radical scavenging. This phenomenon may contribute, at least in part, to the mechanisms by which indapamide facilitated protection of perindopril against recurrent stroke in a recent clinical study (7).

Brain capillary endothelial cells form a metabolic and physical barrier which separates the periphery from the brain to maintain cerebral homeostasis. The lack of fenestrations and the presence of tight junctions differentiate brain microvessel endothelial cells from peripheral microvascular endothelium. While adherent junctions and other junctional proteins contribute to cell-to-cell contacts in the paracellular cleft,