Organization of choroid plexus epithelial and endothelial cell tight junctions and regulation of claudin-1, -2 and -5 expression by protein kinase C

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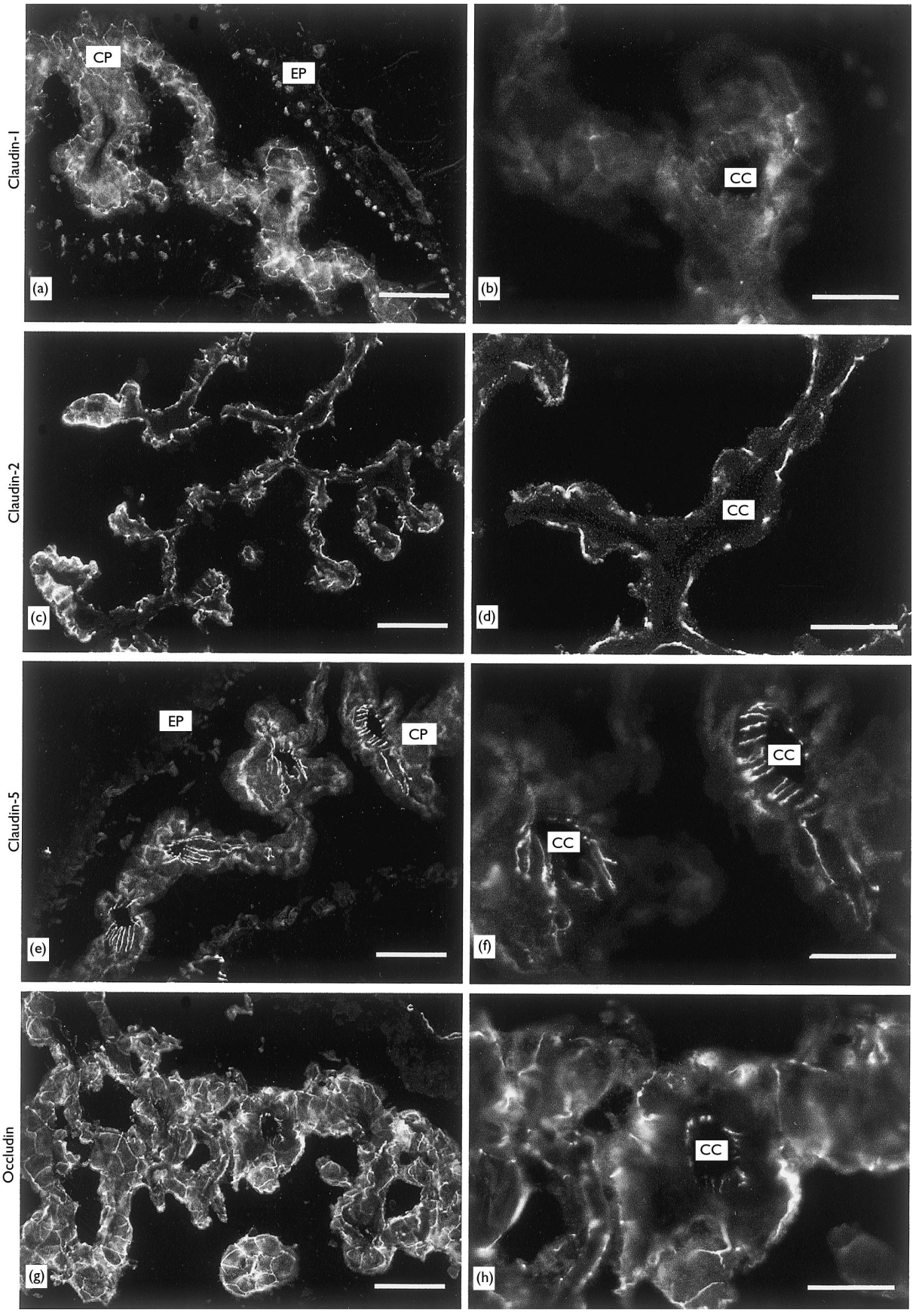
Claudins are components of the tight junctional complex in epithelial and endothelial cells. We characterized the composition of tight junctions in the choroid plexus of the lateral ventricle in the rat brain and tested whether protein kinase C induced changes in their composition. Claudin-1, -2 and -5 were present in the epithelial cells at and near the tight junctions, respectively. In the endothelial cells, claudin-5 was stronger expressed than claudin-1and -2. Twenty-four hours after the phorbolester injection into the ventricle, claudin-1 immunoreactivity of the epithelial cells was increased and spread to the cytoplasm. The claudin-2 and -5 immunoreactivities were reduced. These findings are consistent with an influence of protein kinase C on the composition of the tight junctions in the choroid plexus. *NeuroReport* 11:1427–1431 © 2000 Lippincott Williams & Wilkins.

Key words: Blood–CSF barrier; Claudin; PMA; Tight junction

INTRODUCTION

The choroid plexus constitutes the barrier between the blood and the CSF and is responsible for the production and secretion of the CSF as well as the maintenance of the CSF chemical homeostasis. Recently, we characterized the ventricular ependymal cells and the choroid plexus epithelial cells with regard to their adhesion properties and provided new insights into junctional organization of these types of brain–CSF and blood–CSF interfaces [1]. Tight and adherens junction molecules were demonstrated in the choroid plexus epithelial cells as well as in ependymal cells lining the lateral ventricle. Increased permeability in the ependymal border and the choroid plexus following acute treatment with the phorbolester phorbol-myristate acetate (PMA) is suggested by the findings of an impaired junctional phenotype in these cells [1]. However, the nature of these junctions and their regulation is still not well understood. Recently, a new family of tight junction-associated integral membrane proteins has been identified [2]. These molecules called claudins are currently regarded as most important for junctional tightness [3,4]. Furuse *et al*. [5] have provided new models for the structure of tight junctional strands. They suggested that occludin, the other transmembrane component of the tight junctions, is co-polymerized into the claudin-based strands. Using cultured fibroblasts expressing transfected claudin-1 and freeze-fracture electron microscopy, it was shown that claudin-1 is associated mainly with the P-face [6], whereas claudin-2 and claudin-5 are mainly associated with the E-face [6,7]. The first aim of the present study was to analyze the expression of claudin members in the ventricular ependymal cells and the choroid plexus epithelial cells. The second aim was to analyze the regulation of these junctional proteins, since protein kinase C plays an important role in the regulation of tight junctions [8,9]. We have tested the hypothesis that stimulation of PKC may lead to an altered distribution and/or expression of claudins. To that end, PMA was used since several reports have demonstrated that phorbol esters affect cell-cell contacts via protein kinase C activation [8–12].


Claudin-1
Claudin-2
Claudin-5
Occludin
CP
EP
CC
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)

MATERIALS AND METHODS

Animals: Thirty specific pathogen-free 200 g adult male Sprague–Dawley rats were housed under regular lighting conditions (lights on 06.00–18.00 h) at constant room temperature (23°C) with free access to tap water and standard rat chow (SSNIFF Spezialitäten GmbH). The studies were duly approved; our animal use committee corresponds to the standards of the American Physiological Society.

Immunocytochemistry: The rats were killed by ether anesthesia. The brains were removed and snap-frozen in isopentane at −35°C for immunocytochemistry, which was performed as described previously [1]. Antibodies against claudin-5 [13], claudin-1, and -2 and occludin (Zymed Lab. Inc., San Francisco) were used. Sections were fixed with cold ethanol for 5 min followed by acetone for 1 min, processed for immunocytochemistry, mounted with Aqua Poly/Mount (Polysciences, Inc.) and examined in a Zeiss Axioplan microscope (Zeiss Oberkochen). Controls were performed by omitting the primary antibody.

Microinjections of PMA : The rats were anesthetized with halothane (2% in air) and mounted in a stereotactic frame (Kopf Instruments, USA). A midline incision was made in the skin covering the skull and a 3 mm hole was made using a dental drill (Messner Emtronic, Germany). Microinjections (0.1 µl/2 min) of PMA dissolved in 25% DMSO/0.9% NaCl (1.5 µg) and control injections of 25% DMSO/0.9% NaCl, respectively, were made into the lateral ventricle. The skin was closed with three sutures and the rats were allowed to recover for 24 h [1].

All evaluations were made on coded sections by at least two investigators.

RESULTS

Claudin-1 immunoreactivity: The claudin-1 antibody detected markedly immunoreactive structures between choroid plexus epithelial cells as well as in the cytoplasm of lateral ventricular ependymal cells. Whereas the immunoreactive structures in the choroid plexus epithelial cells were clearly localized at the cellular junctions (Fig. 1a), the immunolabeling of ependymal cells instead appeared in a punctate manner localized in the cytoplasmic compartment (Fig. 1a). Weak immunoreactivity for claudin-1 could be found at the junctions of the endothelial cells of the fenestrated plexus capillaries (Fig. 1b).

Claudin-2 immunoreactivity: The claudin-2 antibody detected markedly immunoreactive structures in choroid plexus epithelial cells (Fig. 1c). At the interfaces of the endothelial cells of the fenestrated plexus capillaries, no claudin-2 immunoreactive material could be found (Fig. 1d).

Claudin-5 immunoreactivity: Weak diffuse claudin-5 immunoreactivity was detected at the interface between the choroid plexus epithelial cells (Fig. 1e). However, no claudin-5 immunoreactive material could be detected between the ependymal cells outlining the lateral ventricle (Fig. 1e). The endothelial cells of plexus capillaries showed strong claudin-5 immunoreactivity, which was restricted to the junctional area (Fig. 1f).

Organization of tight junction proteins in choroid plexus epithelial and endothelial cells: The tight junctions between choroid plexus epithelial cells are formed by the transmembrane molecules claudin-1, claudin-2 and occludin (Fig. 1a,c,g) and the cytoplasmic protein ZO-1 [1]. The anti-claudin-5 immunoreactivity exhibited a diffuse staining pattern around the junctional region in the epithelial cells (Fig. 1e). In contrast, the endothelial cells exhibited a staining pattern of claudin-1/5, occludin (Fig. 1b,f,h) and ZO-1 [1] which was clearly restricted to the junctional region.

PMA treatment: In all PMA-treated brains, the same type of alterations was induced compared to the control brains. Acute PMA-injections led to marked changes in the composition of claudin-1, -2 and -5 in the choroid plexus epithelium. After 24 h, the immunoreactivities of claudin-2 and -5 were strongly decreased in the choroid plexus epithelial cells (Fig. 2c,e), whereas that of claudin-1 was increased compared with its earlier level (Fig. 2a,b). Moreover, claudin-1-immunoreactive material spread to the cytoplasmic compartment of the epithelial cells. Claudin-1 immunoreactivity within the ependymal cells was not influenced by PMA (Fig. 2a). The claudin-1 and -5 immunoreactivities of the tight junctions in endothelial cells of the choroid plexus capillaries were not influenced (data not shown).

DISCUSSION

Tight junction strands are the most apical components of the junctional complex in epithelial and endothelial cells. The epithelial cells of the choroid plexus are specialized compared to other epithelial cells in the body with regard to their function and localization within the brain environment. Plexus epithelial cells as the site of the blood–CSF barrier have been shown to possess tight junctions by means of the freeze-fracture technique [14,15] and by immunocytochemistry [1,16]. The present study adds further information about the tight junctional structure of the choroid plexus epithelial cells. We demonstrate that claudin-1 and -2 are strongly expressed in the choroid plexus epithelial cells and colocalize exactly with the transmembrane tight junction protein occludin and the cytoplasmic protein ZO-1. In contrast, claudin-5 does not appear to be localized exactly within the tight junction, but

Fig. 1. (**a**,**b**) Claudin-1 immunoreactivity in the ventricular system of the rat brain. The pictures demonstrate the staining between the choroid plexus epithelial cells (CP) and the punctate appearance of the claudin-1 immunoreactivity in the ependymal cells (EP) of the lateral ventricle. The choroidal capillaries (cc) exhibit weak claudin-1 immunoreactivity as shown by higher magnification in (b). (**c**,**d**) Claudin-2 immunoreactivity in the ventricular system of the rat brain. The pictures demonstrate the staining between the choroid plexus epithelial cells of the lateral ventricle. The choroidal capillaries (cc) do not have claudin-2 immunoreactivity (d). (**e**,**f**) Claudin-5 immunoreactivity is weak and diffuse in the epithelial cells (EP). In endothelial cells (cc), strong immunoreactivity is found (f). (**g**,**h**) Occludin immunoreactivity is restricted to the junctional area of epithelial as well as of endothelial cells (cc). Bar = 50 µm (a,c,e,g), 25 µm (b,d,f,h).

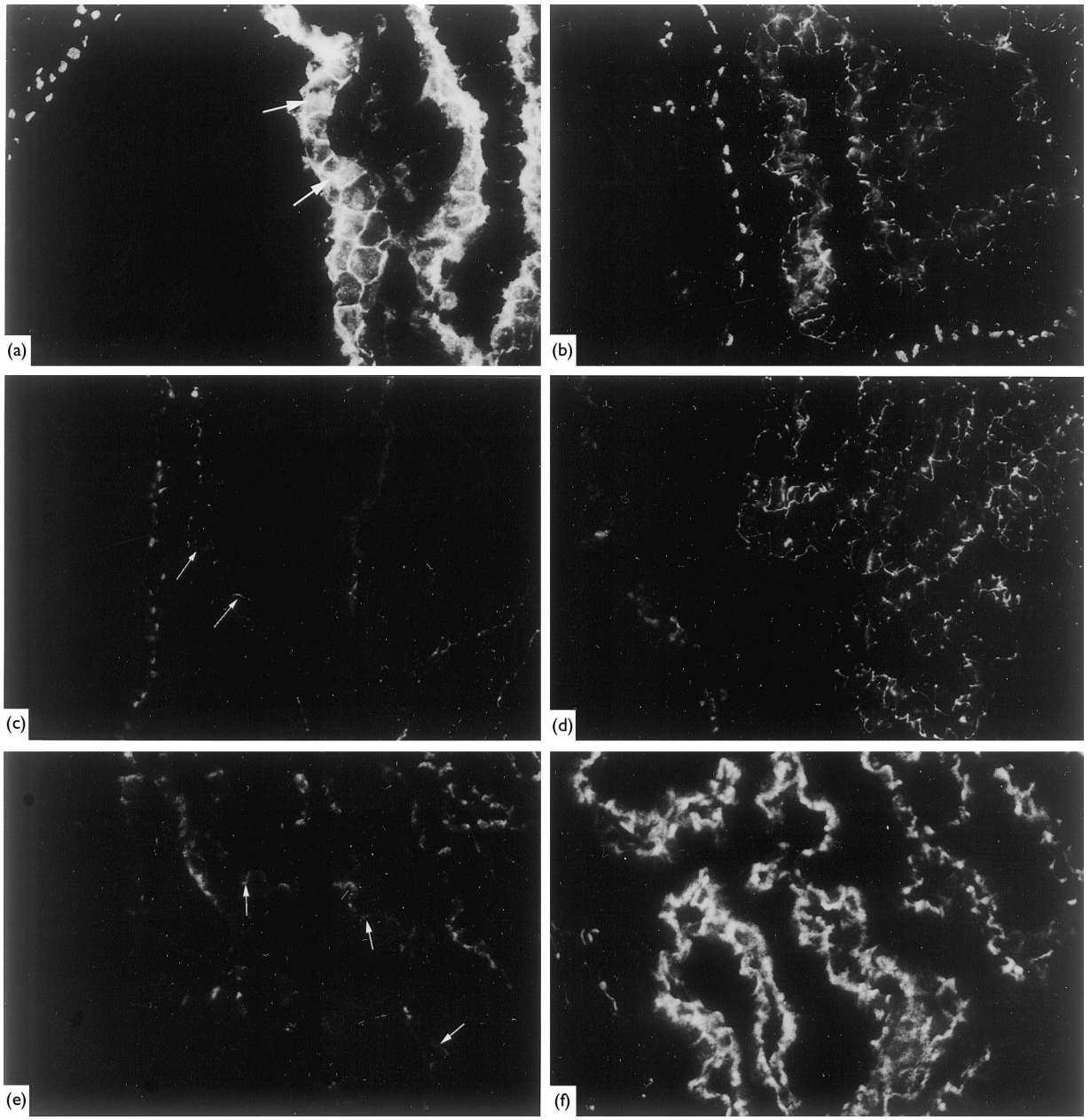


Fig. 2. Acute i.v.t. PMA injections led to an increase in the claudin-1 immunoreactive area and to an altered cellular localization of claudin-1 immunoreactivity in the choroid plexus epithelium. The immunoreactivity is found to be spread into cytoplasmic compartments after PMA treatment (**a**, arrows) compared with the sham-treated animal (**b**). In contrast, claudin-2 (**c**, arrows) and claudin-5 (**e**, arrows) immunoreactivities in the epithelial cells of the choroid plexus decreased markedly after PMA treatment compared with the control animals (**d**,**f**). Magnification ×175.

is rather diffusely distributed in the vicinity of the junctional region.

The ependymal cells establishing the interface between brain and CSF [17,18] do not form any diffusion barrier in mammals. Accordingly, in recent work the ependymal cells were found to express ZO-1 and, weakly, occludin [1]. Claudin-1 was expressed, but not localized at the ependymal junctions. The function of the anti–claudin-1-immunoreactive structures within the cytoplasm is not known. The endothelial cells of the choroid plexus which are known to be fenestrated, were demonstrated to form junctions consisting of ZO-1 [1], occludin, claudin-1 and claudin-5.

The present findings of claudin-1 and -2 immunoreactivities in the choroid plexus epithelial cells give further hints on the nature of the epithelial tight junctions between these specialized cells supporting the function of these molecules for the establishment of the barrier. Transfected fibroblasts expressing claudin-1 have been shown to induce P-face associated tight junction strands which are currently believed to be a characteristic feature of junctional tightness [3,6,11]. Instead, claudin-2, when transfected into fibro-

blasts, induced tight junctions with chains of particles at the E-face and ridges at the P-face with only few particles [6].

In contrast to claudin-1 and -2, claudin-5 was only diffusely distributed in the vicinity of plexus epithelial junctions, but strongly labeled plexus endothelial cell junctions (Fig. 1e,f). Claudin-5 has been described as the endothelial claudin highly associated with the E-face as seen in freeze-fracture experiments [7]. In their recent paper, Furuse *et al.* [5] described models of interaction of heterogenous claudin species in tight junction strands. From earlier studies by Claude [19] it is known that tight junctions do not behave as an absolute seal, but contain aqueous pores that fluctuate between the open and closed state. Indeed, the choroid plexus epithelium also functions as a secretory organ for the CSF. The finding of paracellin-1/claudin-16 as a member of renal tight junctions required for paracellular magnesium resorption gives new aspects to the idea that claudins are involved in the formation of aqueous pores within tight junction strands [20]. Kluge *et al*. [21] found that proteins undergo ultrafiltration via a pattern of tight junction pores with various diameters in choroid plexus epithelium and van Deurs and Koehler [22] described the existence of pores in the barrier-forming tight junctions. Thus, it is tempting to speculate that the tight junctions of the choroid plexus epithelium consist of a mixture of occludin, claudin-1 and -2.

The endothelial cells of the choroid plexus microvessels are known to be fenestrated and highly permeable. At the junctions of these cells, high amounts of claudin-5 were concentrated. Whether the endothelial cell tight junctions are predominantly associated with the E-face, as would be expected from the literature [7], is probable but has not been demonstrated so far.

Phorbol ester treatment led to an increase and redistribution of claudin-1 immunoreactivity and a decrease in claudin-2 and -5 immunoreactivities in choroid plexus epithelial cells. The increased claudin-1 expression can be due to the redistribution and better accessibility of claudin-1 as has been described for occludin [23]. We do not know yet how the increase of claudin-1 and the decrease of claudin-2 fits with the increased leakiness of the epithelial cells suggested to be induced by PMA treatment. The appearance of claudin-1 in the cytoplasm of the epithelial cells could be the result of the dissociation of the protein from the tight junction. However, despite the increased cytoplasmic localization, the immunoreactivity at the junction is also increased. It may be speculated that there exists a junctional complex consisting of occludin and claudin-1/2. From recent investigations [1] it is known that PMA treatment leads to decreased occludin immunoreactivity and thus the predicted junctional complex consisting of occludin and claudin-1/2 may be disturbed. Taken together, these results should be considered in the light of earlier studies in which tight junction permeability in epithelial and endothelial cells could be influenced by phorbol esters *in vitro* [8–12,23–25]. In the present study, we demonstrated that the phorbol ester treatment can regulate the expression and distribution of claudin-1 and -2 as well as of claudin-5. Together with recent findings published elsewhere [1] it therefore seems reasonable that protein kinase C modulation is important for the regulation of junctional proteins *in vivo* and, correspondingly, for that of the tight junction permeability.

REFERENCES

1. Lippoldt A, Jansson A, Kniesel U *et al. Brain Res* **854**, 197–206 (2000).
2. Morita K, Furuse M, Fujimoto K *et al. Proc Natl Acad Sci USA* **96**, 511–516 (1999).
3. Tsukita S and Furuse M. *Trends Cell Biol* **9**, 268–273 (1999).
4. Tsukita S and Furuse M. *Genes Cells* **3**, 569–573 (1998).
5. Furuse M, Sasaki H and Tsukita S. *J Cell Biol* **147**, 891–903 (1999).
6. Furuse M, Sasaki H, Fujimoto K *et al. J Cell Biol* **143**, 391–401 (1998).
7. Morita K, Sasaki H, Furuse M *et al. J Cell Biol* **147**, 185–194 (1999).
8. Izumi Y, Kirose T, Tamai Y *et al. J Cell Biol* **143**, 95–106 (1998).
9. Perez-Moreno M, Avila A, Isles S *et al. J Cell Sci* **111**, 3563–3571 (1998).
10. Citi S and Denisenko N. *Cell Sci* **108**, 2917–2926 (1995).
11. Rubin LL, Hall DE, Porter S *et al. J Cell Biol* **115**, 1725–1736 (1991).
12. Wolburg H, Neuhaus J, Kniesel U *et al. J Cell Sci* **107**, 1347–1357 (1994).
13. Liebner S, Fischmann A, Rascher G *et al. Acta Neuropathol*, in press.
14. Dermietzel R, Meller K, Tetzlaff W *et al. Cell Tissue Res* **181**, 427–441 (1977).
15. Mack A, Neuhaus J and Wolburg H. *Cell Tissue Res* **248**, 619–625 (1987).
16. Gath U, Havoort A, Wegener J *et al. Eur J Cell Biol* **74**, 68–78 (1997).
17. Peters A and Swan RC. *Anat Rec* **194**, 325–345 (1979).
18. Rodriguez P and Bouchaud C. *Neurobiol* **4**, 185–201 (1996).
19. Claude P. *J Membr Biol* **10**, 219–232 (1978).
20. Simon DB, Lu Y, Choate KA *et al. Science* **285**, 103–106 (1999).
21. Kluge H, Hartmann W, Mertins B *et al. J Neurol* **233**, 195–199 (1986).
22. van Deurs B and Koehler JK. *J Cell Biol* **80**, 662–673 (1979).
23. Farshori P and Kachar B. *J Membrane Biol* **170**, 147–156 (1999).
24. Mullin JM, Kampherstein JA, Laughlin KV *et al. Carcinogenesis* **18**, 2339–2345 (1997).
25. Stuart RO and Nigam SK. *Proc Natl Acad Sci USA* **92**, 6072–6076 (1995).