Expression of aquaporin 1 and aquaporin 4 water channels in rat choroid plexus

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Received 19 June 2002; received in revised form 25 October 2002; accepted 5 November 2002

Abstract

The role of aquaporins in cerebrospinal fluid (CSF) secretion was investigated in this study. Western analysis and immunocytochemistry were used to examine the expression of aquaporin 1 (AQP1) and aquaporin 4 (AQP4) in the rat choroid plexus epithelium. Western analyses were performed on a membrane fraction that was enriched in Na+/K+-ATPase and AE2, marker proteins for the apical and basolateral membranes of the choroid plexus epithelium, respectively. The AQP1 antibody detected peptides with molecular masses of 27 and 32 kDa in fourth and lateral ventricle choroid plexus. A single peptide of 29 kDa was identified by the AQP4 antibody in fourth and lateral ventricle choroid plexus. Immunocytochemistry demonstrated that AQP1 is expressed in the apical membrane of both lateral and fourth ventricle choroid plexus epithelial cells. The immunofluorescence signal with the AQP4 antibody was diffusely distributed throughout the cytoplasm, and there was no evidence for AQP4 expression in either the apical or basolateral membrane of the epithelial cells. The data suggest that AQP1 contributes to water transport across the apical membrane of the choroid plexus epithelium during CSF secretion. The route by which water crosses the basolateral membrane, however, remains to be determined.

Keywords: Aquaporin 1; Aquaporin 4; Choroid plexus; Epithelial cell; Cerebrospinal fluid; Secretion

1. Introduction

Choroid plexus epithelial cells are responsible for secreting the majority of cerebrospinal fluid (CSF) into the ventricles of the brain [1,2]. The process of CSF secretion involves the carefully regulated transport of Na⁺, Cl⁻ and HCO₃⁻ from the blood to the ventricles, creating the osmotic gradient that drives the secretion of water. Many of the membrane transport proteins involved in ion transport across the choroid plexus epithelium have now been identified [2]. Less is known, however, about the route by which water is transported into the CSF [2].

In many epithelia, the aquaporins (AQP), a family of water channel proteins, play a major role in water transport [3,4]. Soon after the identification and isolation of aquaporin 1 (AQP1) from erythrocytes, Nielsen et al. [5] demonstrated that AQP1 was expressed in the apical (CSF-facing) membrane of rat fourth ventricle choroid plexus epithelium. The expression of AQP1 in the apical membrane of choroid plexus epithelial cells was also observed by Wu et al. [6] and Masseguin et al. [7]. These data suggest that water transport across the choroid plexus epithelium is transepithelial, with AQP1 contributing to transport from the cell into the CSF across the apical membrane. The route by which water enters the cell at the basolateral (blood-facing) membrane, however, remains to be determined.

Aquaporin 4 (AQP4) is expressed in the basolateral membrane of many epithelia, e.g. kidney collecting duct, colon and trachea [3,8]. AQP4 is also widely expressed throughout the central nervous system [9,10], and is expressed in the basolateral membrane of the ependymal cells that line the ventricles [8]. Thus, AQP4 is a likely candidate for the water transport pathway in the basolateral membrane of the choroid plexus epithelium. There are, however, conflicting reports on AQP4 expression in the choroid plexus. Hasegawa et al. [11] failed to detect AQP4 mRNA using in situ hybridisation in fourth ventricle choroid plexus. Immunocytochemical studies also failed to detect AQP4 in rat choroid plexus [8,12]. By contrast, a recent in situ hybridisation study demonstrated the expres-
sion of mRNA encoding for AQP4 in the epithelial cells of rat lateral ventricle choroid plexus [13].

The aim of the present study was to examine AQP4 expression in fourth and lateral ventricle choroid plexus using Western analysis and immunocytochemistry. To perform the Western analysis, a method was developed for the isolation of a membrane fraction containing both apical and basolateral membrane proteins. The data obtained show that AQP4 is expressed in fourth and lateral ventricle choroid plexus, but does not appear to be expressed in the basolateral membrane of the epithelial cells. A preliminary account of some of these data has been published as an abstract [14].

2. Methods

2.1. Animals and tissue collection

Adult Sprague–Dawley rats were killed by an overdose of halothane (Zeneca Laboratories, UK), and the choroid plexuses removed from the fourth and lateral ventricles of the brain. Tissue samples were then either: (i) snap-frozen in liquid N\textsubscript{2} for use in Western analysis, or (ii) placed in fixative for 30 min for immunofluorescence studies. Brain and kidney tissue were snap-frozen for use as positive controls in Western analyses.

2.2. Preparation of plasma membrane protein samples

Plasma membrane protein samples were prepared from choroid plexus tissue using a protocol modified from Thévenod et al. [15]. Pooled tissue samples were homogenised in homogenisation buffer (280 mM mannitol, 10 mM HEPES, 10 mM KCl, 1 mM MgCl\textsubscript{2}; pH 7.0 with Tris) supplemented with 1% (v/v) protease inhibitor cocktail (Sigma). Homogenisation was performed at 4 \textdegree C using a motor-driven glass-in-Teflon homogeniser. The homogenate was spun at 2500 \times g for 3 min in a microcentrifuge to remove unlysed cells, nuclear material and other cell debris. The supernatant was collected and the pellets were re-homogenised and re-centrifuged as above. This process was repeated a total of four times, and the supernatants were combined to give a post-nuclear supernatant (PNS). The PNS was then spun at 11,000 \times g for 20 min at 4 \textdegree C. The pellet (P1), and a sample of the supernatant (S1) were saved for analysis. The remainder of S1 was then spun at 150,000 \times g for 1.5 h at 4 \textdegree C to yield a plasma membrane pellet (P2). The supernatant (S2) was saved for analysis, whilst P2 was resuspended in a minimal volume of homogenisation buffer and stored at –80 \textdegree C until required. The protein content of each fraction was determined by the Bradford method [16], using bovine serum albumin (BSA; Sigma) as the standard. Western analyses for the \(\alpha\)-subunit of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (an apical membrane marker [17]), and the anion exchanger AE2 (a basolateral membrane marker [18,19]) were performed on each fraction.

2.3. Western analysis

Protein samples were solubilised in 5 × Laemmli sample buffer (0.32 M Tris (pH 6.8), 5% (w/v) sodium dodecyl sulfate, 25% (v/v) glycerol, 1% (w/v) bromphenol blue, 5% (v/v) 2-mercaptoethanol) and incubated at 95 \textdegree C for 4 min. SDS-PAGE was used to separate proteins, on either 7.5% (AE2), 9% (Na\textsuperscript{+}/K\textsuperscript{+}-ATPase) or 10% (AQP1 and AQP4) gels. The quantity of protein loaded onto the gels varied according to the primary antibody used (see figure legends). Proteins were transferred to polyvinylidene difluoride membrane (0.2 \mu m; Bio-Rad Laboratories). After blocking with Tris-buffered saline-Tween (TBST) containing 0.1% (v/v) Tween-20 and 3% (w/v) milk for 1 h at room temperature, the membranes were incubated overnight at 4 \textdegree C with one of the following primary antibodies: (i) a mouse monoclonal to the \(\alpha\)-subunit of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase (1:10,000; Upstate Biotech, USA); (ii) an affinity purified rabbit polyclonal to the COOH-terminal of mouse AE2 (1:10,000; [20]); (iii) an affinity purified rabbit polyclonal antibody against the COOH-terminal of rat AQP1 (1:750; Alpha Diagnostics International, USA); or (iv) an affinity purified rabbit polyclonal antibody against rat AQP4 (1:250; Alpha Diagnostics International). The membranes were washed in several changes of TBST and subsequently incubated for 1 h at room temperature with the secondary antibody (conjugated to horseradish peroxidase). A rabbit anti-mouse IgG was used with the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase primary antibody (1:10,000; Jackson Immunoresearch Laboratories, UK), and a donkey anti-rabbit IgG for AE2, AQP1 and AQP4 (1:10,000; Amersham Pharmacia Biotech, UK). Additional washing steps in TBST were followed by antibody detection using ECL-Plus chemiluminescence (Amersham Pharmacia Biotech) according to the manufacturer’s protocol. Signals were visualised on X-ray films. Possible non-specific binding of the AE2, AQP1 and AQP4 primary antibodies was assessed by preadsorbing the antibody for 1 h with an excess of the respective antigen, i.e. 20:1 for AE2 and 2:1 for AQP1 and AQP4. A crude homogenate of rat kidney and a membrane fraction from rat brain (see Alomone Labs Protocol, http://www.alomone.com) were used as positive controls, in the Western analyses for AQP1 and AQP4, respectively. All experiments were repeated in triplicate.

2.4. Immunofluorescence

Dissected choroid plexus tissue was fixed for 30 min in either (i) 4% (w/v) paraformaldehyde-PBS or (ii) PLP-fixative (2% paraformaldehyde, 75 mM lysine, 10 mM sodium periodate, 37.5 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 6.2 [21]). Sections were subsequently cryoprotected in 30% (w/v) sucrose for a further 30 min. The tissue was embedded in optimum cutting texture compound (R.A. Lamb Ltd., UK) and snap-frozen in N-methylbutane cooled on dry ice. Serial 4 \mu m frozen sections were cut using a Leica CM3050 cryostat.
Leica Instruments, Germany) and thaw-mounted onto gelatin-coated glass slides. In one set of control experiments, AQP4 expression was assessed in sections (4 \mu m, fixed in 4\% paraformaldehyde-PBS) of rat kidney medulla, which were provided by Dr. Cally Ferguson (University of Manchester).

For localisation of AQP1 and AQP4, paraformaldehyde-fixed sections were rehydrated in PBS for 5 min, and then blocked with PBS containing 5\% (w/v) normal goat serum and 1\% (w/v) BSA for 30 min. The blocking solution was removed and replaced with either AQP1 (1:500) or AQP4 (1:500) diluted in the blocking solution, and the slides were then incubated overnight at 4 \degree C. The slides were washed twice in high-salt PBS (2.8\% (w/v) NaCl), followed by two washes with PBS and subsequently incubated with a goat anti-rabbit secondary antibody conjugated to Cy3 (1:400; Jackson Immunoresearch Laboratories) for 1 h at room temperature in the dark. The secondary antibody was diluted in PBS containing 5\% normal goat serum and 1\% BSA. Sections were then washed twice for 5 min in high-salt PBS followed by two washes in PBS, and mounted using Vectashield (Vector Laboratories Ltd., UK).

The AE2 antibody was used to demonstrate protein localisation at the basolateral membrane. Following rehydration of PLP-fixed sections in PBS, sections were treated with 1\% SDS in PBS for 5 min. After washing with PBS, sections were blocked with 1\% BSA in PBS and then incubated overnight at 4 \degree C with AE2 (1:1,000) diluted in PBS. The slides were washed three times in PBS and next incubated with goat anti-rabbit secondary antibody conjugated to fluorescent Cy3 (1:400) for 1 h at room temperature in the dark.

Immunofluorescence was visualised using a Zeiss Axioplan 2 microscope. Images were acquired using a Hamamatsu digital camera and processed using a KS300 software package (Version 3.0; Carl Zeiss, Germany). Non-specific immunoreactivity was assessed by preadsorbing the primary antibody with an excess of the peptide antigen (\pm Ag).

Fig. 1. Immunoblot analysis of fourth ventricle choroid plexus protein fractions for: (A) Na\(^+/\)K\(^-\)-ATPase or (B) AE2. Protein fractions (A, 0.25 \mu g; B, 10 \mu g) from each step of the plasma membrane preparation protocol (PNS, post-nuclear supernatant; S1, 11,000 \times g supernatant; P1, 11,000 \times g pellet; S2, 150,000 \times g supernatant; and P2, 150,000 \times g pellet), were separated by SDS-PAGE. Immunoblotting was then performed with either: (A) the anti-\alpha1-subunit of Na\(^+/\)K\(^-\)-ATPase (0.1 \mu g/ml); or (B) the anti-AE2 antibody (1:10,000). The specificity of the AE2 antibody binding was assessed by preadsorbing the antibody with an excess of antigen (\pm Ag). The positions of molecular mass markers are shown on the left.

Fig. 2. AQP1 and AQP4 immunoreactivity in plasma membrane fractions prepared from choroid plexus tissue. Plasma membrane samples (20 \mu g) were separated by SDS-PAGE on a 10\% acrylamide gel. (A) AQP1 antiserum identified immunoreactive bands in kidney homogenate (K), fourth (IV) and lateral (L) ventricle choroid plexus plasma membrane fractions. Antibody specificity was determined by incubation of the fourth ventricle sample with the antibody preadsorbed with the antigen (\pm Ag). The positions of the molecular mass markers are shown on the left.
3. Results

3.1. Plasma membrane preparation for western analysis

A method was developed for the isolation of a membrane fraction from the choroid plexus epithelium. The protein content of each stage of the preparation (PNS, S1, P1, S2 and P2) was investigated by western analysis. Fig. 1A shows that the Na⁺/K⁺-ATPase antibody detected two bands of between 90 and 105 kDa, in all samples from fourth ventricle choroid plexus tissue with the exception of S2. The expected molecular mass of the α₁ subunit in the choroid plexus is about 104 kDa [22]. The intensity of the bands appeared to be greatest in P2 suggesting that this fraction is enriched in apical

Fig. 3. Immunocytochemical localisation of AQP1 and AQP4 in rat choroid plexus. (A) AQP1 is expressed in the apical membrane of: (i) fourth and (ii) lateral ventricle choroid plexus (arrows indicate the apical membrane); (iii) immunofluorescence from fourth ventricle choroid plexus with AQP1 antibody preadsorbed with an excess of antigen. (B) AQP4 is diffusely expressed in choroid plexus epithelium cells from the (i) fourth and (ii) lateral ventricles; (iii) fourth ventricle choroid plexus with the preadsorbed antibody. (C) (i) Discrete staining of the basolateral membrane of rat kidney collecting duct cells with the AQP4 antibody; (ii) AE2 immunofluorescence of the basolateral membrane (arrows) of fourth ventricle choroid plexus; (iii) AE2 immunofluorescence from fourth ventricle choroid plexus with the preadsorbed antibody. In each panel the bar = 20 µm.
membrane proteins. Similar data were obtained with lateral ventricle choroid plexus (data not shown).

Fig. 1B shows a western blot of protein samples prepared from fourth ventricle choroid plexus tissue, probed with the AE2 antibody. This antibody recognised major bands at \( \sim 70, \sim 95, 140 \) and \( \sim 165 \) kDa, in the PNS, S1 and P2 fractions. Similar results were obtained with the lateral ventricle choroid plexus tissue (data not shown). All of these bands were abolished when the antibody was preadsorbed with the peptide antigen (Fig. 1B; + Ag). The expected molecular mass for AE2 is 165 kDa, however, the detection of multiple bands by this AE2 antibody has previously been reported in salivary gland cells [23]. The strongest staining is seen in P2, suggesting that this fraction is also enriched for basolateral membrane proteins from the choroid plexus epithelium.

3.2. Expression of AQP1 and AQP4 by western analysis

Antibodies raised against the COOH-terminus of rat AQP1 and AQP4 were used to determine the expression of these proteins in choroid plexus plasma membrane fractions. The anti-AQP1 identified two bands, of approximately 27 and 32 kDa, in fourth (IV) and lateral (L) ventricle choroid plexus (Fig. 2A). Bands of similar molecular masses were also observed in the kidney homogenate sample (K), which was included as a positive control. Neither band was detected in the fourth ventricle (Fig. 2A; + Ag) or kidney sample (data not shown), when the primary antibody had been preadsorbed with an excess of the AQP1 antigen. Fig. 2B shows that the AQP4 antibody detected a single protein band of 29 kDa, in the fourth (IV) and lateral (L) ventricle choroid plexuses. A band with a similar molecular mass was also observed in brain (B), the positive control tissue. The 29 kDa band was not observed in the fourth ventricle (Fig. 2B; + Ag) or brain sample (data not shown), if the antiserum had been preadsorbed with an excess of the immunizing peptide.

3.3. Localisation of AQP1 and AQP4 by immunofluorescence

Immunocytochemical methods were used to determine the localisation of AQP1 and AQP4 within the choroidal epithelium. In Fig. 3A, sections of (i) fourth and (ii) lateral ventricle choroid plexus have been probed with anti-AQP1. Distinct fluorescence was observed in the apical membrane of the epithelium in both tissues (arrows). AQP1 did not appear to be expressed in the basolateral membrane of the epithelium, or elsewhere in the choroid plexus. Fig. 3A (iii) shows that when the primary antibody was preadsorbed with an excess of the antigen, there was very little non-specific binding of either the primary or secondary antibody in the fourth ventricle choroid plexus sections (similar data were obtained with the lateral ventricle, data not shown).

Fig. 3B shows AQP4 expression in (i) fourth and (ii) lateral ventricle choroid plexus epithelium. The immunofluorescence was diffusely distributed throughout the epithelial cells in both tissues. This diffuse fluorescence was not a result of non-specific binding of the primary antibody, because it was not observed when the primary antibody was preadsorbed with an excess of antigen in the fourth ventricle (Fig. 3B (iii)) or lateral ventricle (data not shown). Fig. 3C (i) shows sections of rat kidney medulla probed with the AQP4 antibody. A distinct pattern of fluorescence is observed in the basolateral membrane of the collecting duct epithelium. The AE2 antibody was used in further control experiments to demonstrate the position of the basolateral membrane in choroid plexus sections. Fig. 3C (ii) shows the AE2 localisation in the basolateral membrane (arrows) of fourth ventricle choroid plexus. No significant fluorescence was observed in sections incubated with peptide-adsorbed antibody (Fig. 3C (iii)). Similar patterns of AE2 expression were observed in the lateral ventricle choroid plexus (data not shown).

4. Discussion

In this study, we tested the hypothesis that AQP4 is expressed in the basolateral membrane of rat choroid plexus. AQP4 expression was investigated initially using western analysis, and it was necessary to ensure that the protein sample under examination contained basolateral membrane proteins. Experiments were therefore performed to show that the membrane fraction used in the western analysis, contained marker enzymes for both the apical membrane (Na⁺/K⁺-ATPase) and basolateral membrane (AE2). Both proteins were detected in the P2 membrane fraction (Fig. 1), and there appeared to be an enrichment of Na⁺/K⁺-ATPase and AE2 in this fraction compared to the PNS (Fig. 1). Thus, the membrane fraction contains both apical and basolateral membrane proteins from the choroid plexus epithelium. In previous studies of choroid plexus, western analysis has only been used to study the expression of apical membrane proteins [5,24–26]. Thus, the identification of basolateral membrane proteins by western analysis is a useful development in the study of choroid plexus protein expression.

AQP1 was detected by western analysis of the P2 fraction. Two peptides of \( \sim 27 \) and \( \sim 32 \) kDa were identified, and these molecular masses correspond with those of the unglycosylated and glycosylated form of the AQP1 protein [5]. Using immunocytochemical methods, AQP1 was found to be expressed almost exclusively in the apical membrane of the fourth ventricle and lateral ventricle choroid plexus (Fig. 3A). These data therefore confirm those of previous studies of AQP1 expression [5–7].

A single protein band was identified in the western blots for AQP4 in fourth ventricle choroid plexus, lateral ventricle choroid plexus and brain (Fig. 2B). These bands were not
detected when the primary antibody had been preadsorbed with an excess of antigen, indicating that the antibody was interacting specifically with the protein. Furthermore, the molecular mass of the protein detected by the antibody (29 kDa), was similar to that previously reported for AQP4 [8,27,28]. No bands were observed between 30 and 35 kDa in the present study, which is in contrast to some [12,28,29], but not all [8,11,27], previous studies of AQP4 expression.

The western analysis data therefore suggest that AQP4 protein is expressed in choroid plexus tissue from the fourth and lateral ventricle.

Immunocytochemical methods were employed to examine the localisation of AQP4 within the choroid plexus. Diffuse immunofluorescence was detected in the epithelial cells, which was not associated specifically with either the apical or basolateral membranes (Fig. 3B). This pattern of fluorescence was clearly very different to that for AQP1, strongly suggesting that AQP4 is not expressed in the apical membrane. However, it was possible that the diffuse staining could be associated with protein expression in the basolateral membrane, which in the choroid plexus is less well defined than the apical membrane [1]. Immunocytochemistry was therefore used to examine the expression of AE2 (the basolateral membrane marker) in the choroid plexus epithelium. Fig. 3C (ii) shows that both the lateral and basal parts of the basolateral membranes were clearly labelled with the AE2 antibody. Similar patterns of staining have previously been reported for other basolateral transporters, e.g. KCC3 [30]. Other controls were performed to demonstrate that the lack of membrane labelling was not due to inadequacies of the AQP4 antibody used in the present study, i.e. specific labelling of the basolateral membrane of the kidney collecting duct was observed (Fig. 3C (i)), as previously reported [29]. These data suggest that AQP4 is neither expressed in the apical membrane nor the basolateral membrane of choroid plexus epithelial cells.

The diffuse distribution of AQP4 in the choroid plexus cells indicates the possible expression of the protein in the membranes of intracellular organelles. If expressed in organelles, AQP4 could still have been detected in the western blot, if the membrane fraction contains organelar membranes in addition to the apical and basolateral membranes. Little is known about AQP4 expression in intracellular organelles. Nielsen et al. [12], however, did observe AQP4 expression in a membrane fraction prepared from rat cerebellum which was enriched for intracellular organelles. By contrast, AQP6 and AQP8 are known to be expressed in the intracellular organelles of kidney tubule cells [3], and the epithelial cells of the kidney and G.I. tract [31], respectively. AQP6 has been suggested to have a role in the acidification of intracellular vesicles in the kidney, a function that is related more to the permeability of AQP6 to anions than to water [3]. By contrast, AQP8 has been suggested to ensure the rapid osmoequilibration between cytoplasmic and vesicular compartments [31]. A similar role can be envisaged for AQP4 in the choroid plexus epithelial cells.

The demonstration that neither AQP1 nor AQP4 is expressed in the basolateral membrane means that the route by which water crosses this membrane remains to be determined. It is possible that aquaporins other than AQP1 or AQP4 contribute, however, to date there are no reports of the expression of other aquaporins in the choroid plexus. Another possibility is that water crosses the basolateral membrane coupled to the transport of ions or nutrients. Recent work has shown that a variety of solute transporters may mediate water transport [32]. Several of the original observations in support of this hypothesis were made by Zeuthen [33] who reported water transport via K⁺/Cl⁻ cotransporters in the apical membrane of amphibian choroid plexus. These data have not been confirmed for mammalian choroid plexus, but it is interesting to note that KCC3 (the only K⁺/Cl⁻ transporter identified so far in mammalian choroid plexus) is expressed in the basolateral membrane [30].

In conclusion, AQP1 and AQP4 were identified in western analysis of rat choroid plexus. AQP1 was expressed exclusively in the apical membrane, whilst AQP4 was expressed throughout the cytoplasm of the choroid plexus epithelial cells. The route by which water crosses the basolateral membrane during CSF secretion therefore remains unknown.

Acknowledgements

We thank Seth Alper for the gift of AE2 antibody, and Dr. Frank Thevenod for his advice on the preparation of plasma membrane protein fractions. The work was supported by The Wellcome Trust (Grant 055111/Z/98).

References