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Research article

Trafficking and phosphorylation dynamics of AQP4 in histamine-treated human gastric cells

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Background information. AQP4 (aquaporin 4) internalization and a concomitant decrease in the osmotic water permeability coefficient (P_f) after histamine exposure has been reported in AQP4-transfected gastric HGT1 cells.

Results. In the present study we report that AQP4 internalization is followed by an increase in AQP4 phosphorylation. Histamine treatment for 30 min resulted in an approx. 10-fold increase in AQP4 phosphorylation that was inhibited by 1 μM H89, a specific PKA (protein kinase A) inhibitor, but not by PKC (protein kinase C) and CK2 inhibitors. Moreover, measurement of PKA activity after 30 min of histamine treatment showed that PKA activity was approx. 3-fold higher compared with basal conditions. AQP4 phosphorylation was prevented in cells treated with histamine for 30 min after pre-incubation with PAO (phenylarsine oxide), an inhibitor of protein endocytosis. Using an endo–exocytosis assay we showed that, after histamine washed out, internalized AQP4 recycled back to the cell surface, even in cells in which *de novo* protein synthesis was inhibited by cycloheximide.

Conclusions. Phosphorylation experiments, combined with immunolocalization studies, indicated that AQP4 phosphorylation is mediated by PKA and occurs subsequently to its internalization in late endosomes. We suggest that phosphorylation might be a mechanism involved in retaining AQP4 in a vesicle-recycling compartment.

Introduction

AQPs (aquaporins) are water channel proteins that play a central role in the cellular handling of water in various mammalian tissues. At least 13 AQPs, numbered 0 to 12, have been identified in various mammalian tissues. AQP3, AQP7, AQP9 and AQP10 have been shown to transport non-ionic small solutes, such as urea and glycerol, in addition to water, whereas AQP1, AQP2, AQP4, AQP5 and AQP8 are highly selective to water and exclude small solutes (reviewed in Agre et al., 2002; Verkman, 2005). AQP6 has been identified as an intracellular vesicle water channel with anion permeability that is activated by low pH or $HgCl_2$ (Hazama et al., 2002). AQP11 has been localized intracellularly in the proximal tubule, and AQP11-null mice exhibited vacuolization and cyst formation in the proximal tubule, demonstrating that AQP11 is essential for the function of this nephron segment (Morishita et al., 2005).

The physiological significance of membrane channels and transporters lies in their capacity to be regulated. It has been recognized that hormones

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Key words: aquaporin, gastric cell, histamine, phosphorylation, protein kinase A, protein trafficking.

Abbreviations used: AQP, aquaporin; CaMKII, calmodulin-dependent protein kinase II; DRB, 5,6-dichlorobenzimidazole 1-β-D-ribofuranoside; MPR, mannose-6-phosphate receptor; NHE3, Na^+/H^+ exchanger isoform 3; NHS, *N*-hydroxysuccinimide; OAP, orthogonal array of particles; PAO, phenylarsine oxide; PKA, protein kinase A; PKC, protein kinase C; VSV, vesicular-stomatitis virus.

such as vasopressin (Bichet, 1996; Christensen et al., 1998) secretin (Tietz et al., 2003), oestrogen (Oliveira et al., 2005), glucagon (Gradilone et al., 2003, 2005) and hormone peptide YY (Carmosino et al., 2005) regulate the expression and intracellular trafficking of certain aquaporins.

It is known that during signal transduction, the information is transferred on to a protein mainly by phosphorylation, since this is a fast, simple and reversible modification of the protein that can cause a change in its activity and/or localization. The intracellular trafficking and membrane targeting of several water channels, including AQP1 (Han and Patil, 2000), AQP2 (reviewed in Noda and Sasaki, 2005; Valenti et al., 2005) and AQP5 (Parvin et al., 2005), have been shown to be regulated by protein phosphorylation.

The AQP4 water channel is specifically targeted to the basolateral membrane in various epithelial cells, including kidney collecting-duct principal cells and gastric parietal cells (Frigeri et al., 1995; Deen and van Os, 1998; van Hoek et al., 2000).

Gastric parietal cells actively secrete HCl into the stomach. As secreted gastric juice is isotonic with plasma fluid, HCl secretion should be associated with water transport. AQP4 expression in the basolateral membrane of parietal cells suggests that AQP4 may facilitate the water fluxes accompanying gastric secretion. However, in AQP4 knock-out mice, AQP4 deletion was not associated with changes in the rates of basal or stimulated acid or fluid secretion (Wang et al., 2000a). These data do not rule out the possibility that other compensatory mechanisms might be responsible for the absence of obvious defects in overall gastric acid production in AQP4 knock-out mice. To study the possible role of AQP4 in gastric acid secretion, we stably transfected the human gastric cell line HGT-1 (Laboisse et al., 1982), which expresses functional histamine H_2 receptors involved in the stimulation of acid secretion, with the rat AQP4 water channel (Carmosino et al., 2001). Using several experimental approaches, including cell surface biotinylation, osmotic water transport measurements and freeze–fracture analysis, we demonstrated that AQP4 is internalized after relatively long histamine exposure (20 min) in AQP4-transfected gastric HGT-1 cells. These data suggest that the parallel decrease in histamine response and cell surface AQP4 expression are two events involved in the down-regulation of gastric acid secretion. Following these observations, we therefore have investigated if AQP4 phosphorylation is modulated during this process in the present study.

Previous reports indicated that AQP4 can be phosphorylated by PKC (protein kinase C) (Han et al., 1998; Zelenina et al., 2002, Hoffert et al., 2006), protein kinase CK2 (Madrid et al., 2001) and by CaMKII (calmodulin-dependent protein kinase II) (Gunnarson et al., 2005).

In the present work we investigated whether AQP4 phosphorylation occurs during histamine treatment of AQP4-expressing gastric cells. We have provided strong evidence showing that short-term phosphorylation of AQP4 occurs after 30 min of histamine exposure. The possible role of PKA (protein kinase A)-mediated AQP4 phosphorylation in retaining AQP4 in a vesicle-recycling compartment, and thus regulating AQP4 surface expression, is discussed.

Results

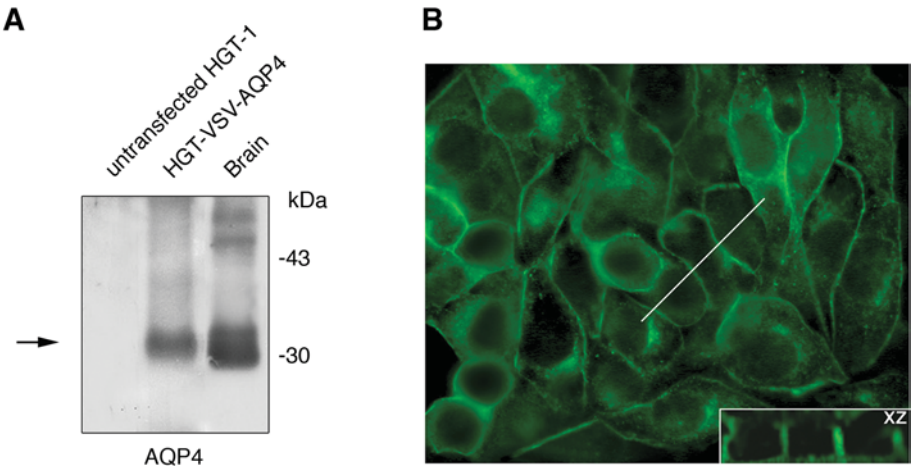
Internalized AQP4 is recycled back to the plasma membrane after histamine washout

We have previously shown that AQP4 is internalized after a long exposure to histamine in AQP4-transfected gastric HGT-1 cells (Carmosino et al., 2001). Based on these results, in the present study we have analysed the possible intracellular signals mediating this effect. As phosphorylation is often a key signal triggering internalization of cell surface receptors and integral proteins, this possibility was investigated first. To this end, we generated a clonal cell line stably transfected with VSV (vesicular-stomatitis virus)-tagged M1 AQP4 isoform to immunoprecipitate AQP4 using anti-VSV antibodies. This was done because some of the putative consensus sequences for AQP4 phosphorylation are located at the C-terminus, therefore the available anti-(C-terminus AQP4) antibodies would not recognize phosphorylated AQP4 at these sites.

Western blotting experiments showed that anti-AQP4 antibody stained a single band with the expected molecular mass for the VSV-tagged AQP4 (34 kDa) in HGT-VSV-AQP4 cells. Due to the VSV tag, the molecular mass of AQP4 is slightly higher than that of AQP4 expressed in the rat brain

Figure 1 | Characterization of VSV-tagged AQP4-transfected gastric cells

(**A**) Western blotting of cell homogenates probed with anti-AQP4 antibody. (**B**) Confocal image of HGT-VSV-AQP4 cells. Analysis (inset) of an *x–z* reconstruction (indicated by the white line) confirmed that VSV-tagged AQP4 was localized on the basolateral membrane domain.



membranes used as positive control. No proteins were detected in untransfected HGT-1 cells (Figure 1A).

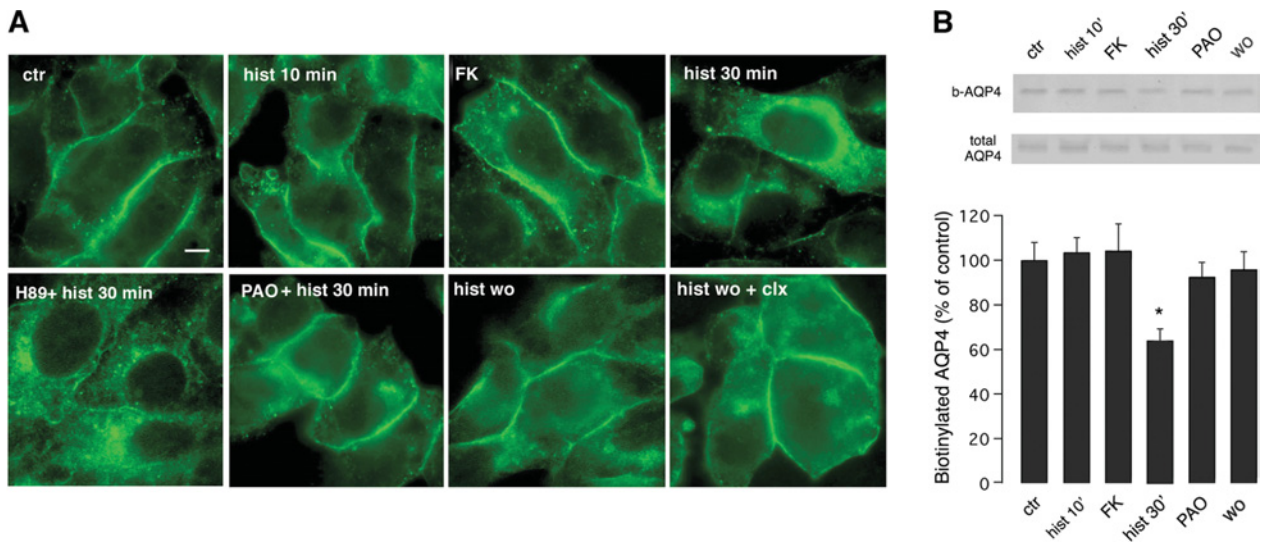
Confocal analysis of HGT-VSV-AQP4 cells was carried out using a polyclonal antibody against a peptide corresponding to the C-terminus of rat AQP4 (Valenti et al., 1996) to verify whether the VSV epitope tag at the N-terminus of the protein alters AQP4 processing and distribution. As shown in Figures 1(B) and 1(B, inset), VSV-tagged AQP4 was localized on the basolateral membrane domain displaying a distribution similar to that observed in native parietal cells (Carmosino et al., 2001).

AQP4 localization was analysed in HGT-VSV-AQP4 cells stimulated with histamine (500 μM) or with forskolin (100 μM). Histamine or forskolin stimulation (10 min) did not modify the basolateral localization of AQP4 (Figure 2A; hist 10 min, FK). Stimulation with forskolin for 30 min did not change the basolateral expression of AQP4 (data not shown). In contrast, a considerable decrease in cell surface AQP4 expression with a parallel increase in intracellular staining was observed in cells exposed to histamine for 30 min (Figure 2A; hist 30 min). Interestingly, in cells treated with histamine for 30 min in the presence of H89, a specific PKA inhibitor, AQP4 was found in intracellular vesicles, indicating that internalization is a PKA-independent process (Figure 2A; H89 + hist 30 min). Treatment with H89 alone did not alter the basolateral localization of AQP4 (data not shown). AQP4 internalization after 30 min of histamine stimulation was prevented by pre-incubation with 40 μM PAO (phenyl arsine oxide), an inhibitor of protein internalization including cell surface receptors (Gibson et al., 1989) (Figure 2A; PAO + hist 30 min). After 1 h of histamine washout, AQP4 relocated to the basolateral membrane (Figure 2A; hist wo). The same result was obtained when the histamine washout was carried out in the presence of cycloheximide (1 μg/ml), an inhibitor of protein synthesis, indicating that AQP4 can be recycled in the absence of *de novo* protein synthesis (Figure 2A; hist wo + clx). AQP4 distribution in the same experimental conditions described was analysed by cell surface biotinylation. Obtained results confirmed that after 30 min of histamine treatment, the AQP4 expression on the cell surface decreased significantly by $34.3 \pm 5.1\%$ (Figure 2B).

AQP4 endocytosis and recycling was evaluated by AQP4 endo–exocytosis assay. The experimental methods are shown in Figure 3(A) and relative results in Figure 3(B). Confluent monolayers were incubated with the cleavable sulfo-NHS (*N*-hydroxysuccinimide)-SS–biotin. Cell surface NHS-SS–biotin was cleaved in the presence of the membrane-impermeant reducing agent glutathione, which allowed endocytosed AQP4 to be detected and semi-quantified by

Figure 2 | Effect of histamine and forskolin on AQP4 localization

(**A**) Immunofluorescence localization of AQP4 in HGT-VSV-AQP4 cells treated with forskolin (FK), histamine (hist) in the presence of H89, PAO and cycloheximide (clx) for the times indicated. Scale bar, 5 μm. (**B**) Representative experiment of cell surface biotinylation. Band intensities of biotinylated AQP4 were semi-quantified by densitometric analysis and are shown as a percentage of the intensity of the band detected in the control sample. Western blot analysis (total AQP4) of equal amounts of the solubilized starting material (cell lysate after biotinylation) from each condition confirmed the presence of comparable amounts of AQP4 in all samples.



its resistance to cleavage under this condition. Using this biochemical assay, AQP4 recycling in different experimental conditions can be quantitatively evaluated. Under basal conditions, a certain amount of biotinylated AQP4 (corresponding to endocytosed AQP4 at rest) can be precipitated from cell lysates, indicating that a constitutive AQP4 endocytosis occurs in the absence of an acute stimulus (Figure 3B; CTR). In contrast, the amount of endocytosed AQP4 increased significantly by $52 \pm 8.2\%$ after 30 min of 500 μM histamine stimulation (Figure 3B; HIST). These data are in agreement with the immunofluorescence results reported in Figure 2(A). Histamine washout is expected to allow internalized biotintylated AQP4 to recycle back to the cell surface, making AQP4 sensitive to glutathione cleavage. Therefore, after a 30 min stimulation, histamine was washed out for 1 h and cells were treated with glutathione followed by AQP4 precipitation.

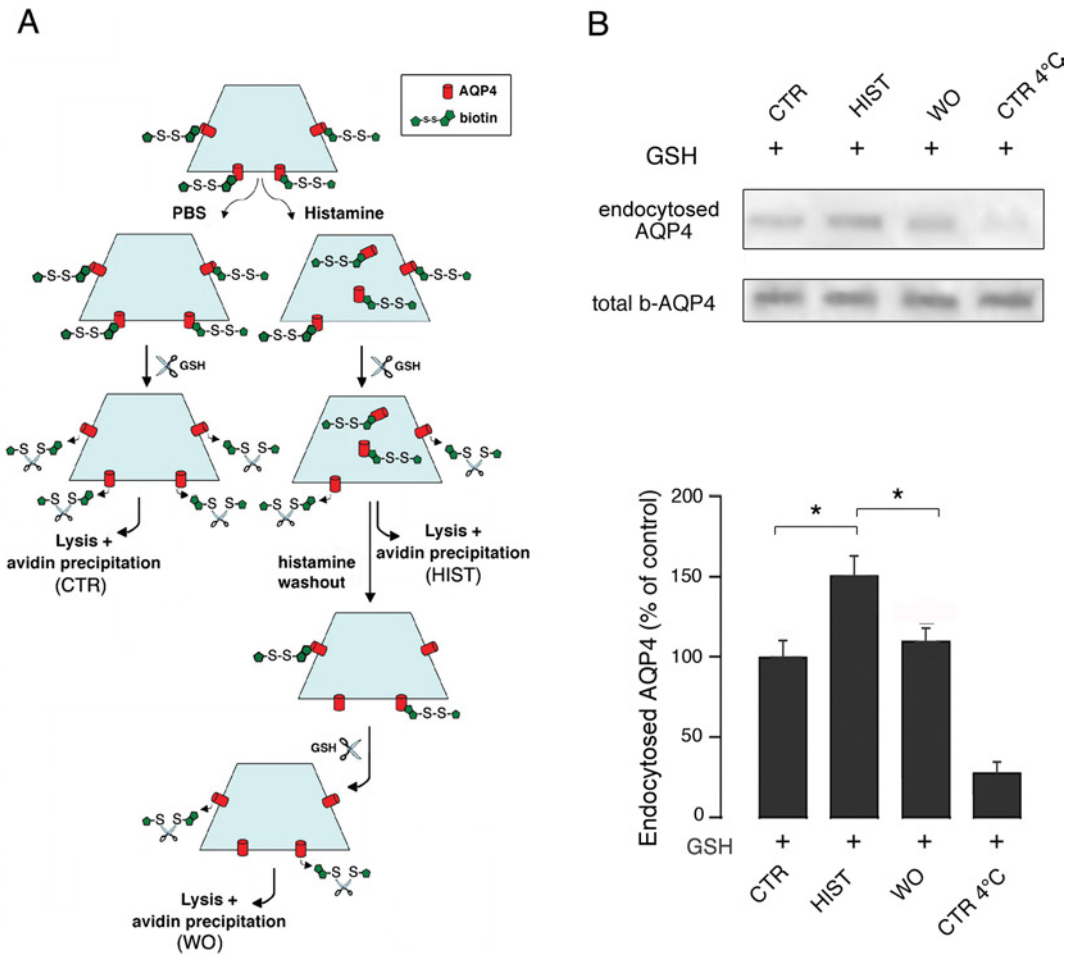
Under these conditions, a significant decrease in AQP4 signal by $42 \pm 6.8\%$ was observed, compared with histamine-treated cells, indicating that internalized AQP4 was recycled back to the plasma membrane after histamine washout (Figure 3B; WO + GSH). In parallel, two controls were performed: (a) after incubation with sulfo-NHS-SS–biotin, cells were kept at 4°C to block basal endocytosis and then treated with glutathione to verify the biotin cleavage (Figure 3B; CTR 4°C + GSH); (b) incubation with sulfo-NHS-SS–biotin not followed by stripping for normalization with total biotinylated AQP4 (Figure 3B; total b-AQP4). The intensity of bands for total biotinylated AQP4 were comparable in all experimental conditions tested, indicating that AQP4 does not enter the degradative pathway.

Histamine-induced AQP4 internalization is associated with an increase in AQP4 phosphorylation

To investigate the phosphorylation dynamics of AQP4 during histamine treatment, AQP4 was immunoprecipitated using the anti-VSV tag antibody from confluent monolayers of HGT-VSV-AQP4 cells metabolically labelled with $[^{32}P]P_i$. In basal conditions, AQP4 is weakly phosphorylated (Figures 4A and 4B; Ctr). Compared with the control, histamine

Figure 3 | Endo–exocytosis assay

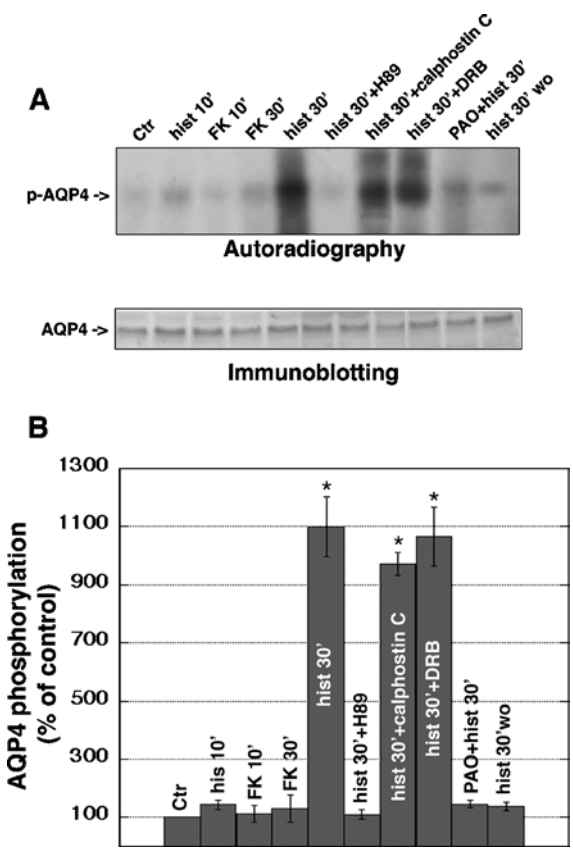
(**A**) Principal steps of the endo–exocytosis assay performed in HGT-VSV-AQP4 cells using the NHS-SS–biotin surface labelling technique (see the Materials and methods section for details). CTR, control; HIST, histamine; WO, washout. (**B**) Detection and semi-quantitation of endocytosed AQP4. Upper panel: representative Western blot of endocytosed biotinylated AQP4 probed with anti-AQP4 antibodies (endocytosed AQP4). For signal normalization, total biotinylated AQP4 was probed with anti-AQP4 antibodies (total b-AQP4). Results are representative of three independent experiments. Lower panel: densitometric profile (means ± S.E.M, $n = 3$) of the 34 kDa band expressed as a percentage of the control. $^*P < 0.001$ (Student's t test for unpaired data).



treatment (500 μM) for 10 min or forskolin treatment (100 μM) for 10 to 30 min did not significantly alter the AQP4 phosphorylation state (Figures 4A and 4B; hist 10′, FK 10′, FK 30′). In contrast, 30 min of histamine stimulation resulted in an approx. 10-fold increase in phosphorylated AQP4, which was abolished by 1 μM H89, a specific PKA inhibitor (Figures 4A and 4B; hist 30′, hist 30′ + H89). The effect of histamine stimulation on AQP4 phosphorylation was also tested in the presence of specific PKC and CK2 inhibitors. Cells were pre-incubated with either 5 μM photo-activated calphostin C or 5 μM DRB (5,6-dichlorobenzimidazole 1-β-D-ribofuranoside), in order to inhibit PKC and CK2 respectively, prior to treatment with histamine for 30 min. No significant reduction of histamine-induced AQP4 phosphorylation was observed in both of the conditions described (Figures 4A and 4B; hist 30′ + calphostin C; hist 30′ + DRB). Interestingly, both AQP4 internalization (Figure 2A) and

Figure 4 | Phosphorylation experiments in intact HGT-VSV-AQP4 cells

(**A**) Upper panel: autoradiography of phosphorylated AQP4 (p-AQP4) in different experimental conditions. Experimental conditions are indicated on top of each lane. Lower panel: equal amounts of AQP4 immunoprecipitated from HGT-VSV-AQP4 cells were immunoblotted and probed with anti-AQP4 antibodies to verify the protein loading. (**B**) Means ± S.E.M. of the densitometric analysis performed with the 34 kDa AQP4 band obtained by autoradiography and corresponding to phosphorylated AQP4 from three independent experiments. $^*P < 0.0001$ (Student's *t* test for unpaired data). Ctr, control; FK, forskolin; hist, histamine; wo, washout; 10′, 10 min; 30′, 30 min.



phosphorylation (Figures 4A and 4B; PAO + hist 30′) were prevented after 30 min of histamine treatment in cells pre-incubated with 40 μM PAO.

After 1 h of histamine washout with fresh medium, AQP4 phosphorylation returned to the levels observed in the control condition (Figures 4A and 4B; hist 30′ wo).

These results suggest that PKA is the kinase that is likely to be responsible for AQP4 phosphorylation, and that AQP4 endocytosis is the crucial prerequisite for its phosphorylation by PKA.

Since histamine activates a signal transduction cascade mediated by PKA and/or PKC (Wang et al., 2000b), PKA and PKC activity were measured *in vitro* using the MESACUP protein kinase assay kit with cell homogenates from HGT-VSV-AQP4 cells (see the Material and methods sections for details). This kind of assay allows the simultaneous detection of increases in phosphorylation activities due to both PKA and PKC.

Phosphorylation activity associated with HGT-VSV-AQP4 cell lysates increased by approx. 3-fold after forskolin treatment, whereas 10 min and 30 min of histamine treatment resulted in an increase in phosphorylation activity by approx. 2- and 3-fold respectively (Figure 5). In these experimental conditions, phosphorylation activity was completely inhibited by 1 μM H89, thus indicating that PKA is the kinase activated by histamine stimulation.

To test whether AQP4 is an *in vitro* substrate for PKA, *in vitro* phosphorylation experiments were carried out. Equal amounts of immunoprecipitated AQP4 were incubated with [γ-^{32}P]ATP in the presence of purified PKA catalytic subunit. Phosphorylated proteins were separated by SDS/PAGE and revealed by autoradiography. As shown in Figure 6, AQP4 was phosphorylated in the presence of the catalytic subunit of PKA, and this effect was strongly reduced in the presence of the PKA inhibitor H89. This is the first evidence that AQP4 is an *in vitro* substrate for PKA. When immunoprecipitated AQP4 was incubated with activated PKC, AQP4 was phosphorylated and this effect was prevented in the absence of PKC activators (Figure 6). These results are in agreement with the study by Han et al. (1998) that AQP4 is also an *in vitro* substrate for PKC.

AQP4 phosphorylation by PKA occurs subsequently to its internalization in late endosomes

Having established that AQP4 phosphorylation is a PKA-dependent event (Figure 4, hist 30′ + H89), whereas its internalization is not (Figure 2, H89 + hist 30 min), the next step was to identify the intracellular compartment where AQP4 is internalized

Figure 5 | *In vitro* kinase activity measurements in HGT-VSV-AQP4 cells

After each experimental treatment, cells were homogenized and phosphorylation activity was determined *in vitro* using the MESACUP protein kinase assay kit (see the Material and methods section for details). Phosphorylation activity is expressed as a percentage of the activity found in untreated cells. $^{*}P < 0.0001$ (Student's *t* test for unpaired data). Ctr, control; FK, forskolin; hist, histamine; 10′, 10 min; 30′, 30 min.

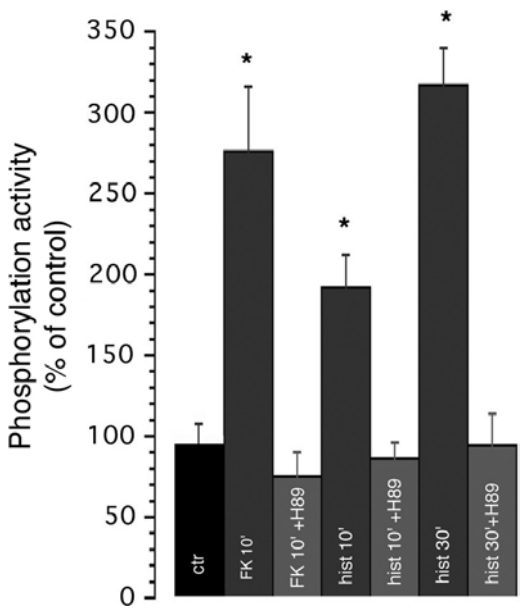
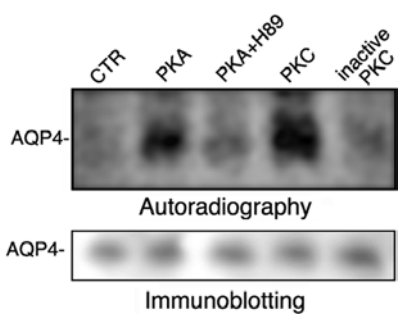


Figure 6 | AQP4 phosphorylation *in vitro*

Equal amounts of immunoprecipitated AQP4 were incubated with [γ-^{32}P]ATP in the presence of purified PKA catalytic subunit and activated PKC. Phosphorylated proteins were separated by SDS/PAGE and revealed by autoradiography. The same autoradiography gels were probed by Western blotting using anti-AQP4 antibody.



after histamine treatment. Co-immunolocalization experiments, observed by analysis with laser-scanning confocal microscopy, were performed using different markers for intracellular organelles. Most of the AQP4 internalized after 30 min of histamine treatment was found in the same vesicular compartment where MPR (mannose-6-phosphate receptor), a marker for late endosomes, was expressed (Figure 7; hist, merge). No co-localization was found using the Golgi marker giantin or with the lysosomal-associated membrane glycoprotein AC17 (data not shown), indicating that, after 30 min histamine treatment, internalized AQP4 does not enter the degradative pathway.

Discussion

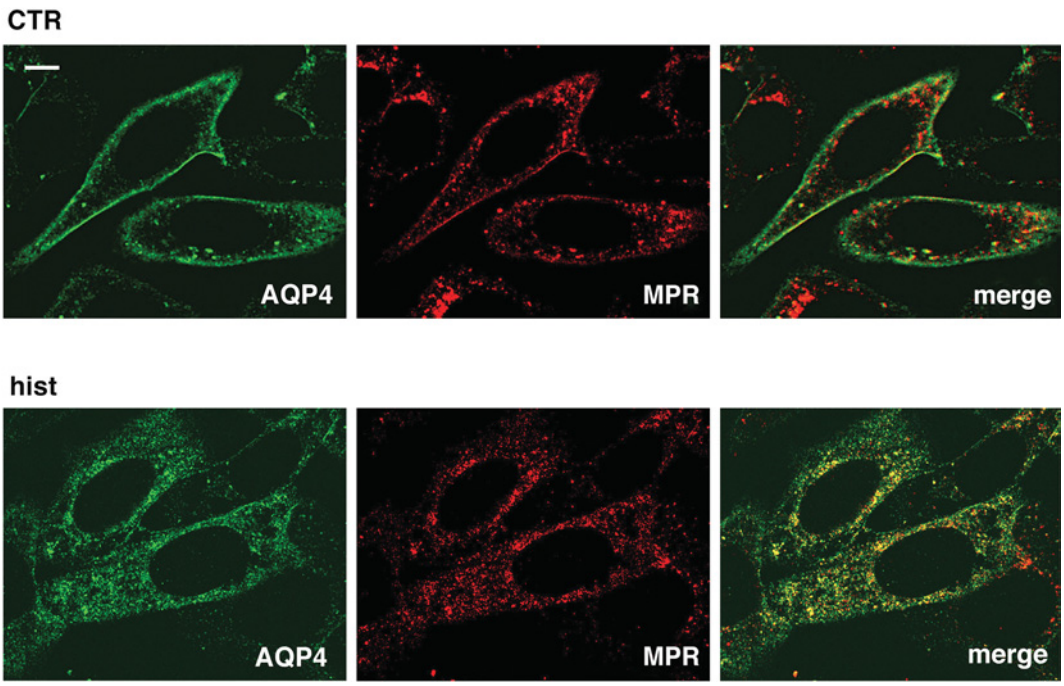
The rapid vectorial transcellular water movement in epithelial cells expressing AQPs is generally accomplished by the expression of the same or different AQPs on both apical and basolateral membranes. The general assumption regarding the water permeability properties of polarized cells expressing AQPs is that AQPs expressed in the basolateral membrane allow water absorption or secretion without representing a limiting barrier. It is generally believed in fact that typical basolaterally located AQPs in epithelial cells, such as AQP4 or AQP3, can account for the high constitutive osmotic water permeability and would not be subjected to a dynamic short-term regulation.

However, early findings suggest that this concept needs to be re-evaluated. We have previously demonstrated that OAPs (orthogonal arrays of particles), formed by AQP4 in the plasma membrane, rearranged after a relatively long histamine exposure as a consequence of AQP4 internalization in AQP4-transfected gastric HGT-1 cells (Carmosino et al., 2001). Moreover, Silberstein et al. (2004) reported that the organization of principal cell OAPs in Brattleboro rat kidney was perturbed by vasopressin V_2 receptor stimulation. AQP4 also can be dynamically regulated in a kidney epithelial cell line by activation of PKC and dopamine (Zelenina et al., 2002).

Reversible protein phosphorylation is the most common mechanism by which the function of a protein can be dynamically regulated. Whether AQP4 can be regulated by phosphorylation similar to other AQPs is the object of intense debate. Sequence

Figure 7 | Histamine treatment results in the internalization of AQP4 into late endosomes

AQP4-transfected cells were fixed and double-labelled with anti-AQP4 antibody and anti-MPR antibody (late-endosome marker). Anti-AQP4 antibody was visualized with the Alexa Fluor 488®-conjugated anti-rabbit IgG (green), anti-MPR antibody with the Alexa Fluor 568®-conjugated anti-mouse IgG (red) and co-localization in merge panels (yellow). Slides were analysed by confocal fluorescent microscopy. Representative microscopy fields from three independent experiments are shown. Scale bar, 5 μm.



analysis of the AQP4 molecule reveals several potential phosphorylation sites for PKA, CaMKII, PKC and CK2 (Kreegipuu et al., 1999). A few reports suggest that AQP4 can actually be phosphorylated by different kinases. Recently, phosphoproteomic analysis of rat inner medullary collecting duct treated with vasopressin indicated that, in renal cells, AQP4 is phosphorylated at its C-terminus on Ser^{321} by PKC. This phosphorylation site is found in a PDZ-binding motif involved in membrane localization of AQP4 in perivascular astrocytes (Hoffert et al., 2006).

Han et al. (1998) showed that PMA, a PKC activator, increases AQP4 phosphorylation and inhibits its activity. Moreover, the decrease in AQP4 water permeability observed by Zelenina et al. (2002) in renal epithelial cells is mediated by AQP4 phosphorylation at Ser^{180}, found in the consensus site for PKC. On the other hand, it has been demonstrated that, in MDCK (Madin–Darby canine kidney) cells, AQP4 phosphorylation by CK2 modulates the trafficking and degradation of AQP4 by directing AQP4 from an endosomal compartment to the lysosomes through interactions with different clathrin-adaptor protein complexes (Madrid et al., 2001). Moreover, the ability of AQP4 to form OAPs in the kidney principal cells was increased approx. 2-fold when Ser^{111} was mutated to glutamic acid, a mutation that mimics constitutive phosphorylation of this residue. It suggests that the putative PKA phosphorylation site Ser^{111} is involved in OAP formation (Silberstein et al., 2004). However, the short-term effect of dDAVP (1-desamino-[8-D-arginine]vasopressin) to increase OAP formation in the kidney described by the same authors reflects Ser^{111} phosphorylation, has not been evaluated.

Following these observations, in the present study we analysed whether AQP4 is phosphorylated after histamine treatment when expressed in human gastric cells. Using several approaches, such as immunolocalization analysis and cell surface biotinylation

experiments, we have shown that AQP4 is internalized after histamine treatment, relocates in late endosomes and recycles back to the plasma membrane after histamine washout.

The analysis of AQP4 phosphorylation dynamics during AQP4 internalization and recycling clearly shows that AQP4 internalization observed after 30 min histamine treatment was associated with a strong increase in AQP4 phosphorylation.

AQP4 phosphorylation induced by 30 min of histamine treatment was inhibited by 1 μM H89. At this concentration H89 shows a high and selective affinity for PKA compared with other kinases, including PKC and CK2 (Engh et al., 1996). Moreover, calphostin C and DRB, specific inhibitors of PKC and CK2 respectively, did not affect histamine-induced AQP4 phosphorylation.

The measurement of PKA and PKC activities after 30 min of histamine treatment showed that the activity of these kinases was approx. 3-fold higher compared with basal conditions. However, this phosphorylation activity was completely blocked by incubation with 1 μM H89. This evidence indicated that PKA is the kinase most likely to be activated by histamine treatment and involved in AQP4 phosphorylation.

The novel finding of the present work is that AQP4 phosphorylation occurs subsequently to its internalization in late endosomes. This conclusion is supported by several observations: (i) although PKA is already activated after 10 min of histamine or forskolin treatment, AQP4 phosphorylation occurred after only 30 min of histamine treatment, a time at which AQP4 was localized mostly in an intracellular compartment (late endosomal compartment); (b) inhibition of AQP4 internalization by PAO prevented AQP4 phosphorylation, despite the 30 min of histamine treatment; and (c) the PKA inhibitor H89 inhibited AQP4 phosphorylation, but did not prevent AQP4 internalization.

Based on our results, we suggest that PKA-mediated AQP4 phosphorylation might be involved in retaining AQP4 in a vesicle-recycling compartment, therefore escaping the degradative pathway. Our findings are supported by several observations that phosphorylation is not absolutely required for receptor internalization, but it plays an important role in the recycling of internalized receptors.

Phosphorylation of the plasma membrane NHE3 (Na^+/H^+ exchanger isoform 3) by PKA is a necessary event for assembly of AP2 (adaptor protein 2) around NHE3 followed by invagination, severing and discharge of a clathrin-coated vesicle that trafficks NHE3 to the endosomal compartment (Hu et al., 2001). Madrid et al. (2001) reported that AQP4 phosphorylation by CK2 redirects AQP4 from an endosomal compartment to a degradative pathway. Our data obtained from the endo–exocytosis assay demonstrated that internalization of AQP4 following histamine treatment promotes the recycling of AQP4 back to the cell surface without entering the degradative compartment. It is likely that AQP4 can recycle several times back and forth from the plasma membrane in the absence of *de novo* protein synthesis, as shown for AQP2 (Katsura et al., 1997). Indeed, we show here that after 1 h of histamine washout, AQP4 relocated to the basolateral membrane, even in the presence of cycloheximide, an inhibitor of protein synthesis. According to this result, AQP4 did not co-localize with lysosome maker AC17, but with the late-endosome marker MPR, suggesting that AQP4 is recycled to the plasma membrane from this compartment. This is in agreement with the recent proposal of long recycling pathways for vasopressin receptors (Innamorati et al., 2001).

It has been observed that PAO inhibits cell surface receptors internalization, including histamine H_2 receptors (Smit et al., 1995), suggesting that AQP4 might be internalized together or concomitantly with the H_2 receptors after 30 min of histamine stimulation. This hypothesis, however, requires further study and is currently under investigation.

Rat AQP4 possesses one potential consensus site for phosphorylation by PKA at Ser^{111}. Another putative site for PKA phosphorylation, Ser^{21}, is only present in human AQP4 (Kreegipuu et al., 1999).

Further strategies, including disruption of the PKA consensus sites, are, of course, necessary to identify the unique amino acid residue responsible for AQP4 phosphorylation in this physiological event.

To conclude, the present work provides the first evidence that AQP4 is phosphorylated after a physiological stimulus such as histamine treatment of human gastric cells, suggesting a possible molecular explanation for the mechanisms involved in the hormone-regulated AQP4 internalization/trafficking in epithelial cells.

Materials and methods

Cell transfection

The cDNA encoding the rat M1 AQP4 isoform was amplified by PCR from a rat kidney cDNA library and cloned in the expression vector pCB6. This construct was made and kindly provided by Dr Jean Merot (Inserm U533, Nantes, France). A VSV-tagged AQP4 construct was generated as described previously (Madrid et al., 2001). Transfections were performed using Lipofectin® (Life Technology). HGT-1 cells were grown at 37°C under a humidified atmosphere of 5% CO_2 in DMEM (Dulbecco's modified Eagle's medium), supplemented with 20 mM sodium bicarbonate, 20 mM Hepes and 10% fetal calf serum without antibiotics. Cells were transfected with 5 μg of recombinant plasmid. Stably transfected HGT-1 cells clones, referred to as HGT-VSV-AQP4 cells, were selected in a medium containing geneticin (500 μg/ml; Life Technology) for 10–15 days.

Preparation of cell homogenates, crude membranes and Western blotting

Cells were grown in 25 cm^2 flask to confluency, scraped off, pelleted and resuspended in 30 μl of lysis buffer [50 mM Tris/HCl (pH 8), 110 mM NaCl, 0.5% Triton X-100, 0.5% Igepal, 2 mM PMSF, 2 μg/ml leupetin and 2 μg/ml pepstatin], vigorously vortexed and incubated at 4°C for 60 min. The cell lysate was clarified with a brief centrifugation and resuspended in Laemmli buffer. Crude brain membranes were prepared as described previously (Carmosino et al., 2001).

Membranes or cell homogenates were solubilized in Laemmli buffer, heated to 60°C for 10 min and subjected to SDS/PAGE (13% gel). Gels were transferred on to Immobilon-P membrane (Millipore), blocked in blotting buffer [150 mM NaCl, 20 mM Tris/HCl (pH 7.4) and 1% Triton X-100] containing 5% non-fat dried milk for 1 h. The membranes were incubated with the anti-AQP4 polyclonal antibody (1:500) for 2 h at room temperature in blotting buffer, and washed in several changes of the same blotting buffer. The membranes were incubated with goat anti-rabbit IgG alkaline-phosphatase-conjugated (1:5000; Sigma) and the proteins revealed with 1-Step™ NBT/BCIP (Pierce).

Immunofluorescence and confocal analysis

For immunofluorescence experiments, cells were grown on glass coverslips and subjected to the different experimental conditions described and then fixed in ice-cold methanol for 5 min. Immunofluorescence was performed as described previously (Carmosino et al., 2000). Cells were labelled with the polyclonal antibody against AQP4 (1:200) and then stained with anti-(rabbit IgG Alexa Fluor 488®) (1:1000). The slides were examined with a Leica photomicroscope equipped for epifluorescence, and digital images were obtained by a cooled CCD (charge-coupled-device) camera interfaced to the microscope (Princeton Instruments). In co-localization experiments, the monoclonal antibody anti-MPR (1:100) was used as primary antibody. Anti-(mouse IgG Alexa Fluor 568®) (1:1000) was used as secondary antibody. Cells were examined by laser-scanning confocal microscopy (MRC-1024, Bio-Rad) equipped with a Kr/Ar-mixed gas laser. Specialized software for acquisition and processing of confocal images (Laser Sharp MRC 1024) was used for image analysis and processing.

Phosphorylation of AQP4 in intact cells

Confluent monolayers of HGT-VSV-AQP4 cells were grown in 20 mm Petri dishes. Cells were metabolically labelled by incubation with 250 μCi/ml [^{32}P]P_i (NEN) for 2 h in 1 ml of phosphate-free and serum-free DMEM at 37°C in a 5% CO_2 atmosphere. Medium was removed and cells were washed several times with PBS at 37°C and left untreated or treated under the different experimental conditions described.

Cells were rapidly solubilized in 400 μl of ice-cold incubation buffer [150 mM NaCl, 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM PMSF, 2% Triton X-100 and 0.5% Igepal] for immunoprecipitation. Insoluble material was discarded and the lysates were subjected to immunoprecipitation with anti-VSV monoclonal antibody (Sigma) conjugated to Protein A–Sepharose CL-4B for 3 h at 4°C. Immunocomplexes were washed four times in buffer [150 mM NaCl, 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 1 $CaCl_2$, 1 mM PMSF, 0.4% Triton X-100 and 0.1% Igepal], mixed with 30 μl of Laemmli buffer, heated at 60°C for 15 min and resolved on 13% polyacrylamide gels. Gels were dried and exposed to Kodak X-Omat AR film Lightning Plus intensifying screens at −80°C. To evaluate that identical amounts of AQP4 were immunoprecipitated from each sample, gels were routinely probed with anti-AQP4 antibodies by Western blotting.

In vitro phosphorylation of AQP4

HGT-VSV-AQP4 cells were grown to confluence in a 150 cm^2 Petri dish. Cells were washed twice in PBS and solubilized in 1.8 ml of incubation buffer for immunoprecipitation. AQP4 was immunoprecipitated by incubation with 20 μl of anti-VSV monoclonal antibody (Sigma) as described above. Phosphorylation of immunoprecipitated AQP4 by PKA was carried out using 20 units/ml of PKA catalytic subunit (Calbiochem) in the absence or in the presence of 10 μM H89.

The reaction for PKA activity was carried out in a solution containing 25 mM Tris/HCl (pH 7.0), 3 mM $MgCl_2$, 0.5 mM EDTA, 1 mM EGTA and 5 mM 2-mercaptoethanol. For each experimental condition, 30 μCi of [γ-^{32}P]ATP (10 Ci/mmol) (PerkinElmer) was added. Phosphorylation of immunoprecipitated AQP4 by PKC was performed in the presence of 1.7 units/ml of PKC (Calbiochem). The final concentrations in the reaction mixture for the PKC assay were: 25 mM Tris/HCl (pH 7.0), 3 mM $MgCl_2$, 0.5 mM EDTA, 1 mM EGTA, 5 mM 2-mercaptoethanol, 50 μg/ml phosphatidylserine, and 100 nM PMA. A negative control was performed in the absence of PKC activators. Assays were carried out at 30°C for 10 min, and the reaction stopped placing samples on ice. Immunocomplexes were then washed twice in washing buffer for immunoprecipitation then solubilized in 25 μl of Laemmli's buffer, heated at 60°C for 15 min and separated by SDS/PAGE. Gels were dried and exposed to Kodak X-Omat AR film Lightning Plus intensifying screens at −80°C for various times. To evaluate that identical amounts of AQP4 were loaded, gels were routinely probed with anti-AQP4 antibodies by Western blotting.

PKA and PKC activity assays

Subconfluent monolayers of HGT-VSV-AQP4 cells were grown in 25 cm^2 culture flasks. Cells were washed several times with PBS at 37°C and maintained under basal conditions or treated

under the different experimental conditions. Cells were scraped from the flasks and resuspended in ice-cold homogenizing buffer [50 mM Tris-/HCl (pH 7.5), 5 mM EDTA, 10 mM EGTA, 50 mM 2-mercaptoethanol and 1 mM PMSF] and sonicated for 30 s on ice. Cell homogenates were clarified at 1000 *g* for 15 min at 4°C. PKA and PKC activities were measured in cell homogenates using a colorimetric assay kit according to the manufacturer's instructions (MESACUP protein kinase assay).

Cell surface biotinylation

To semi-quantify the expression levels of AQP4 in the plasma membrane, cell surface biotinylation experiments were performed as described previously (Carmosino et al., 2001).

Briefly, HGT-VSV-AQP4 cells grown on Petri dishes were treated under different experimental conditions. Cells were then incubated with sulfo-NHS-SS–biotin (Pierce) and lysed. Equal volumes of streptavidin–agarose beads were added to the lysates. Steptavidin-bound complexes were pelleted and washed three times with lysis buffer. Biotinylated proteins were eluted in Laemmli buffer by boiling for 10 min, resolved by SDS/PAGE, electroblotted on to Immobilon-P and probed with anti-AQP4 antibody. Band intensities were quantified by densitometric analysis using NIH (National Institutes of Health) Images software.

AQP4 endo–exocytosis assay

For the endocytosis assay, confluent monolayers were incubated with the cleavable sulfo-NHS-SS–biotin (0.5 mg/ml; Sigma) in PBS with Ca^{2+}/Mg^{2+} for 30 min at 4°C. Free sulfo-NHS-SS–biotin was blocked by washing cells twice at 4°C with 0.1 M glycine in PBS with Ca^{2+}/Mg^{2+} and then with ice-cold PBS with Ca^{2+}/Mg^{2+}. To evaluate endocytosis, cell monolayers were treated under the different experimental condition at 37°C. All cell monolayers were again chilled on ice to stop membrane trafficking. Cell surface biotin bound to membrane proteins was cleaved by washing cells twice at 4°C with glutathione strip buffer (50 mM glutathione, 75 mM NaCl, 75 mM NaOH and 10% fetal bovine serum in water), whereas the internalized biotinylated proteins were protected from glutathione cleavage. Cells were then washed twice for 15 min at 4°C with iodoacetamide buffer [50 mM iodoacetamide/1% BSA in PBS, (pH 7.4)] to quench residual glutathione and cap free sulfhydryl groups present on proteins. Monolayers were then solubilized in Triton X-100 extraction buffer [150 mM NaCl, 20 mM Tris/HCl (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% (v/v) Triton X-100, 0.1% Igepal, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 PMSF and 1 mg/ml iodoacetamide] and extracts were clarified by centrifugation (13 000 *g* for 10 min).

Equal amounts of cell lysates were equilibrated with streptoavidin–agarose beads (Pierce) at 4°C overnight. Beads were washed three times in Triton X-100 buffer and biotinylated proteins were released from the beads by incubation with 100 mM dithiothreitol in Laemmli's buffer. Proteins were separated by SDS/PAGE, electroblotted into Immobilon-P and probed with anti-AQP4 antibody (1:500).

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