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The phosphatase inhibitor okadaic acid induces AQP2 translocation independently from AQP2 phosphorylation in renal collecting duct cells

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SUMMARY

Phosphorylation by kinases and dephosphorylation by phosphatase markedly affect the biological activity of proteins involved in intracellular signaling. In this study we investigated the effect of the serine/threonine phosphatase inhibitor okadaic acid on water permeability properties and on aquaporin2 (AQP2) translocation in AQP2-transfected renal CD8 cells. In CD8 cells both forskolin alone and okadaic acid alone increased the osmotic water permeability coefficient P_f by about 4- to 5-fold. In intact cells, in vivo phosphorylation studies revealed that forskolin stimulation resulted in a threefold increase in AQP2 phosphorylation. In contrast, okadaic acid treatment promoted only a 60% increase in AQP2 phosphorylation which was abolished when this treatment was performed in the presence of 1 μM H89, a specific protein kinase A (PKA) inhibitor. Nevertheless, in this latter condition, confocal microscopy analysis revealed that AQP2 translocated and fused to the apical membrane. Okadaic acid-induced AQP2 translocation was dose dependent having its maximal effect at a concentration of 1 μM. In conclusion, our results clearly indicate that okadaic acid exerts a full forskolin-like effect independent from AQP2 phosphorylation. Thus AQP2 phosphorylation is not essential for water channel translocation in renal cells, indicating that different pathways might exist leading to AQP2 apical insertion and increase in P_f.

Key words: Aquaporin2, Okadaic acid, Phosphorylation, Actin cytoskeleton, Phosphatase

INTRODUCTION

Vasopressin plays a key role in the modulation of water permeability in mammalian kidney. The ability of vasopressin to increase water transport is mediated by translocation of the specific vasopressin-sensitive water channel AQP2, from intracellular vesicles to the apical membrane (Knepper and Inoue, 1997; Sasaki et al., 1998). The initial mechanism of vasopressin action involves the hormone binding to the V2 receptor located on the basolateral cell surface of collecting duct principal cells and subsequent activation of cAMP-dependent PKA. It has been shown that a cAMP-dependent phosphorylation of AQP2 occurs at a serine level (ser-256) located in the cytoplasmic C terminus, suggesting a relation to vasopressin-regulated AQP2 trafficking (Kuwahara et al., 1995; Lande et al., 1996; Nishimoto et al., 1999). Recent studies demonstrated that mutated AQP2 (S256A) expressed in LLCPK1 cells did not translocate to the plasma membrane after vasopressin stimulation, suggesting a role for ser-256 phosphorylation in vasopressin-stimulated AQP2 trafficking (Katsura et al., 1997). In addition, Fushimi et al. (1997) by expressing the same mutant in the LLCPK1 cells, also suggested that AQP2 phosphorylation may represent an important mechanism in regulated exocytosis of the AQP2 water channel. However, the authors also found that the increment of phosphorylated AQP2 (p-AQP2) after vasopressin stimulation was not proportional to the cell surface expression of AQP2. In addition an apparent phosphorylation of the AQP2 occurred in the absence of cAMP stimulation. The authors concluded that AQP2 phosphorylation might be one of the regulatory steps leading to translocation of the water channel.

In this study we examined the effects of okadaic acid (OA) on AQP2 trafficking in AQP2-transfected renal CD8 cells (Valenti et al., 1996). Okadaic acid is a tumor promoter which inhibits the activity of protein phosphatases 1 and 2A in intact cells (Bialojan and Takai, 1988; Haystead et al., 1989). We demonstrated that OA increases the water-permeability coefficient P_f to an extent comparable to vasopressin or forskolin stimulation. The possible involvement of the actin cytoskeleton in this OA effect is discussed. A brief account has been given previously (Valenti et al., 1998a).

MATERIALS AND METHODS

Materials

Phosphorus-32, as orthophosphoric acid-HCl free (10 mCi/ml) was

obtained from NEN. Protein A-Sepharose CL-4B, okadaic acid (sodium salt), anti-rabbit IgG FITC conjugate, anti-rabbit IgG TRITC conjugate, phalloidin-FITC labeled and forskolin were purchased from Sigma; H89, dihydrochloride from Calbiochem; CellTracker™ Orange (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine) CMTRM from Molecular Probes. Specificity of AQP2 antiserum generated against a syntethic peptide corresponding to AQP2 C terminus has been described previously (Valenti et al., 1996).

Cell culture

The study was performed on CD8 cells (Valenti et al., 1996), a cell line established by stably transfecting the RC.SV3 rabbit cortical collecting duct cells (Vandewalle et al., 1989) with cDNA encoding rat AQP2. CD8 cells respond to vasopressin or forskolin with a 4- to 6-fold increase in the osmotic water permeability coefficient, P_f and redistribution of AQP2 from an intracellular compartment to the apical membrane. CD8 cells were grown at 37°C, as described, in a hormonally defined medium (Valenti et al., 1996). Confluent monolayers were used at days 3-5 after plating.

Immunofluorescence studies in CD8 cells

CD8 cells were grown on coverslips for 3-5 days to confluence. In one set of experiments employing okadaic acid (OA) cells were stimulated with 10^{-4} M forskolin (FK) or with 1 μM OA for 15 minutes at 37°C in PBS or left under resting condition. In experiments employing the {N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, HCl} (H89) cells were preincubated with 1 μM H89 for 1 hour in culture medium prior to being treated with 1 μM OA or 10^{-4} M forskolin (FK) for 15 minutes in the presence of H89. Cells were fixed in ice-cold methanol for 5 minutes, washed twice in PBS (CM) and saturated for 15 minutes with 0.1% gelatin in PBS (PBS-gelatin). Cells were incubated with affinity-purified AQP2 antibodies for 2 hours at room temperature, washed three times in PBS-gelatin, and stained with goat anti-rabbit FITC-conjugated IgG or with goat anti-rabbit TRITC-conjugated IgG for 1 hour. After three washes in PBS, coverslips were mounted in 50% glycerol in 0.2 M Tris-HCl, pH 8.0, containing 2.5% *n*-propyl gallate as an antiquenching agent. For actin cytoskeleton visualization, cells were fixed in 4% paraformaldehyde for 20 minutes and stained with phalloidin-FITC (1:400 dilution). Slides were examined with a Leica photomicroscope equipped for epifluorescence and digital images were obtained using a cooled CCD camera (Princeton Instruments, NJ). Alternatively, cells were examined by laser scanning confocal microscopy (MRC-1024, Bio-Rad) equipped with a Krypton/Argon mixed gas laser. A specific software for acquisition and processing of confocal images (LaserSharp MRC 1024) was used for image analysis, processing and 3-D reconstruction. Reconstruction of the xz vertical sections was performed with the NIH image software.

Osmotic water permeability measurements by total internal reflection (TIR) microfluorimetry

Confluent CD8 monolayers grown on coverslips were used to measure the osmotically-induced cell-volume changes under different experimental conditions. Measurements were carried out by TIR microfluorimetry. Application of the TIR procedure to CD8 cells and calculation of P_f values have been previously described (Valenti et al., 1996). Experimental conditions included: control cells (CTR), 30 μM H89 for 1 hour followed by 10^{-4} M FK treatment for 15 minutes at 37°C (H89+FK), 10^{-4} M FK for 15 minutes at 37°C (FK); OA 1 μM for 15 minutes (OA); (5) 1 μM OA + 10^{-4} M FK for 15 minutes (OA+FK).

Data for each experiment were obtained from the same field of cell monolayer.

Quantification of the effect of okadaic acid on AQP2 translocation by image analysis

To quantify the effect of okadaic acid on AQP2 redistribution in CD8 cells, AQP2 immunofluorescence intensity was analyzed in different experimental conditions as described previously (Valenti et al., 1998b). Briefly, basal planar images obtained by confocal immunofluorescence were analyzed using the Image Tool software that assigns a score of 255 to the brightest fluorescence detectable in the image and a value of 1 to the least fluorescence detectable. All images had exactly the same pixel number in order to determine differences in the distribution of fluorescence between the different experimental groups. At least 4 randomly chosen boxes from different fields of the coverslips (corresponding to approximately 20 cells each) were analyzed from at least three separate experiments. Background fluorescence was measured and normalized. Images were processed and the distribution of pixel intensity as a function of their frequency was determined for each image and relative parameters as standard deviation, skewness and kurtosis were determined. Skewness is a measure of the symmetry of a profile about the mean pixel intensity value. Kurtosis describes the randomness of the shape of the profile relative to that of a perfectly random pixel intensity distribution. Statistical analyses were performed using the unpaired *t*-test.

In vivo phosphorylation of AQP2

Confluent monolayers of CD8 cells were grown on 20 mm cell culture Petri dishes. Cells were metabolically labeled by incubation with 250 μCi/ml of [^{32}P]orthophosphoric acid (NEN) for 2 hours in 1 ml of phosphate-free DMEM at 37°C in a 5% CO_2 atmosphere. In the experiments employing the PKA inhibitor H89, the drug was included during the second hour of metabolic labeling at 1 μM concentration. Medium was removed and cells were washed several times with PBS (CM) at 37°C. Cells were rapidly solubilized in ice-cold incubation buffer for immunoprecipitation (150 mM NaCl, 20 mM Tris, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 2% Triton X-100, 0.5% Nonidet P40, PMSF 1 mM, pH 7.4). Insoluble material was discarded and the lysates were subjected to immunoprecipitation with AQP2 antibodies conjugated to Protein A-Sepharose CL-4B for 3 hours at 4°C. Immunocomplexes were washed 4 times in washing buffer (150 mM NaCl, 20 mM Tris, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.4% Triton X-100, 0.1% Nonidet P40, PMSF 1 mM, pH 7.4), mixed with 30 μl of Laemmli buffer, heated at 60°C for 15 minutes and resolved in a 13% polyacrylamide slab gel. Gels were stained, dried and exposed to Kodak X-Omat AR film Lightning Plus intensifying screens at –80°C. To evaluate that identical amounts of AQP2 were immunoprecipitated from each sample, gels were routinely probed also with AQP2 antibodies by western blotting. Experimental conditions included: (1) control condition (CTR) (2) 1 μM H89 for 1 hour followed by 1 μM OA treatment for 15 minutes at 37°C (OA+H89); (3) 1 μM H89 for 1 hour followed by 10^{-4} M FK for 15 minutes at 37°C (H89+FK); (4)10^{-4} M FK for 15 minutes at 37°C; (5) OA for 15 minutes at concentration reported in the figure legends (OA); (6) 1 μM OA+ 10^{-4} M FK for 15 minutes (OA+FK).

RESULTS

Effect of okadaic acid on AQP2 localization and on forskolin-induced water transport

The effect of OA on AQP2 redistribution was analyzed by indirect immunofluorescence and by laser confocal microscopy. CD8 cells grown on coverslips were stimulated with 10^{-4} M FK or left under basal conditions or treated with increasing concentrations of OA. Previous studies demonstrated that 10^{-4} M FK was the maximal active concentration in promoting AQP2 translocation and P_f increase in CD8 cells (Valenti et al., 1996, 1998b). Cells were fixed and immunostained with AQP2 antibodies. Under control conditions, AQP2 was located in intracellular vesicles (Fig.

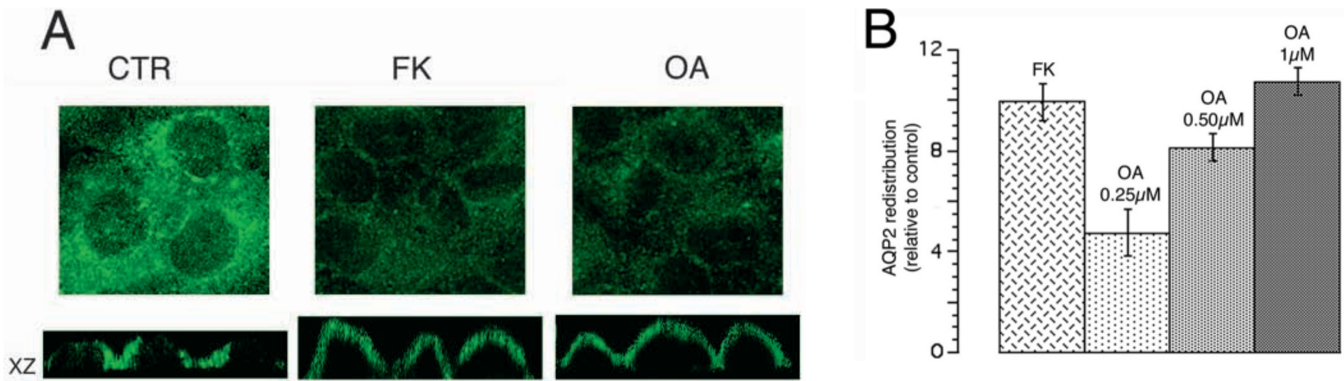

Fig. 1. Effect of okadaic acid on AQP2 localization in CD8 cells and quantification by image analysis. (A) CD8 cells grown on coverslips were incubated with 1 μM okadaic acid for 15 minutes (OA) or stimulated with 10^{-4} M forskolin for 15 minutes (FK) or left under basal conditions (CTR). Cells were fixed, immunostained with AQP2 antibodies and analyzed by laser confocal microscopy. Treatment with okadaic acid resulted in a redistribution of AQP2 similar to that obtained with forskolin stimulation. Analysis of an xz reconstruction confirmed that while in control conditions the cells appeared flat with a mostly intracellular AQP2 staining (CTR xz), stimulation with forskolin resulted in increased staining in the apical membrane and an increase in cell height (FK xz). A comparable effect was seen upon preincubation with 1 μM okadaic acid alone (OA xz); (×700). (B) Semiquantification of AQP2 redistribution by image analysis (see Materials and Methods for details). Each column represents the mean ± s.e. of the parameters obtained from four images of a representative experiment. The effect of OA on AQP2 translocation was dose-dependent. 1 μM okadaic acid promoted AQP2 translocation similar to that observed with maximal forskolin stimulation. Similar results were obtained in at least three independent experiments.

1A, CTR), while treatment with FK induced a drastic reduction of intracellular AQP2 staining (Fig. 1A, FK) due to the relocation of AQP2 to the apical membrane (Valenti et al., 1996, 1998b). Analysis by laser confocal microscopy of an xz reconstruction confirmed that, while in control condition the cells appeared flat (calculated cell height 6 μm in average) with a mostly intracellular AQP2 staining (Fig. 1A, CTR xz), stimulation with FK resulted in an increased staining in the apical membrane and an increase in cell height (calculated cell height 10 μm in average; Fig. 1A, FK xz). A comparable effect was seen upon preincubation with 1 μM OA alone (Fig. 1A, OA). As for FK-stimulated condition, CD8 cells examined at a basal focal plan displayed a strong reduction in AQP2 intracellular staining (Fig. 1A, OA). Confocal analysis confirmed that similarly with FK stimulation, this treatment resulted in a relocation of the AQP2 to the apical membrane and increase in cell height (calculated cell height 9 μm in average; Fig. 1B, OA xz).

The effect of OA on AQP2 translocation was dose-dependent. Semiquantitative image analysis in fact demonstrated that 1 μM OA promoted the maximal redistribution. Fig. 1B reports the effect of increasing concentrations of OA on AQP2 redistribution. For semiquantitation of AQP2 redistribution, confocal planar images taken at the basal cell level were analyzed using the Image Tool software as previously described (Valenti et al., 1998a) and reported in Materials and Methods. Images were processed and the distribution of pixel intensity (corresponding to the AQP2 staining in intracellular vesicles at the basal focal plan) as a function of their frequency was determined for each image and relative parameters as standard deviation, skewness and kurtosis were determined. The statistical parameter of kurtosis can be regarded as an index of AQP2 redistribution (Valenti et al., 1998b). Statistical analyses were performed using the unpaired t-test. As shown in Fig. 1B, 1 μM OA was as effective as FK in inducing AQP2 redistribution. Higher concentrations of OA did not promote further redistributions (data not shown). Based on these results 1 μM OA was used for functional studies.

To test the effect of OA on the water-permeability properties of CD8 cells, we measured the time course of cell swelling in response to changes in perfusate osmolality by TIR microfluorimetry (Fig. 2). Measurements were performed under basal conditions, after stimulation with FK, in the presence or in the absence of H89 pretreatment, or with OA and in presence of FK and OA together. Incubation of CD8 cells for 15 minutes with 10^{-4} M FK, a direct activator of adenylate cyclase, increased the rate of cell swelling in response to a 200 mOsm NaCl gradient (Fig. 2A), and the calculated P_f was about 4-fold higher than that calculated under basal condition (Fig. 2B). A similar effect on both the rate of cell swelling and the P_f was also observed after incubation of CD8 cells with 1 μM OA for 15 minutes (Fig. 2A,B). Preincubation of CD8 cells with H89, abolished the increase in P_f induced by FK (Fig. 2A,B). Interestingly, when cells were stimulated with FK in the presence of OA, a partial additive effect on the rate of cell swelling was observed (Fig. 2A). This treatment resulted in an increase in P_f of about 7-fold (Fig. 2B). These data confirm that OA stimulates AQP2 relocation to the apical membrane leading to a 4-fold increase in P_f.

The effect of OA might indicate that phosphorylation of AQP2 itself could be an essential step in the AQP2 relocation pathway. Alternatively inhibition of phosphatases might act indirectly by altering the balance of phosphorylation of essential components of the transport machinery.

In vivo phosphorylation experiments

To better define the OA effect, in vivo phosphorylation experiments were performed in order to evaluate the phosphorylation state of AQP2 in intact cells after treatment with agents expected to modulate the rate of AQP2 phosphorylation. Confluent monolayers of CD8 cells were metabolically labeled with [^{32}P]orthophosphate, incubated under various experimental conditions followed by AQP2

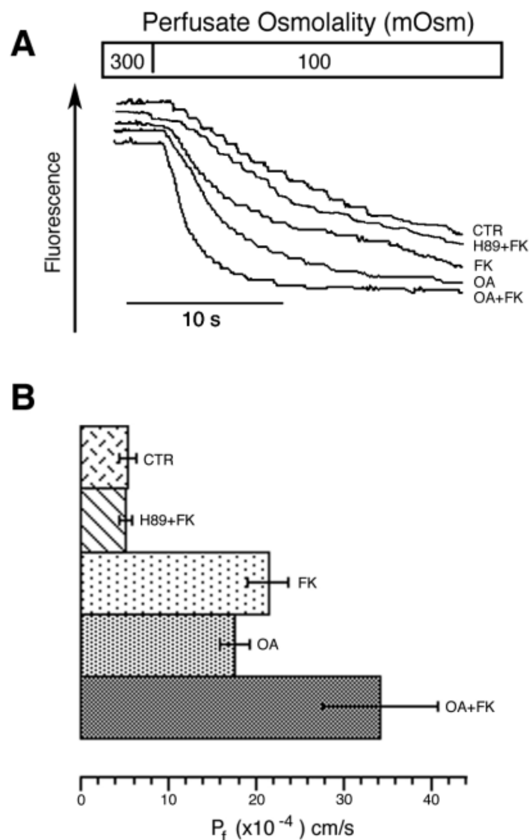

Fig. 2. Effect of okadaic acid on water permeability properties of CD8 cells. (A) Representative TIR time courses of CD8 cells in response to a 200 mOsm inwardly directed NaCl gradient, at 10°C. Forskolin stimulation (FK, 10^{-4} M for 15 minutes) determined a dramatic increase in the rate of cell swelling compared with basal conditions (CTR). A similar effect was also observed after incubation of CD8 cells with okadaic acid (OA, 1 μM) for 15 minutes. Pretreatment of CD8 cells with H89 abolished the increase of cell swelling induced by forskolin stimulation (H89+FK). Incubation with both agents (OA+FK) resulted in a partially additive effect on the rate of cell swelling. (B) Mean values ± s.e. (n=3) of osmotic water permeability coefficients P_f.

immunoprecipitation (see Materials and Methods). Fig. 3A reports the effect of increasing concentrations of OA on AQP2 phosphorylation. A slight increase (about 60%, Fig. 3A′) in AQP2 phosphorylation was only observed when OA was employed at the concentration of 1 μM whereas 0.25 or 0.5 μM OA did not significantly modify the amount of immunoprecipitated p-AQP2 (Fig. 3A,A′). Higher OA concentrations did not promote further increase of p-AQP2 in intact cells (data not shown). In contrast, short-term FK stimulation resulted in a nearly threefold increase in p-AQP2 (Fig. 3A,A′) confirming previous findings obtained in renal cells (Fushimi et al., 1997; Katsura et al., 1997; Nishimoto et al., 1999). In vivo phosphorylation studies was undertaken to investigate whether OA might potentiate the effect of FK on AQP2 phosphorylation.

Fig. 3B shows the autoradiography of immunoprecipitated phosphorylated AQP2 of a representative experiment. In control conditions, AQP2 appeared to be weakly phosphorylated (CTR), whereas stimulation with 10^{-4} M FK increased the rate of AQP2 phosphorylation by about threefold as assessed by densitometric analysis of the phosphorylated 29 kDa band (Fig. 3B′, FK). Pretreatment of intact cells with H89 abolished the increase of AQP2 phosphorylation induced by FK (Fig. 3B, B′; H89+FK). Incubation with 1 μM OA alone induced an increase in AQP2 phosphorylation of about 60%. The strongest signal for phosphorylated AQP2 was obtained when stimulation with FK was performed in the presence of

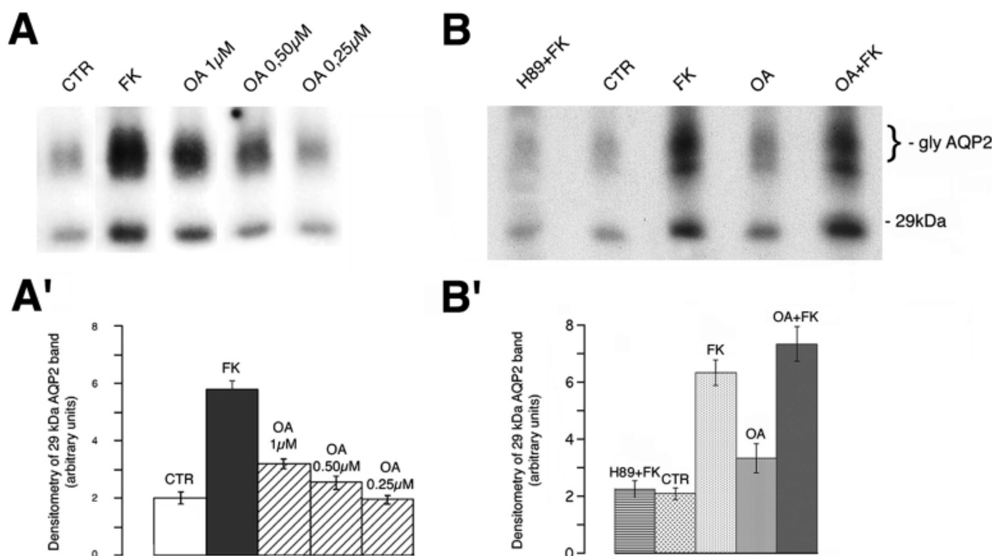

Fig. 3. In vivo phosphorylation of AQP2 in intact CD8 cells. (A) For each experimental condition identical amounts of CD8 cells grown to confluence on Petri dishes were metabolically labeled with [^{32}P]orthophosphate (250 μCi/ml) for 3 hours. Cells were then lysed and AQP2 was immunoprecipitated using AQP2 antibodies and revealed by autoradiography. Experimental conditions included: control condition (CTR), stimulation with 10^{-4} M forskolin (FK), treatment with okadaic acid 1 μM (OA 1 μM), 0.5 μM (OA 0.5 μM) and 0.25 μM (OA 0.25 μM). AQP2 phosphorylation was slightly increased (60%) in 1 μM OA treated cells. (A′) Densitometric profile (Mean values ± s.e., n=3) of the 29 kDa phosphorylated AQP2 band. (B) CD8 cells grown on Petri dishes were metabolically labeled with [^{32}P]orthophosphate (250 μCi/ml) for 3 hours. Cells were then stimulated with 10^{-4} M forskolin (FK) or 1 μM okadaic acid (OA) or with the two agents together (OA+FK). Forskolin stimulation was also performed in H89-pretreated CD8 cells (H89+FK). AQP2 was immunoprecipitated with the specific antibody and phosphorylated AQP2 revealed with autoradiography. The strongest signal for phosphorylated AQP2 was obtained when stimulation with forskolin was performed in the presence of okadaic acid. In this experimental condition the effect of the two agents appeared to be additive. Similar results were obtained in three independent experiments. (B′) Densitometric profile (mean values ± s.e., n=3) of the 29 kDa phosphorylated AQP2 band.

OA. In this experimental condition the effect of the two agents appeared to be additive, the bulk of phosphorylated AQP2 being due to FK stimulation (Fig. 3B,B′; OA+FK).

Phosphorylation of AQP2 is not required for okadaic acid-induced AQP2 translocation

To determine whether the slight increase of AQP2 phosphorylation induced by OA treatment might per se be responsible for AQP2 translocation and fusion, we analyzed the effect of OA on AQP2 trafficking in the presence of 1 μM H89 to exclude the contribution of PKA during OA treatment. Fig. 4A reports confocal immunofluorescence micrographs of horizontal sections through the basal focal plan of CD8 cells and the vertical focal plan (xz). In CD8 cells treated with OA in the presence of 1 μM H89, AQP2 was clearly relocated in the apical membrane as assessed by confocal vertical reconstruction (Fig. 5, OA+H89 xz). A confirmation of this effect came from a 3-D analysis shown in the inset: while in control conditions the AQP2 is not detectable on the apical surface, in OA+H89 treated cells AQP2 reveals a clear apical surface localization. In addition, a vertical confocal plan reconstruction (Fig. 4, OA+H89 xz) demonstrated the increased staining in the apical membrane and an increase in cell height in these experimental conditions compared with control conditions, where the cells appear flat with a mostly intracellular AQP2 staining (Fig. 4, CTR xz). Notably, in vivo phosphorylation studies confirmed that the amount of phosphorylated AQP2 immunoprecipitated from OA+H89 treated cells is even decreased compared with that immunoprecipitated from control CD8 cells (Fig. 4B) indicating that okadaic acid-induced recruitment of AQP2 to the apical membrane occurs independently of AQP2 phosphorylation.

Visualization of actin network in okadaic acid treated cells

Actin cytoskeleton is also controlled by kinase and phosphatase activities (Hartwig et al., 1992). In amphibian bladder and in rat inner medullary collecting duct it has been reported that vasopressin significantly depolymerized F-actin indicating that a reorganization of apical actin network might be crucial in promoting fusion of AQP2-bearing vesicles (Holmgren et al., 1992; Simon et al., 1993). Therefore we explored the possibility that OA treatment might induce actin rearrangement leading to AQP2 translocation in renal CD8 cells. Visualization of actin network with phalloidin-FITC and colocalization with AQP2 in CD8 cells treated with either FK or OA are reported in Fig. 5. Control CD8 cells showed a well-organized meshwork of actin filament (Fig. 5A′) as visualized by confocal analysis in cells treated with phalloidin-FITC. The 3-D reconstruction (Fig. 5A) in control CD8 cells double-labeled with phalloidin-FITC (green) and AQP2 (red) clearly showed the absence of AQP2 on the apical pole of the cell which is otherwise visible on the basal plan. On the other hand, very few organized actin filaments were visible in FK treated cells (Fig. 5B′) or in OA treated cells (Fig. 5C′). The 3-D reconstruction of OA-treated (Fig. 5C) CD8 cells double-labeled with phalloidin-FITC (green) and AQP2 (red) clearly show the absence of organized actin filaments (green) and a massive fusion of AQP2 on the entire apical surface (red). In FK-stimulated cells (Fig. 5B) the 3-D reconstruction also showed a deep disorganization of the actin cytoskeleton (green) and a fusion of AQP2 on the apical surface (red) although AQP2 staining appeared more polarized and concentrated in a smaller apical pole area.

Therefore, both FK and OA stimulation seem to affect the organization of actin network. To establish whether this effect is a prerequisite for AQP2 translocation, H89-pretreated CD8 cells were stimulated with FK or with OA and double labeled with phalloidin-FITC and with AQP2 antibodies. Interestingly, compared with control condition (Fig. 6 CTR), in H89-treated CD8 cells, FK stimulation does not affect the actin cytoskeleton organization (Fig. 6 H89+FK, green) and does not promote AQP2 redistribution (Fig. 6, H89+FK red and inset). In contrast, in H89-treated CD8 cells, OA treatment still results in actin cytoskeleton disorganization (Fig. 6 H89+OA, green) with concomitant decrease of AQP2 staining at the basal plan (Fig. 6H89+OA, red and inset) consistent with a relocation of AQP2 toward the apical surface.

DISCUSSION

The major finding of our study is that okadaic acid, a potent and specific inhibitor of protein phosphatase 1 and 2A, stimulates osmotic water permeability by inducing AQP2 redistribution to the apical membrane in AQP2-transfected collecting duct CD8 cells. In CD8 cells okadaic acid was as effective as forskolin, a direct activator of the catalytic subunit of adenylate cyclase, on both processes. Okadaic acid can enter the cells and increase the phosphorylation state of many proteins within minutes and has no effect on intracellular ATP levels and no unexpected toxic effects in short-term incubation (Cohen et al., 1990). However, this forskolin-like effect was independent from AQP2 phosphorylation. In fact, in vivo phosphorylation studies revealed that okadaic acid promoted only a 60% increase in AQP2 phosphorylation which was abolished when this treatment was performed in the presence of 1 μM H89, a specific PKA inhibitor. In contrast, forskolin stimulation resulted in about a threefold increase in p-AQP2. Confocal microscopy analysis revealed that AQP2 translocated and fused to the apical membrane in okadaic acid treated cells even when H89 was included during the treatment. These results suggest that besides the short-term regulation of AQP2 insertion by phosphorylation at ser256, increased serine/threonine phosphorylation of an unidentified protein(s) other than AQP2, might promote AQP2 translocation toward the apical membrane.

The presence of both PKA and phosphatase activities in purified AQP2 endosomes was demonstrated by in vitro phosphorylation studies confirming the finding obtained by Harris et al. (Lande et al., 1996). In situ phosphorylation-dephosphorylation of AQP2 may occur in endosomes in response to hormonal stimulation. Phosphorylation events are controlled not only by the balance of kinase and phosphatase activity, but also by where these enzymes are located in the cells. Subcellular targeting within the cells increases the selectivity of multifunctional serine/threonine phosphatases and kinases by favoring their accessibility to substrate proteins (Faux and Scott, 1996). This might be the case for the AQP2 protein whose phosphorylation state might be in situ modulated.

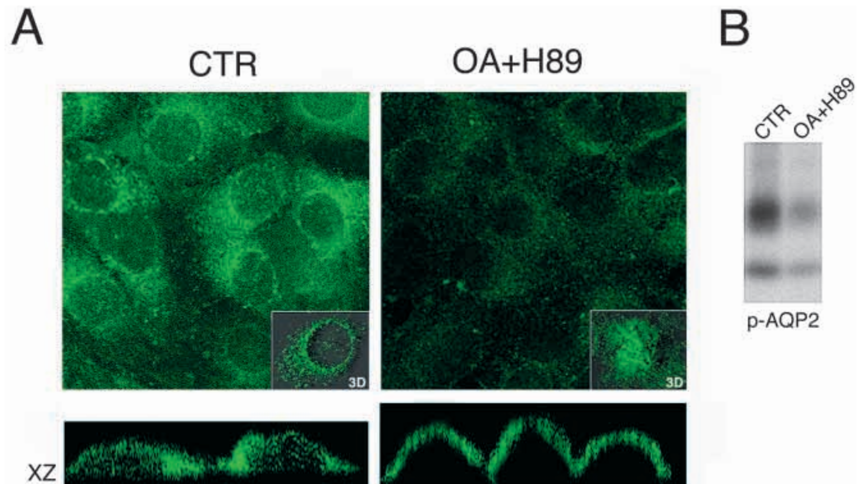

Fig. 4. Effect of okadaic acid on AQP2 redistribution and phosphorylation, in H89 pretreated CD8 cells. (A) CD8 cells grown on coverslips were incubated with 1 mM H89 for 1 hour in the culture medium and then treated with 1 mM okadaic acid in the presence of 1 μM H89 for 15 minutes (OA+H89). Control experiments with untreated cells were done in parallel (CTR). Cells were then fixed, immunostained with AQP2 antibodies and analyzed by laser confocal microscopy. In basal conditions (CTR) AQP2 staining is predominantly located in intracellular vesicles (×500). In H89-pretreated cells incubated with 1 μM OA, AQP2 staining is relocated toward the apical membrane showing an increase in cell height as shown in a confocal xz plan (xz OA+H89). Confocal 3-D reconstruction (3-D inset OA+H89) clearly demonstrate that AQP2 is redistributed in the apical surface whereas AQP2 is not detectable on the apical surface in control cells (3-D inset CTR). (B) Intact CD8 cells were metabolically radiolabeled with [^{32}P]orthophosphate (250 μCi/ml) for 3 hours and either preincubated with 1 μM H89 for 1 hour and treated with 1 μM okadaic acid for 15 minutes (OA+H89) or left under basal conditions (CTR). Immunoprecipitation of AQP2 followed by autoradiography demonstrate that the amount of phosphorylated AQP2 (p-AQP2) in OA+H89 treated cells is decreased compared with that which is immunoprecipitated from untreated cells. Similar results were obtained in at least three independent experiments.

On the other hand, in vivo phosphorylation studies performed in intact cells probably reflect the activity of a more complicated system regulating the phosphorylation state of the AQP2 water channel at various steps. The intriguing question is to what extent is phosphorylation involved in AQP2 trafficking. In this work, several strategies were undertaken to answer this question. A crucial observation was that the combination of forskolin and okadaic acid produced a partial additive effect on the increase in the osmotic water-permeability coefficient. This implies that maximal concentration of forskolin does not promote the complete redistribution of AQP2 protein from the intracellular pool to the apical membrane. This effect might be due to the continued recycling of AQP2-bearing vesicles which could be controlled by AQP2 dephosphorylation. However, experiments using the fluid-phase fluorescent marker FITC-dextran would not favour the hypothesis that okadaic acid could act through an inhibition of endocytosis (data not shown). In addition, functional studies indicated that the half-time for the stimulating effect of okadaic acid on cell swelling was similar to that of forskolin, making it unlikely that inhibition of AQP2-endocytosis can explain the effect of okadaic acid. There is much evidence, based on the expression of AQP2 deficient of serine 256 in LLCPK1 cells, to support a key role of AQP2 phosphorylation in activating AQP2 trafficking (Fushimi et al., 1997; Katsura et al., 1997; Nishimoto et al., 1999). However Fushimi et al. (1997) observed that the increase in p-AQP2, in cells transfected with wild type AQP2, was not proportional to the increase of cell surface expression of AQP2, thus indicating that phosphorylation of AQP2 might be one of a series of regulatory steps and is not sufficient for inducing exocytosis. The results obtained in CD8 cells indicate that okadaic acid treatment

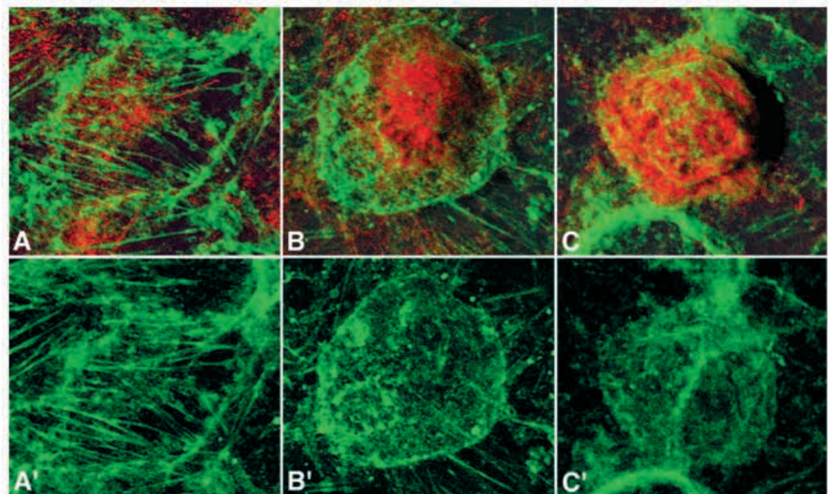

Fig. 5. Effect of okadaic acid treatment and forskolin stimulation on actin cytoskeleton. Colocalization of F-actin with AQP2 in CD8 cells. CD8 cells grown on coverslips were stimulated with 10^{-4} M forskolin for 15 minutes or treated with 1 μM OA for 15 minutes or left under control conditions. Fixed cells were stained with phalloidin-FITC (green color) and AQP2 was revealed with anti-rabbit antibodies (red color). Control CD8 cells showed a well-organized meshwork of actin filament (A′) as visualized by confocal analysis in cells treated with phalloidin-FITC. The 3-D reconstruction (A) in control CD8 cells double-labeled with phalloidin-FITC and AQP2 clearly shows the absence of AQP2 on the apical pole of the cell which is visible on the basal plan. By contrast, very few organized actin filaments are visible in forskolin-stimulated cells (B′) or in okadaic acid-treated cells (C′). The 3-D reconstruction of okadaic acid-treated (C) CD8 cells double-labeled with phalloidin-FITC and AQP2 clearly shows the absence of organized actin filaments and a massive fusion of AQP2 on the entire apical surface. In forskolin-stimulated cells (B) the 3-D reconstruction also shows a deep disorganization of the actin cytoskeleton and a fusion of AQP2 to the apical surface, although AQP2 staining appears more polarized and concentrated in a smaller apical pole area (×2000).

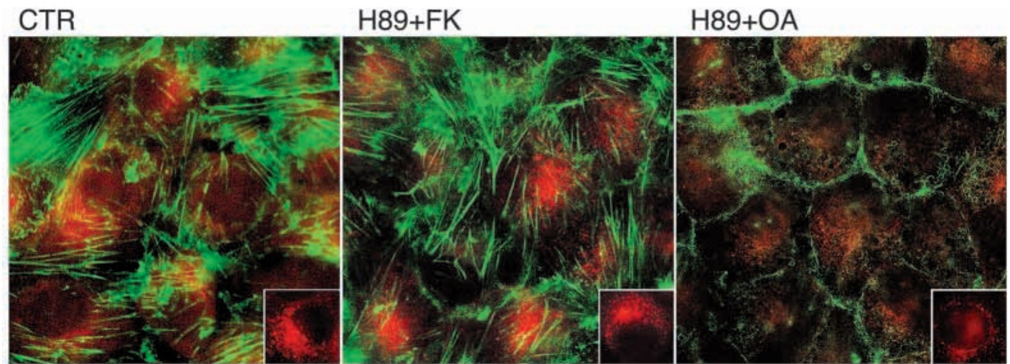

Fig. 6. Double labeling of F-actin and AQP2 in H89-pretreated CD8 cells: effect of okadaic acid and forskolin. H89-pretreated CD8 cells were stimulated with 10^{-4} M forskolin for 15 minutes or treated with 1 μM okadaic acid for 15 minutes. H89-treated, as well as control (CTR) CD8 cells, were fixed and stained with phalloidin-FITC (green color) and AQP2 was revealed with TRITC-conjugated anti-rabbit antibodies (red color). Control CD8 cells show a well-organized meshwork of actin filament (CTR) and a mostly intracellular AQP2 staining (CTR red color, inset). A similar staining for F-actin and for AQP2 is obtained in H89-pretreated CD8 cells stimulated with forskolin (H89+FK). In contrast, very few organized actin filaments are visible in H89-pretreated CD8 cells stimulated with okadaic acid (H89+OA). In this latter condition, a decrease in the intracellular staining for AQP2 at the basal focal plan is observed (H89+OA inset) (×800).

results in a slight (60%) increase in AQP2 phosphorylation in intact cells vs the threefold increase in p-AQP2 induced by maximal forskolin stimulation. Moreover, when H89, a selective PKA inhibitor, was present during okadaic acid treatment, no increase in p-AQP2 was observed whereas confocal laser microscopy demonstrated that AQP2 translocated and fused with the apical membrane supporting the existence of two functionally distinct pools of AQP2-bearing vesicles in renal CD8 cells, one of which is not under the control of 'acute' AQP2 phosphorylation. This hypothesis is sustained by the observation of the partial additive effect of okadaic acid and forskolin in increasing the osmotic permeability coefficient P_f. The observation that okadaic acid stimulates water transport and AQP2 translocation in the absence of any measurable increase in AQP2 phosphorylation, clearly supports the presence of an alternative AQP2 phosphorylation-independent pathway to stimulate AQP2 sorting to the apical membrane. An increased level of protein phosphorylation induced by okadaic acid treatment could mobilize AQP2-containing vesicles unmasking a translocation mechanism that may function normally. The actin cytoskeleton is also regulated by kinases (Hartwig et al., 1992). Therefore a possible explanation for the effect of okadaic acid is that, by altering the phosphorylation state of several proteins, okadaic acid induces cytoskeletal rearrangements that promote vesicle fusion with the apical plasma membrane. In this respect it is known that synaptic vesicles are organized into two distinct functional pools, a large reserve pool in which vesicles are restrained by the actin cytoskeleton and a releasable pool in which vesicles approach the presynaptic membrane and fuse upon stimulation (Benfenati et al., 1999). In nerve terminals, synaptic vesicle clustering depends on the state of protein phosphorylation. In fact, there is strong evidence that synaptic vesicles are immobilized in resting terminals by the binding capacity of dephospho-synapsin I and, during nerve activity, phosphorylation of synapsin I allows the vesicle to be free for fusing (Greengard et al., 1993; Nielander et al., 1997). In frog motor-nerve terminals, it has been shown that vesicle clusters were disrupted by okadaic acid treatment suggesting that the increased level of protein phosphorylation mobilizes synaptic vesicles otherwise clustered in a cytoskeletal 'cage' (Betz and Henkel, 1994). Actin rearrangement induced by okadaic acid treatment in the absence of forskolin stimulation might explain AQP2 translocation in renal cells as well. In amphibian bladder and in rat inner medullary collecting duct it has also been reported that vasopressin significantly depolymerized F-actin indicating that a reorganization of apical actin network might be crucial in promoting fusion of AQP2-bearing vesicles (Holmgren et al., 1992; Simon et al., 1993). Visualization of actin network with phalloidin-FITC in CD8 cells double labeled with AQP2 and treated with either forskolin or okadaic acid would favour this hypothesis (Fig. 5). Compared with untreated cells, okadaic acid treated cells showed very few organized actin filaments and a massive fusion of AQP2 on the apical surface. This effect was clear 10 minutes after okadaic acid administration which correlates closely with the early time course of AQP2 translocation. Okadaic acid effect on actin network was still visible in H89-pretreated CD8 cells, a condition in which AQP2 translocates toward the apical membrane. In contrast, forskolin stimulation does not induce apparent modification in the actin network organization in H89-pretreated CD8 cells, and does not promote AQP2 redistribution as well as AQP2 phosphorylation. This suggest that okadaic acid acts primarily on the cellular machinery that facilitates the movement of AQP2 and other proteins to the cell surface rather than directly on AQP2 protein itself. Moreover, these data indicate that a reorganization of apical actin network might be a prerequisite for promoting redistribution of AQP2-containing vesicles either for forskolin and for okadaic acid effects. In addition, recent findings obtained in adipocytes expressing a regulated exocytosis of the glucose transporter GLUT4, indicate that okadaic acid stimulates GLUT4 translocation (Lawrence et al., 1990; Rondinone and Smith, 1996) and its phosphorylation at ser-488 located at the carboxy-terminal. However, GLUT4 mutated at ser-488 still translocates to the apical membrane after okadaic acid treatment, suggesting that okadaic acid might promote translocation independently of the phosphorylation site of GLUT4 (Marsh et al., 1998). This result would fit with the data obtained in the present work.

In conclusion, the finding that okadaic acid increases water transport through AQP2 translocation independently of AQP2 phosphorylation suggests that different intracellular pools of AQP2-bearing vesicles under different regulatory mechanisms might exist in renal collecting duct epithelial cells. This hypothesis is substantiated by the observation that the effects

of OA and forskolin on P_f were in part additive. This provocative finding suggested that increased serine/threonine phosphorylation of an unidentified protein(s) distinct from AQP2 itself, was involved in the activation of AQP2 trafficking. In this respect this study indicates that exposure of renal cells to okadaic acid appears to produce selective depletion of a subpopulation of AQP2-bearing vesicles suggesting that phosphatases, probably controlling actin filament organization, play a role in AQP2 compartimentalization. Future studies may yield novel information on the possible similarity of AQP2-containing vesicles and synaptic vesicles which are known to be organized into two distinct functional pools regulated in distinct ways by kinases and the actin cytoskeleton.

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