Protein Kinase A Anchoring Proteins Are Required for Vasopressin-mediated Translocation of Aquaporin-2 into Cell Membranes of Renal Principal Cells*

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The antidiuretic hormone arginine-vasopressin (AVP) regulates water reabsorption in renal collecting duct principal cells by inducing a cAMP-dependent translocation of water channels (aquaporin-2, AQP-2) from intracellular vesicles into the apical cell membranes. In subcellular fractions from primary cultured rat inner medullary collecting duct (IMCD) cells, enriched for intracellular AQP-2-bearing vesicles, catalytic protein kinase A (PKA) subunits and several protein kinase A anchoring proteins (AKAPs) were detected. In non-stimulated IMCD cells the majority of AQP-2 staining was detected intracellularly but became mainly localized within the cell membrane after stimulation with AVP or forskolin. Quantitative analysis revealed that preincubation of the cells with the synthetic peptide Ht31, which prevents the binding between AKAPs and regulatory subunits of PKA, strongly inhibited AQP-2 translocation in response to forskolin. Preincubation of the cells with the PKA inhibitor H89 prior to forskolin stimulation abolished AQP-2 translocation. In contrast to H89, S-Ht31 did not affect the catalytic activity of PKA. These data demonstrate that not only the activity of PKA, but also its tethering to subcellular compartments, are prerequisites for cAMP-dependent AQP-2 translocation.

The antidiuretic action of arginine-vasopressin (AVP) is mediated by renal collecting duct principal cells. The water channel aquaporin-2 (AQP-2), which is exclusively expressed in principal cells, belongs to a family of water channel proteins (aquaporins), which greatly increase the osmotic water permeability of biological membranes (1, 2). In resting principal cells AQP-2 is localized on intracellular vesicles. The antidiuretic hormone AVP causes its redistribution from the intracellular compartment to the apical cell membrane and thereby facilitates water reabsorption (3, 4). Mutations in the genes coding for the vasopressin V2 receptor or AQP-2 cause nephrogenic diabetes insipidus, a disease characterized by a massive loss of water through the kidney (5–9).

The binding of AVP to vasopressin V2 receptors (10) on principal cells stimulates cAMP synthesis via the Gs/adenyl cyclase system. Cyclic AMP activates protein kinase A (PKA), which in turn phosphorylates AQP-2 at Ser-256 (11, 12). The functional consequence of this modification is not clear: Kuwahara et al. (11) found a minor increase in AQP-2 water permeability in oocytes injected with AQP-2 mRNA, while Lande et al. (13) did not find an increased water permeability in isolated AQP-2-bearing vesicles after phosphorylation. Elimination of the PKA phosphorylation site of AQP-2 or incubation with the PKA inhibitor H89 prevents AQP-2 translocation in LLC-PK1 cells stably transfected with the water channel (14). These data indicate that the phosphorylation of AQP-2 is important for its subcellular localization rather than for its function. The finding that isolated AQP-2-bearing vesicles contain endogenous PKA activity (13) led to the premise that PKA might be anchored to the vesicle membranes by protein kinase A anchoring proteins (AKAPs), whose function is to localize PKA in close proximity to its substrates (15–19). In the present work it was investigated whether the compartmentalization of PKA by AKAPs is obligatory for AQP-2 translocation. A recently described primary cell culture model of rat IMCD cells (20) was utilized to address this question.

**EXPERIMENTAL PROCEDURES**

Preparation of Peptides—The synthetic peptide Ht31, derived from the human AKAP Ht31 (residues 493–515; Ref. 21), assumes an amphipathic helix structure that binds regulatory PKA subunits. This inhibits binding of regulatory PKA subunits to AKAPs and consequently the tethering of PKA to subcellular compartments. The inactive control peptide Ht31-P, containing prolines at positions 502 and 507, does not assume the amphipathic helix structure and therefore cannot prevent binding of PKA regulatory subunits to AKAPs. For detection of AKAPs with radioactive regulatory RII subunits of PKA in RII overlay experiments, Ht31 and Ht31-P were used without stearate. S-Ht31 and S-Ht31-P were used with stearate. S-Ht31 and S-Ht31-P are coupled to stearate residues and thus rendered membrane-permeable (22); these peptides were used in experiments with intact IMCD cells. All peptides, including AVP, were synthesized on a 433A peptide synthesizer (Applied Biosystems, Weiterstadt, Germany) on Rapp resin columns (Rapp Polymere, Tuebingen, Germany) using Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry. Stearic acid was added to the free N terminus of the protected peptides before cleavage from the resin. The peptides were purified and analyzed by high performance liquid chromatography on Polyenca A300 columns (Bischoff, Leonberg, Germany) and electrospray mass spectrometry (TSQ 700, Finnigan MAT, Bremen, Germany).

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‡‡ The abbreviations used are: AVP, arginine-vasopressin; AQP-2, aquaporin-2; IMCD, inner medullary collecting duct; AKAP, protein kinase A anchoring protein; PKA, protein kinase A; RII, type II regulatory subunit of PKA; H89, N-[2-(isopropinamidamino)ethyl]-5-isoquinolinesulfonamide; LS, low speed pellet; HS, high speed pellet; PAGE, polyacrylamide gel electrophoresis.
Soden, Germany) as indicated.

**Immunofluorescence Microscopy and Quantification of Immunofluorescence Intensities—**AQP-2 was visualized by immunofluorescence microscopy as described (20, 23). Fluorescence signals (xy images) were detected by confocal microscopy with a laser scanning microscope (LSM 410; Carl Zeiss, Jena, Germany). For quantification, the immunofluorescence and transmission images of each cell were scanned with the confocal microscope. These two images were used for determination of the intracellular and cell membrane fluorescence signal intensities with an image analysis software (KS400, Kontron Electronic). Cytoplasm, cell membranes, and nuclei of each cell were marked on the transmission image. The masks obtained were transferred to the immunofluorescence images and immunofluorescence signal intensities were measured in the marked areas. Intracellular and cell membrane signal intensities were related to the signal intensity of the nucleus (subtraction of unspecific immunofluorescence signals derived from the secondary antibody). Then the intracellular-cell membrane signal intensity ratios were calculated. For all groups mean and S.E. values were calculated. Statistically significant differences were determined using the Student’s t test and one way analysis of variance. In addition to xy scans, z scans were carried out to investigate the fluorescence at different levels in z direction.

**Preparation of IMCD Cell Fractions Enriched for Cell Membranes or Intracellular Vesicles, Western Blotting, and Detection of AKAPs—**IMCD cells were either untreated or stimulated with AVP (1 μM). Fractions enriched for cell membranes (low speed pellet (LS), 17,000 × g) or intracellular vesicles (high speed pellet (HS), 200,000 × g) were prepared as described (24, 25). AQP-2 was detected by Western blot with the antisera H27 (1:5,000; Ref. 20). Catalytic PKA subunits were detected with a specific antisera kindly provided by Dr. G. Schwob (1:4,000; University of Gottingen, Germany; Refs. 20 and 28). Alkaline phosphatase-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany) was used as secondary antibody (1:2,500). AKAPs were detected by the RII overlay procedure described previously (27). The overlays were carried out in the presence of either the active peptide Ht31 (10 μM) or the control peptide Ht31-P (10 μM; see above).

**Phosphorylation of Membrane Proteins with [γ-32P]ATP—**HS pellets (25 μg; see above) were resuspended in phosphate-buffered saline and incubated in the presence of [γ-32P]ATP (0.1 mM, 10 Ci/mmol; NEN Life Science Products, Köln, Germany) and exogenous catalytic PKA subunits (20 units/ml; Promega, Mannheim, Germany; Ref. 13). H89, Me2SO, or synthetic peptides were added as indicated. The proteins were separated by 10% SDS-PAGE. Radioactive proteins were detected by autoradiography.

**RESULTS**

**Disruption of the Binding between PKA and AKAPs or Inhibition of PKA Prevents AQP-2 Translocation to the Cell Membranes of Rat IMCD Cells—**Rat IMCD cells were cultured for 6 days as described (20). Prior to stimulation with AVP (1 μM, 30 min) or forskolin (100 μM, 15 min), they were incubated with the membrane-permeable peptide S-Ht31 (100 μM, 30 min), which inhibits the binding of PKA to AKAPs, or with the corresponding (inactive) control peptide S-Ht31-P (100 μM, 30 min). Subsequently, the distribution of AQP-2 was determined by immunofluorescence microscopy. Fig. 1A shows a mainly intracellular distribution of AQP-2 in nonstimulated control cells. After stimulation of the cells with forskolin (Fig. 1B), the AQP-2 staining was mainly present at the basolateral cell membranes as shown by both xy and z scans (20). Preincubation of the cells with S-Ht31 prior to forskolin stimulation strongly inhibited this redistribution (Fig. 1C), whereas preincubation with the control peptide S-Ht31-P inhibited the redistribution of AQP-2 only weakly (Fig. 1D). Preincubation with the PKA inhibitor H89 abolished AQP-2 redistribution (Fig. 1E).

To quantify the effect of the synthetic peptides and of H89, intracellular and cell membrane fluorescence signal intensities were related to nuclear fluorescence signal intensities by laser scanning microscopy (Fig. 2A), and the ratios of intracellular/ cell membrane fluorescence signal intensities were determined (Fig. 2B; see “Experimental Procedures”). Fig. 2B shows two groups of ratios: ratios > 1 were determined for nonstimulated control cells (1.77 ± 0.08; mean ± S.E.) and for forskolin-stimulated cells preincubinated with H89 (1.75 ± 0.05) or S-Ht31 (1.58 ± 0.06), indicating a primarily intracellular localization of AQP-2. Ratios < 1 were determined for forskolin-stimulated cells (0.31 ± 0.02) and for cells preincubated with S-Ht31-P (0.60 ± 0.05) prior to forskolin stimulation, indicating a pre-
A

| Treatment of IMCD cells | absolute fluorescence intensity |
|-------------------------|--------------------------------|
| non-stimulated          | 72.7 ± 5.5                     |
| forskolin               | 35.9 ± 2.5                     |
| S-Ht31 + forskolin      | 79.5 ± 5.2                     |
| S-Ht31-P + forskolin    | 79.7 ± 5.3                     |
| H89 + forskolin         | 73.7 ± 5.2                     |

B

![Graph showing fluorescence intensity ratios](image)

**Fig. 2.** Quantitative analysis of the effects of the synthetic peptides S-Ht31 and S-Ht31-P and the PKA inhibitor H89 on forskolin-induced AQP-2 translocation in IMCD cells. Cells were treated as indicated in Fig. 1, A–E. Immunofluorescence staining of AQP-2 was visualized and quantitatively analyzed by laser scanning microscopy (for details, see “Experimental Procedures”). A, intracellular and cell membrane fluorescence signal intensities were related to the nuclear fluorescence signal intensities for 44 cells under each set of conditions (8-bit scale; mean ± S.E.; three independent experiments). B, the ratios of intracellular/cell membrane fluorescence signal intensities were calculated. Ratios > 1 indicate a predominantly intracellular localization of AQP-2, and ratios < 1 a predominant localization at the cell membrane. Statistically significant differences (p < 0.05) between the ratios are given. Differences between the ratios obtained for nonstimulated cells and forskolin-stimulated cells preincubated with H89 or S-Ht31 were not significantly different.

**Fig. 3.** Phosphorylation of proteins from fractions enriched for intracellular vesicles with [γ-32P]ATP. IMCD cell fractions enriched for intracellular vesicles (HS fraction, 25 μg) were incubated with [γ-32P]ATP in the presence of exogenous catalytic PKA subunits. The proteins were separated by SDS-PAGE, and the gel was autoradiographed (for details, see “Experimental Procedures”). In addition to catalytic PKA subunits the PKA inhibitor H89 (30 μM), MeSO (DMSO) (1%), in which synthetic peptides were dissolved or the synthetic peptides S-Ht31, S-Ht31-P, Ht31, or Ht31-P were added to the reaction mixture as indicated.

**Detection of AQP-2 and Catalytic PKA Subunits in IMCD Cell Fractions Enriched for Intracellular Vesicles or Cell Membranes—**Western blotting was used to demonstrate the presence of AQP-2 and catalytic PKA subunits in fractions from IMCD cells enriched for either cell membranes (LS) or intracellular vesicles (HS; Fig. 4). Catalytic PKA subunits were detected in both fractions, and no change in distribution between the two fractions was observed after stimulation with AVP (and + AVP in Fig. 4) or forskolin (not shown). In contrast, the amount of AQP-2 decreased in the intracellular vesicle fraction after stimulation, indicating a shift to the cell membrane as described previously (20).

**Presence of AKAPs in IMCD Cell Fractions Enriched for Intracellular Vesicles or Cell Membranes—**RII overlay assays of total IMCD cell homogenate and subcellular fractions enriched for cell membranes (LS) or intracellular vesicles (HS) with radioactively labeled RII subunits were carried out (see “Experimental Procedures”). The overlay in Fig. 5A was performed in the presence of the control peptide Ht31-P (or in the absence of peptide, not shown), and that in Fig. 5B in the presence of Ht31. The remaining bands (55 kDa) in Fig. 5B corresponded to regulatory RII subunits. The peptide Ht31 inhibits all binding of the radioactive RII subunits to the membrane-bound proteins, excepting the 55-kDa protein. Thus the bands visible in Fig. 5A represent AKAPs. One AKAP (35 kDa) was found only in the fraction enriched for cell membranes (LS).
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**Fig. 4. Detection of AQP-2 and catalytic PKA subunits (cPKA) in IMCD cell fractions enriched for cell membranes (LS) or intracellular vesicles (HS).** IMCD cells were left unstimulated or stimulated with arginine-vasopressin (AVP; 1 μM) before subcellular fractionation (24, 25). Proteins (30 μg per lane) were separated by SDS-PAGE, blotted onto nitrocellulose, and glycylated, and nonglycosylated AQP-2 (upper and lower bands, respectively) was detected with a specific antiserum (20, 29). Catalytic PKA subunits were detected with an antiserum kindly provided by Dr. G. Schwöch (University Göttingen, Germany; Ref. 28).

**Fig. 5. Detection of AKAPs in subcellular IMCD cell fractions.** Total cell homogenates (lanes 1 and 4) and fractions enriched for cell membranes (LS, lanes 2 and 5) or intracellular vesicles (HS, lanes 3 and 6) were obtained from nonstimulated or AVP-stimulated (−AVP and +AVP, 1 μM) IMCD cells (25). Proteins (120 μg per lane) were separated by SDS-PAGE (10%) and blotted onto nitrocellulose membranes. Regulatory RII subunits of PKA were α2-P-phosphorylated by incubation with γ[32P]ATP and catalytic PKA subunits and hybridized to the filter-bound proteins (27). A, the hybridization was carried out in the presence of the control peptide Ht31-P (10 μM). B, the hybridization was carried out in the presence of the peptide Ht31, which inhibits the binding of regulatory RII subunits to AKAPs (10 μM). and several others (70–80, 95, and >120 kDa) only in the fraction enriched for intracellular vesicles (HS). No change in distribution of AKAPs between the two fractions was observed after stimulation with AVP (− and + AVP in Fig. 5) or forskolin (not shown).

**DISCUSSION**

AKAPs are targeting proteins, which anchor PKA in close proximity to its substrates (15–19). Membrane-permeable peptides (myristylated or stearated) comprising the amphiphilic helix region of Ht31 (21) effectively compete for PKA-AKAP interaction (17). These peptides have been shown to relieve cAMP-dependent inhibition of interleukin-2 transcription (17) and to arrest sperm motility (22). In this study, synthetic H31-derived peptides coupled to stearic acid (S-H31) were used to demonstrate that the targeting of PKA to subcellular compartments via AKAPs is a prerequisite for cAMP-mediated AQP-2 translocation (Figs. 1 and 2).

Incubation of primary cultured IMCD cells with S-H31 strongly inhibited AQP-2 translocation, although less efficiently than incubation with the PKA inhibitor H89 (Fig. 2). The peptide, in contrast to H89, did not inhibit PKA activity in an in vitro phosphorylation assay (Fig. 3). It is therefore highly unlikely that S-Ht31 inhibits PKA activity in IMCD cells. A minor fraction of intracellular AQP-2 may be phosphorylated by the nonnethered, cytosolic PKA which may explain why S-Ht31 inhibits translocation not completely, in contrast to H89. The control peptide S-Ht31-P did not inhibit PKA activity at a low concentration (10 μM) in the in vitro phosphorylation assay, but abolished the activity at the concentration used for incubation of IMCD cells (100 μM, Fig. 3). The apparent discrepancy between complete inhibition of PKA activity in vitro and the small inhibitory effect of S-Ht31-P on AQP-2 translocation in IMCD cells (Figs. 1 and 2) may arise because the intracellular peptide concentration is most probably lower than that of the growth medium. Therefore, inhibition of PKA appears dependent on the amount of S-Ht31-P that actually enters the cell.

The finding that several different AKAPs and catalytic PKA subunits are present in IMCD cell fractions enriched for intracellular vesicles (HS) that contain AQP-2 is consistent with these proteins residing on the same vesicles (Figs. 4 and 5). Since additional AKAPs were, however, also found in the fraction enriched for cell membranes (LS; Fig. 5), it is also possible that the AKAPs required for AQP-2 translocation are present on other cellular compartments, like cell membranes or the cytoskeleton.

There was no decrease in the amount of catalytic PKA subunits or AKAPs in the HS fraction after AVP stimulation, in contrast to AQP-2, which redistributed to the cell membrane (Figs. 4 and 5). Furthermore, the distribution of proteins of about 55 kDa, probably representing regulatory PKA subunits, did not change after stimulation (Fig. 5). The HS fractions may contain minor amounts of cytosol or particulate subcellular compartments other than AQP-2-bearing vesicles, veiling small differences in the distribution of PKA or AKAPs after stimulation (for example in HEK293 cells 90% of PKA RII α- and β-subunits are cytosolic (28)). On the other hand, no redistribution of other proteins than AQP-2 to the cell membrane after stimulation of IMCD cells with AVP or forskolin has been described to date, although a co-enrichment of AQP-2-bearing vesicles and Rab3 proteins (29) and the colocalization of AQP-2, synaptobrevin II, dynin, and dynactin on vesicles have been demonstrated (30, 31, 32). Therefore, delivery of AQP-2 to the apical cell membrane may possibly be followed by immediate recycling of other vesicle-associated proteins.

Recently it was shown that PKA anchoring to AKAPs facilitates glucagon like peptide-1-mediated insulin secretion in primary islets and RINm5F cells (33), underlining the significance of PKA compartmentalization in exocytosis. Members of the cytoskeleton-associated ERM family (ezrin, radixin, and moesin) of proteins (34) have been identified as AKAPs (35). Ezrin seems to be directly involved in the control of gastric acid secretion by parietal cells (35), a cAMP-dependent exocytosis analogous to the translocation of AQP-2 in kidney principal cells. The expression of ezrin in kidney collecting duct epithelium has been demonstrated (36), and we recently showed the presence of moesin in principal cells (37). Ezrin and/or moesin might play a similar role in AQP-2 translocation to that of ezrin in acid secretion. This possibility is being investigated at present. Furthermore, we are attempting to identify the AKAPs detected by the overlay technique in the IMCD cell fraction enriched for intracellular vesicles. Vesicle-associated AKAPs may play a general role in cAMP-dependent exocytic events, including those mentioned above, and also mast cell degranulation or thyroglobulin secretion.

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