AQP2 exocytosis in the renal collecting duct – involvement of SNARE isoforms and the regulatory role of Munc18b

Giuseppe Procino[1], Claudia Barbieri[1], Grazia Tamma[1], Leonarda De Benedictis[2], Jeffrey E. Pessin[3], Maria Svelto[1] and Giovanna Valenti[1,*]

[1]Department of General and Environmental Physiology, University of Bari, Via Amendola 165/A, Bari 70126, Italy
[2]Department of Pharmacology and Human Physiology, University of Bari Medical School, Bari 70124, Italy
[3]Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY 10461, USA
*Author for correspondence (e-mail: g.valenti@biologia.uniba.it)

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Summary

Vasopressin regulates the fusion of the water channel aquaporin 2 (AQP2) to the apical membrane of the renal collecting-duct principal cells and several lines of evidence indicate that SNARE proteins mediate this process. In this work MCD4 renal cells were used to investigate the functional role of a set of Q- and R-SNAREs, together with that of Munc18b as a negative regulator of the formation of the SNARE complex. Both VAMP2 and VAMP3 were associated with immunoisolated AQP2 vesicles, whereas syntaxin 3 (Stx3), SNAP23 and Munc18 were associated with the apical plasma membrane. Co-immunoprecipitation experiments indicated that Stx3 forms complexes with VAMP2, VAMP3, SNAP23 and Munc18b. Protein knockdown coupled to apical surface biotinylation demonstrated that reduced levels of the R-SNAREs VAMP2 and VAMP3, and the Q-SNAREs Stx3 and SNAP23 strongly inhibited AQP2 fusion at the apical membrane. In addition, knockdown of Munc18b promoted a sevenfold increase of AQP2 fused at the plasma membrane without forskolin stimulation.

Taken together these findings propose VAMP2, VAMP3, Stx3 and SNAP23 as the complementary set of SNAREs responsible for AQP2-vesicle fusion into the apical membrane, and Munc18b as a negative regulator of SNARE-complex formation in renal collecting-duct principal cells.

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Key words: Aquaporin2, Vasopressin, VAMP, SNARE, Syntaxin, Munc18, Exocytosis

Introduction

In renal collecting-duct principal cells aquaporin 2 (AQP2) insertion into the apical membrane, in response to circulating vasopressin, allows massive water reabsorption from the pro-urine to the interstitium thus maintaining body-fluid homeostasis (Nielsen et al., 1995a). Secretagogue-stimulated AQP2 exocytosis is mediated by a G-protein-coupled receptor, which in turn activates adenylate cyclase and PKA. AQP2 phosphorylation by PKA at amino acid residue Ser256, together with an increase in the concentration of Ca^{2+} [Ca^{2+}], is thought to be essential to promote fusion of AQP2-containing vesicles from a subapical storage compartment into the apical plasma membrane (Chou et al., 2000; Fushimi et al., 1997; Kamsteeg et al., 2000; Nishimoto et al., 1999; Noda and Sasaki, 2006; Valenti et al., 2005; Van Balkom et al., 2002).

The soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein target receptor (SNARE) hypothesis has been proposed to explain vesicle to membrane fusion in all the steps of the secretory pathway (Jahn and Scheller, 2006). On the basis of this model, vesicle-associated membrane proteins (VAMPs) or synaptobrevins are mainly associated with secretory vesicles. Syntaxins, localized at the target membrane, can form a four-helix bundle along with synaptosome-associated proteins (SNAPs), such as SNAP23 or SNAP25. The latter participate in the formation of the SNARE complex by contributing two SNARE motifs, while the two remaining SNARE motifs are provided by VAMP and syntaxin.

In non neuronal cells, a clear role of the SNARE machinery in hormone-regulated exocytosis has been already demonstrated for the insulin-sensitive GLUT4 transporter (Cheatham et al., 1996; Olson et al., 1997). In renal cells, fusion of AQP2-containing vesicles with the apical plasma might be mediated by a mechanism for exocytosisis, similar to that described in neuronal cells. The presence of SNARE proteins in collecting-duct principal cells, indeed, suggests that, for AQP2-vesicle fusion to occur, SNARE proteins are required. VAMP2 and VAMP3 have been found associated with AQP2-storage vesicles in rat kidney inner medulla (Barile et al., 2005; Franki et al., 1995; Liebenhoff and Rosenthal, 1995; Marples et al., 1995). Syntaxins isoforms have a discrete distribution in the epithelial cells along the renal tubules. In particular Stx3 and Stx4 are restricted to the apical and basolateral domains, respectively, thus indicating a role in the establishment of cell polarity (Li et al., 2002). Moreover, Stx3 expression has been shown to be upregulated during kidney development, suggesting a role in vesicle transport to the apical membrane and maintenance of polarized phenotype (Lehtonen et al., 1999a; Lehtonen et al., 1999b).

Stx2 is expressed at a low level in the collecting duct and its polarity is cell-type dependent: it is basolateral in the cortical

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principal cells but apical in the medullary collecting duct, which suggests a distinct role in the trafficking pathway of cortical and medullary principal cells (Li et al., 2002).

SNAP23 is an ubiquitously expressed SNAP25-related protein that is involved in fusion of vesicles with the plasma membrane in non-neuronal cells. SNAP23 binds to Stx2, Stx3 and Stx4 (Ravichandran et al., 1996), all of which are involved in exocytic trafficking pathways outside the brain. In collecting-duct principal cells, SNAP23 has been found previously both in the apical plasma membrane and in AQP2 vesicles (Inoue et al., 1998).

Yeast genetics has provided evidence that SNARE-complex formation is regulated by proteins of the Sec1 family (Aalto et al., 1992; Ferro-Novick and Jahn, 1994; Halachmi and Lev, 1996). Munc18 is the mammalian analog of the Sec1 protein (Garcia et al., 1994; Hata et al., 1993; Hodel et al., 1994; Pevsner et al., 1994a) that binds to syntaxins. According to the proposed mechanism explaining their role as regulators of SNARE complex formation, Munc18 binds with high affinity to specific syntaxins in a 'closed' conformation, thus preventing their ability to form a physical complex with their cognate SNARE partners (Dulubova et al., 1999; Misura et al., 2000). A conformational switch to an 'open' conformation would allow the formation of the SNARE core complex (Dulubova et al., 1999). Displacement of Munc18 from syntaxin might be regulated by either phosphorylation (Fletcher et al., 1999; Fujita et al., 1996; Liu et al., 2007; Reed et al., 1999; Shuang et al., 1998) or by other proteins such as tomosyn (Fujita, Y. et al., 1998) or Munc13 (Betz et al., 1998).

Three isoforms of Munc18 have been suggested to regulate vesicle fusion to the plasma membrane in different cell types. Munc18a, the neuronal isoform, binds to Stx1, Stx2 and Stx3 thus regulating neurotransmission (Garcia et al., 1994; Hata et al., 1993; Pevsner, 1996; Pevsner et al., 1994b; Verhage et al., 2000). Munc18b is expressed at the apical plasma membrane in the epithelial cells (Hata and Sudhof, 1995; Katagiri et al., 1995; Tellam et al., 1995), where it colocalizes with Stx3 (Lehtonen et al., 1999b; Riento et al., 1996). A third isoform, Munc18c, which is ubiquitously expressed in mammalian cells, preferentially interacts with Stx2 or Stx4 (Grusovin et al., 2000) and has been suggested to be a regulator of insulin-dependent GLUT4 trafficking in adipocytes (Tamori et al., 1998; Tellam et al., 1997; Tellam et al., 1995; Thurmond et al., 1998).

The precise role of Munc18 in regulating exocytosis is still unclear. Although it has been reported that, in yeast, fusion cannot proceed to the formation of the SNARE complex in the absence of Sec proteins (Bryant and James, 2001; Sato et al., 2000), it has been suggested that, in mammals, Munc18c acts as a negative regulator of GLUT4-containing vesicles in 3T3-L1 adipocytes (Kanda et al., 2005; Tamori et al., 1998; Thurmond et al., 1998). A possible role of the Munc18b isoform has been proposed in the regulation of apical membrane transport in CaCo-2 epithelial cells (Riento et al., 1998; Riento et al., 2000), exocytosis of H^+-ATPase in rat inner medullary collecting-duct cells (Nicoletta et al., 2004) and in gastric-acid secretion (Liu et al., 2007).

Here, we describe the identification of a set of SNARE proteins required for the fusion of AQP2 vesicles to the apical membrane. Moreover, we provide compelling evidence for a negative regulatory role of Munc18b in the formation of the apical SNARE complex that catalyzes the fusion between AQP2 vesicles and the apical membrane in mouse collecting-duct cells.

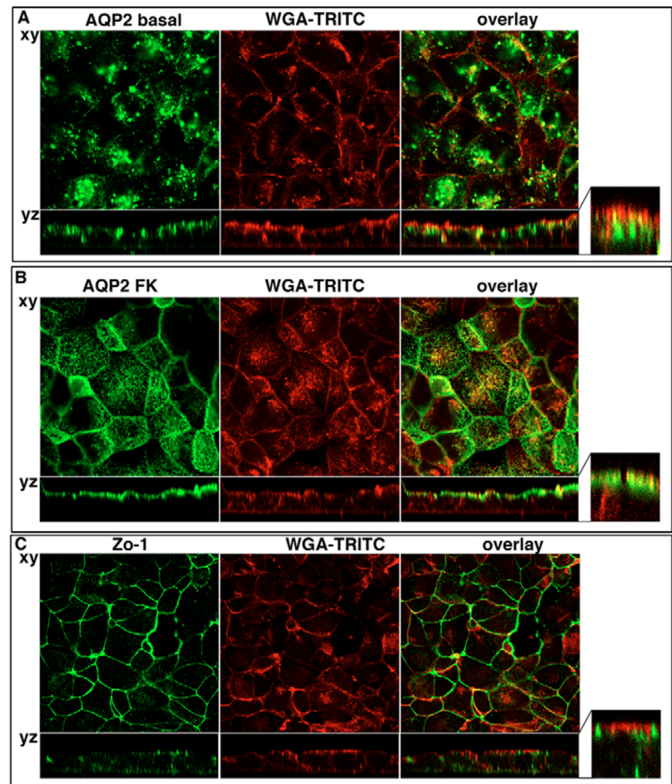

Fig. 1. AQP2 redistribution in response to FK in MCD4 cells. AQP2 trafficking was analyzed in polarized MCD4 cells grown on permeable support to full confluence. Cells were stained with antibodies against AQP2 and ZO-1 (both in green) and counterstained with WGA-TRITC (red) to visualize the plasma membrane. Confocal scans were taken in both the *xy* and *yz* plane. (A) In resting cells, AQP2 was mainly detectable in sub-apical vesicles. (B) After FK treatment, AQP2 localized to the apical membrane with a substantial co-localization with the plasma membrane marker. (C) MCD4 cells cultured on permeable support to full confluence display a positive staining for the tight junction marker ZO-1, indicating a full polarization.

Results

AQP2 trafficking in MCD4 cells

We have recently established a new cell culture model of mouse kidney cortical collecting-duct MCD4 cells (Iolascon et al., 2007), stably transfecting the M-1 cell line (Stoos et al., 1991) with cDNA that encodes human AQP2. When grown for 2-3 days after confluence on permeable supports, MCD4 cells form a polarized epithelium, as demonstrated by positive immunoreactivity for the tight-junction marker ZO-1 (Fig. 1C). In unstimulated cells, although most of AQP2 was located intracellularly, a significant amount of AQP2 was also detectable at the plasma membrane (not shown). This could be due to the high basal activity of adenylate cyclase that results in an increase in cell surface expression of AQP2. The higher adenylate cyclase activity can be due to stimulation of the EP4 receptor by prostaglandin E2 (PGE2) endogenously produced by M-1 cells (Nasrallah et al., 2001; Sandrasagra et al., 2004). To maintain low levels of cAMP at resting condition, cells were preincubated with the cyclooxigenase inhibitor indomethacin before each experiment. After this treatment AQP2 was mainly found in intracellular vesicles localized underneath the apical plasma membrane stained with TRITC-conjugated wheat-germ agglutinin (WGA-TRITC) (Fig. 1A, overlay). In cells stimulated with forskolin (FK), AQP2 strongly

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translocated to the apical plasma membrane. Confocal analysis confirmed AQP2 fusion with the apical plasma membrane because AQP2 staining completely colocalizes with the plasma membrane marker WGA-TRITC (Fig. 1B, overlay).

SNARE and Munc18 isoform expression and localization in MCD4 cells

Next, we evaluated SNARE protein involvement in regulated exocytosis of AQP2 in MCD4 cells. Confocal laser-scanning microscopy (CLSM) analysis performed on polarized cells that had been grown on permeable supports as well as western blot analysis confirmed the expression of SNARE proteins and SNARE-regulating proteins Munc18. As shown in Fig. 2, both VAMP2 and VAMP3 localized in intracellular vesicles as well as in the plasma membrane (Fig. 2A,B). Western blot analysis of subcellular fractions confirmed that VAMP2 and VAMP3 were expressed both in the plasma membrane fraction and in the intracellular vesicles (Fig. 2A',B'). The detected immunoreactive bands co-migrated with the respective homologue that was found in mouse kidney homogenate.

CLSM confirmed that, in MCD4 cells, Stx3 was strictly localized at the apical plasma membrane, whereas Stx4 antibody mainly stained the baso-lateral membrane (Fig. 2C,D). In agreement with this observation, western blotting analysis revealed that both Stx3 and Stx4 were more abundant in the plasma membrane fraction than in intracellular vesicles (Fig. 2C',D').

SNAP23 was found both at the apical plasma membrane and in intracellular vesicles (Fig. 2E). Western blot analysis of subcellular fractions confirmed the presence of SNAP23 in all the subcellular fractions isolated from MCD4 cells. The SNAP23 immunoreactive band migrated at the same molecular mass than that detected by the same antibody in mouse kidney (around 28 kDa). The antibody also stained a higher molecular-mass band in MCD4 cells, which was, however, not present in the mouse kidney sample (Fig. 2E').

Munc18b was selectively expressed at the apical plasma membrane similar to the apical distribution of the plasma membrane marker WGA-TRITC (Fig. 2F, red). Western blotting analysis performed on MCD4 homogenate revealed a clear band of the expected mass (~70 kDa) for Munc18b. The same band was also detected in a homogenate prepared from mouse kidney. Subcellular fractionation followed by immunoblotting showed that Munc18b was almost equally distributed between the plasma-membrane-enriched and the intracellular-vesicle-enriched fractions (Fig. 2F'). A similar pattern of expression and subcellular distribution was found for Munc18c (Fig. 2G,G').

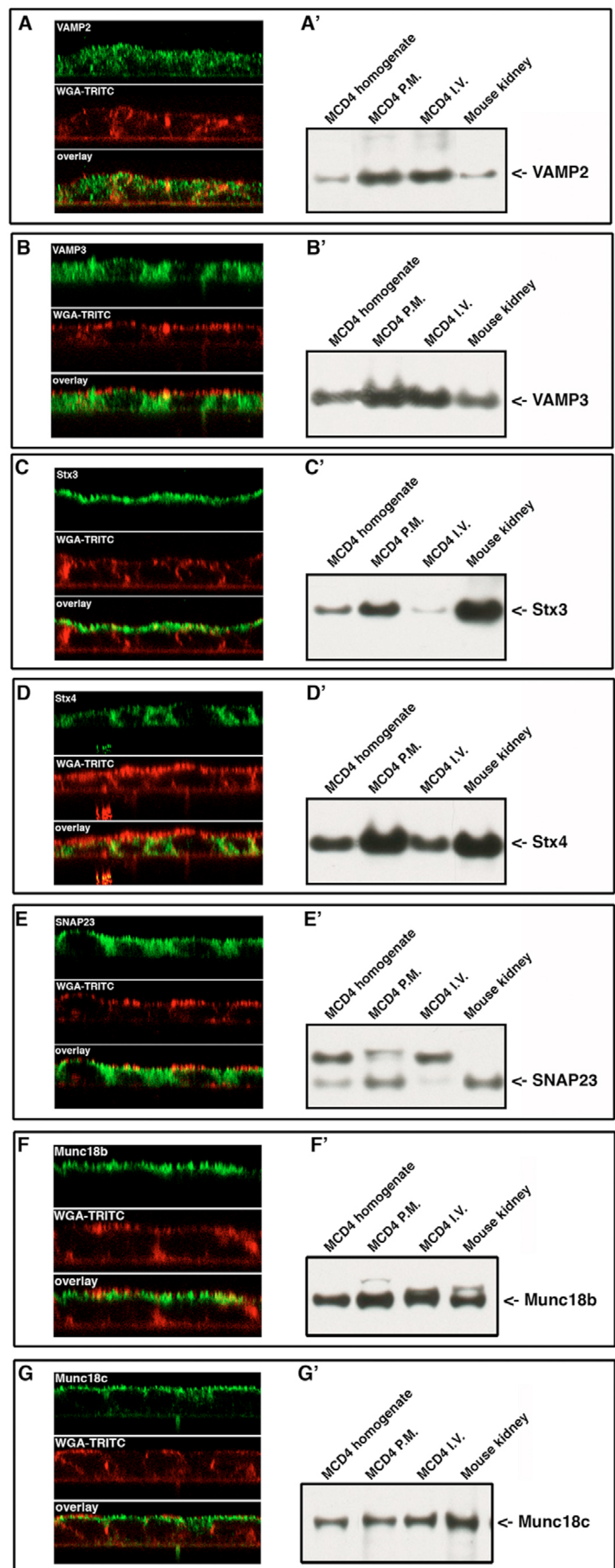

Fig. 2. Subcellular distribution of SNARE and SNARE-regulator Munc18 proteins in MCD4 cells. Subcellular localization of SNARE and Munc18 isoforms was analyzed in MCD4 cells by CLSM and western blotting. (A-G) Polarized MCD4 cells were fixed and stained with antibodies against VAMP2, VAMP3, Stx3, Stx4, SNAP23, Munc18b and Munc18c (all in green) and counterstained with WGA-TRITC (red). Confocal scans were taken in the *yz* plane to analyze the apical vs basolateral distribution of each protein. (A'-G') MCD4 total homogenates, plasma membrane (P.M.), intracellular vesicles (I.V.) and mouse kidney homogenates were separated by NuPAGE electrophoresis and analyzed by western blotting using the same antibodies as above. VAMP2 and VAMP3 were found almost equally distributed between plasma membranes and intracellular vesicles, whereas Stx3, Stx4 localized at the apical and basolateral membrane respectively. SNAP23 was abundantly expressed at both the plasma membrane and intracellularly. Munc18b and Munc18c were both associated with the apical plasma membrane. Same results were obtained in at least three independent experiments.

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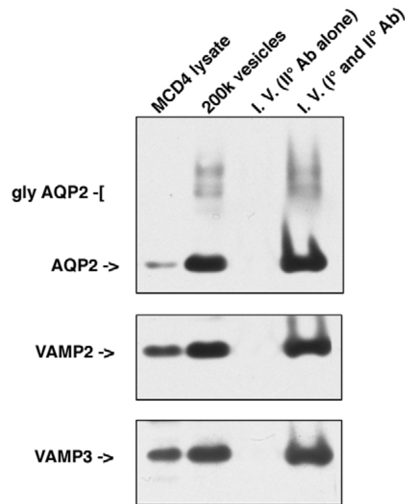

Fig. 3. Immunoisolated AQP2-expressing vesicles contain VAMP2 and VAMP3. Immunoblotting of MCD4 lysate, total intracellular vesicles (200k vesicles), immunoisolated AQP2-bearing vesicles (I° and II° Ab) and negative control (II° Ab alone) probed with antibodies against AQP2, VAMP2 and VAMP3. AQP2, VAMP2 and VAMP3 were expressed and enriched in the immunoisolated vesicles. Results are representative of three independent experiments.

VAMP2 and VAMP3 are both expressed in AQP2-containing vesicles

Recently, proteomic analysis has shown the expression of both VAMP2 and VAMP3 in AQP2 vesicles immunoisolated from rat kidney (Barile et al., 2005). To verify whether both proteins are associated with AQP2-bearing intracellular vesicles in MCD4 cells, equal amounts (30 μg) of MCD4 lysate, intracellular vesicles and immunoisolated AQP2-containing vesicles were separated by NuPAGE electrophoresis and probed with antibodies against AQP2, VAMP2 and VAMP3. The results indicate a dramatic enrichment of AQP2 in the intracellular-vesicle fraction (Fig. 3). Purified AQP2-bearing vesicles that were obtained by using anti-AQP2-antibody-coupled magnetic beads resulted in a very good yield based on the strong signal for AQP2 obtained by western blotting. No AQP2 was detectable when the AQP2 antibody was omitted during the preparation. Both VAMP2 and VAMP3 proteins were found enriched in the AQP2-containing vesicles, which suggests that – also in this cell line – VAMP2 and VAMP3 are expressed together with AQP2 in the same vesicles. To confirm the validity of the immuno-isolation, we show that the plasma membrane marker Na^+/K^+-ATPase is present at low levels in immunoisolated vesicles (and not enriched with respect to total intracellular vesicles that were used as starting material), whereas immuno-isolation yields strong enrichment of AQP2 (supplementary material Fig. S1).

SNARE-complex formation in MCD4 cells

We next tested the hypothesis that Stx3 and SNAP23 expressed on the apical membrane, and VAMP2 and/or VAMP3 on the AQP2-bearing vesicle, could form a physical SNARE complex in MCD4 cells. To address this question, we performed co-immunoprecipitation experiments in MCD4 cells under basal condition or after FK stimulation. Lysates were incubated with antibodies against Stx3 in order to precipitate Stx3 together with its interacting partners. Immunocomplexes were precipitated with protein A-Sepharose and analyzed by western blotting with antibodies against Stx3, VAMP2, VAMP3, SNAP23, Munc18b and Munc18c. The results and the relative densitometric analysis are reported in Fig. 4A,B. Stx3 co-precipitated with substantial amounts of VAMP2, VAMP3, SNAP23 and Munc18b.

A comparable amount of Stx3 was immunoprecipitated from quiescent MCD4 cells or from those after FK stimulation. Whereas the amount of co-precipitated VAMP2 and VAMP3 was unaffected by the FK stimulation, the amount of co-precipitated SNAP23 was

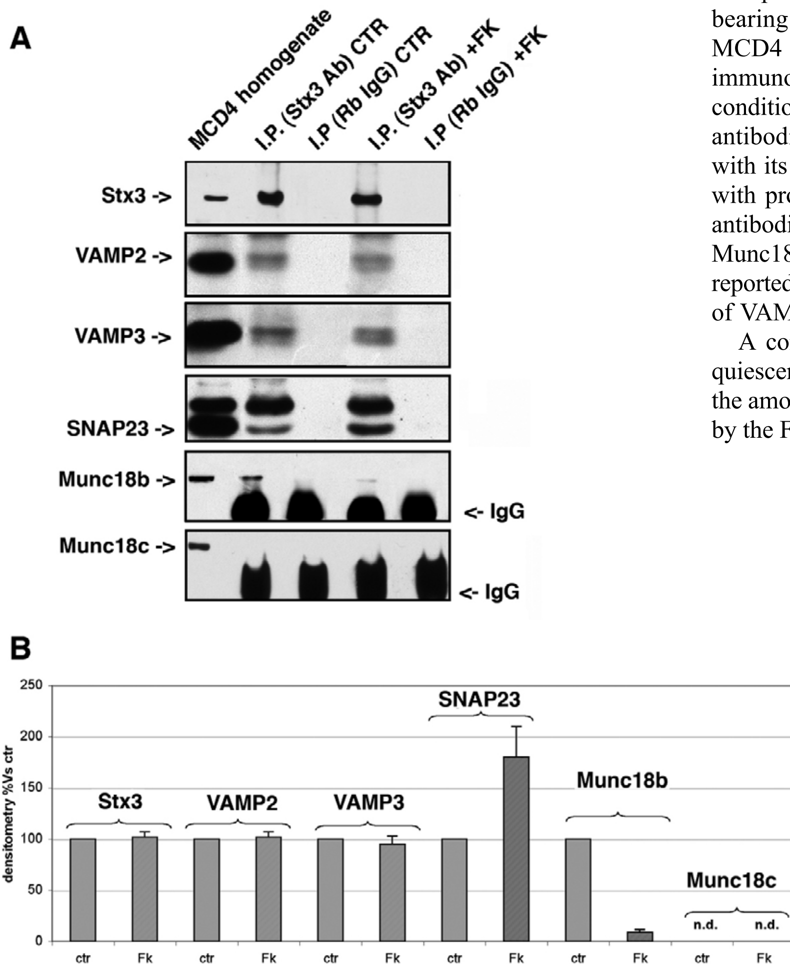

Fig. 4. Apical SNARE complex in MCD4 cells. Anti-Stx3 antibodies or normal rabbit IgG were used for co-immunoprecipitation studies from MCD4 cell lysates. (A) Immunocomplexes were resolved by NuPAGE and immunoblotted with antibodies against Stx3, VAMP2, VAMP3, SNAP23, Munc18b and Munc18c. Anti-Stx3 antibody was very efficient in immunoprecipitating Stx3 protein from MCD4 lysates and no bands were seen in samples incubated with normal rabbit serum (Rb IgG). Immunoblotting performed on the same samples revealed that equal amounts of VAMP2 and VAMP3 co-precipitated with Stx3 in both resting or FK-stimulated MCD4 cells, whereas the association of SNAP 23 or Munc18b with Stx3 was FK-dependent. No band for Munc18c was detectable in Stx3 immunoprecipitates, even after long exposure.
(B) Densitometric analysis of the amount of each protein precipitated from contol- (ctr; set as 100%) or FK-stimulated cells. Experiments were performed three times with comparable results.

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substantially increased after FK stimulation (180±30% of the amount of unstimulated cells, n=3). Conversely, the intensity of the Munc18b band was strongly reduced after FK treatment (9±2.5% of the intensity of the Munc18b band for unstimulated cells, n=3). No Munc18c-corresponding band was observed when the same analysis was performed using antibodies against Munc18c, regardless of whether cells were resting or stimulated. None of the co-precipitated proteins were detected when non-immune serum was used. Na^+K^+ ATPase, EEA1 and calnexin, although abundantly expressed in the starting material, were not precipitated by anti-Stx3 antibodies (data not shown).

These results strongly support the hypothesis of a stable association between Munc18b and Stx3 at the apical plasma membrane in unstimulated MCD4 cells, and a dissociation of this complex after FK stimulation. This result indicates that once Munc18b is displaced from Stx3, SNAP23 can form a binary intermediate acceptor complex, allowing Stx3 to interact with VAMP2 and VAMP3.

Effect of selective SNAREs and Munc18 knockdown on AQP2 translocation to the apical surface

The results of the co-precipitation experiments raise two important questions. The first is whether the SNARE complex formed by Stx3, VAMP2, VAMP3 and SNAP23 can mediate the fusion of AQP2-containing vesicles with the apical membrane. The second is whether Munc18 displacement from Stx3 might be part of a physiological cascade that promotes the association of SNAREs and, hence, AQP2 exocytosis. To address both questions, the protein levels of SNAREs and Munc18 isoforms were reduced by transfecting specific small interfering RNA (siRNA) targeting the respective genes, and the effect of this RNA interference (RNAi) on AQP2 localization was evaluated. Cell-surface biotinylation was used to semi-quantify the effect RNAi had on AQP2 expression at the apical plasma membrane.

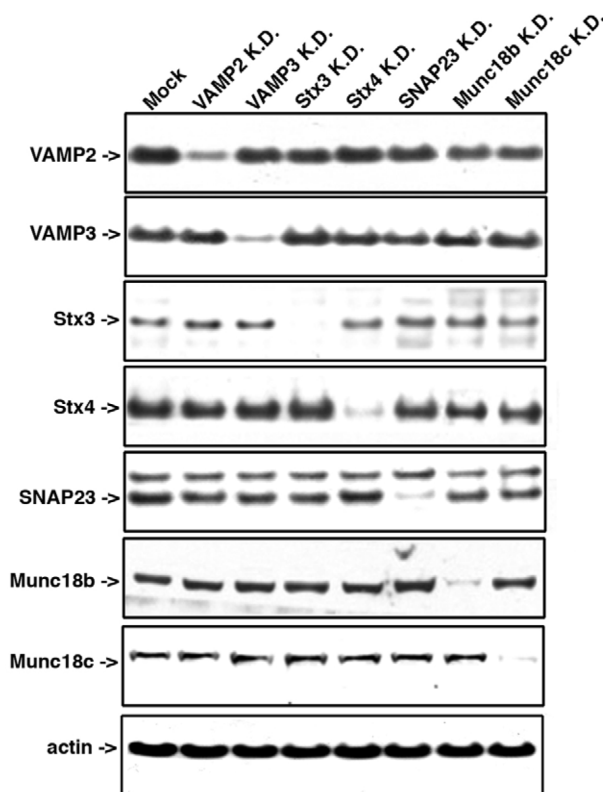

Fig. 5. Selective SNARE and Munc18 isoforms knockdown (K.D.). (A) Endogenous VAMP2, VAMP3, Stx3, Stx4, SNAP23, Munc18b and Munc18c were selectively knocked down using specific siRNAs. Cells were transfected with siRNA specifically targeting each protein (indicated on top of each lane) or with control siRNA (Mock), lysed and analyzed at 48 hours after transfection. 15 μg of each cell lysate were probed with all antibodies against SNARE and Munc18 isoforms (indicated on the left) to confirm the specificity of the knockdown. Protein expression levels were decreased by at least 75%. Actin was used as a loading control.

Using SMARTpool siRNA (Dharmacon), the protein levels of VAMP2, VAMP3, Stx3, Stx4, SNAP23 Munc18b and Munc18c were reduced between 75% and nearly 100% in MCD4 cells (western blot analysis of lysates from MCD4 cells treated with 100 nM siRNA for 48 hours), as illustrated in Fig. 5. siRNA-induced downregulation of each protein was highly specific because only the target protein was reduced 48 hours after siRNA treatment. No change in cell number, shape and viability, and no substantial downregulation of other proteins was seen under any experimental condition.

Fig. 6A,A' and the relative densitometric analysis shown in Fig. 6B,B' demonstrate the effect of RNAi on the amount of AQP2 detectable at the apical surface in both unstimulated (ctr) and FK stimulated cells. In mock-transfected cells, FK stimulation resulted in a nearly fourfold increase of biotinylated AQP2 at the apical surface when compared with unstimulated cells (set as 100%). In cells in which the amount of VAMP2 or VAMP3 was decreased through RNAi, the amount of biotinylated AQP2 was significantly reduced even without FK treatment. Under the same experimental conditions, FK stimulation promoted only a small increase of AQP2 at the cell surface. Interestingly, when VAMP2 and VAMP3 were knocked down at the same time, the effect on AQP2 exocytosis was even more dramatic: AQP2 at the cell surface in resting cells was down to 42% of the amount quantified in mock-transfected cells, and FK stimulation induced only a negligible increase.

SNAP 23 knockdown profoundly affected AQP2 fusion at the cell surface. Under this condition, biotinylated AQP2 was reduced in basal state and a small increase was induced by FK stimulation. Almost complete inhibition of AQP2 expression at the apical membrane was observed in MCD4 cells when Stx3 was knocked down. Strikingly, in Munc18b knockdown cells, the surface expression of AQP2 was dramatically increased (sevenfold) without FK treatment. FK stimulation promoted an additional substantial increase of AQP2 at the cell surface. By contrast, Munc18c knockdown, caused a smaller, although substantial, increase of AQP2 at the cell surface in resting cells. However, the amount of AQP2 detected at the cell surface after FK stimulation was comparable with that measured in mock-transfected cells. Knockdown of basolateral Stx4 had no effect on AQP2 exocytosis thus confirming specificity of siRNA. At the concentration used, none of the siRNAs affected cell viability or total AQP2 content (Fig. 6A,A', total AQP2). Therefore, the Munc18b knockdown results in accumulation of existing AQP2 at the apical plasma membrane without stimulating new AQP2 synthesis. Consistent with these data, visualization of AQP2 by immunofluorescence and analysis by CLSM in MCD4 cells treated with siRNA confirmed that, in Munc18b-silenced cells, the amount of AQP2 at the plasma membrane was dramatically increased (Fig. 7A). By contrast, the Munc18c knockdown had a much weaker effect on AQP2

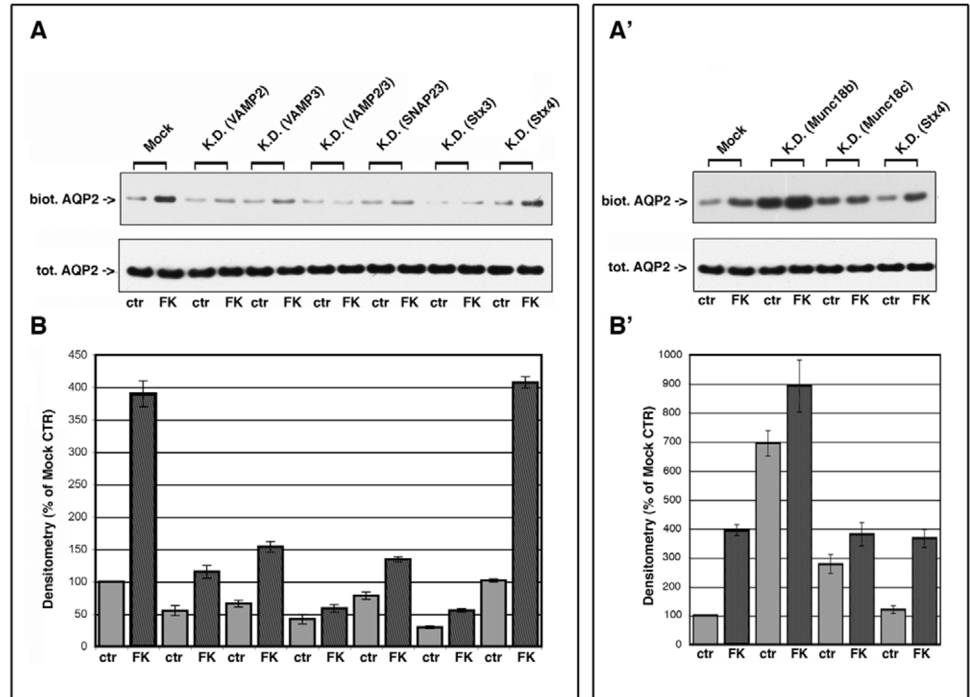

Fig. 6. Effect of SNARE and Munc18 knockdown on AQP2 translocation to the apical surface. Cells silenced for SNARE (left panel) or Munc18 (right panel) isoforms, were subjected to apical surface biotinylation in resting condition (ctr) of after FK stimulation (FK). The amount of biotinylated AQP2 was estimated by western blotting (biot. AQP2). siRNA transfection did not affect the total amount of AQP2 detectable in each sample (tot. AQP2). (C) Densitometric analysis of the 29 kDa biotinylated AQP2 band. Results are expressed as mean values ± s.e. of the values obtained in three independent experiments. The amount of AQP2 detected in mock-transfected cells in resting condition was set as 100%.

expression at the plasma membrane (Fig. 7B). Together, these data strongly support a physiological role of the SNARE proteins VAMP2, VAMP3, Stx3 and SNAP23 in the vesicle fusion events that lead to translocation of AQP2 to the apical membrane in MCD4 cells. They also support the hypothesis that Munc18b acts as a negative regulator of AQP2-vesicle fusion.

Discussion

The results described here provide the first compelling evidence for the functional involvement of VAMP2 and/or VAMP3, Stx3 and SNAP23 in AQP2-vesicle fusion with the apical membrane, and for Munc18b to act as a negative regulator of SNARE-complex formation in the renal collecting-duct principal cells. A number of studies have previously indicated VAMP2 and VAMP3 as the R-SNARE associated with the AQP2-storage compartment, suggesting their involvement in the vesicle to membrane fusion event (Barile et al., 2005; Franki et al., 1995; Gouraud et al., 2002; Nielsen et al., 1995b). However, full information regarding the role of SNAREs proteins and regulators, and the specific isoforms involved in AQP2 exocytosis in kidney, was missing. Here, we used AQP2-expressing collecting-duct MCD4 cells to obtain further insight. We found that, both VAMP2 and VAMP3 sorted and highly enriched in immuno-isolated AQP2-bearing vesicles. Of note, VAMP2 and VAMP3 knockdown dramatically reduced the amount of AQP2 expressed at the apical plasma membrane under both unstimulated and FK-stimulated conditions. Moreover, AQP2 apical expression was almost completely abolished when cells were silenced for both VAMP2 and VAMP3, suggesting that the lack of one of the two VAMP proteins can be partially rescued by the other one. These data are in agreement with previous findings, which

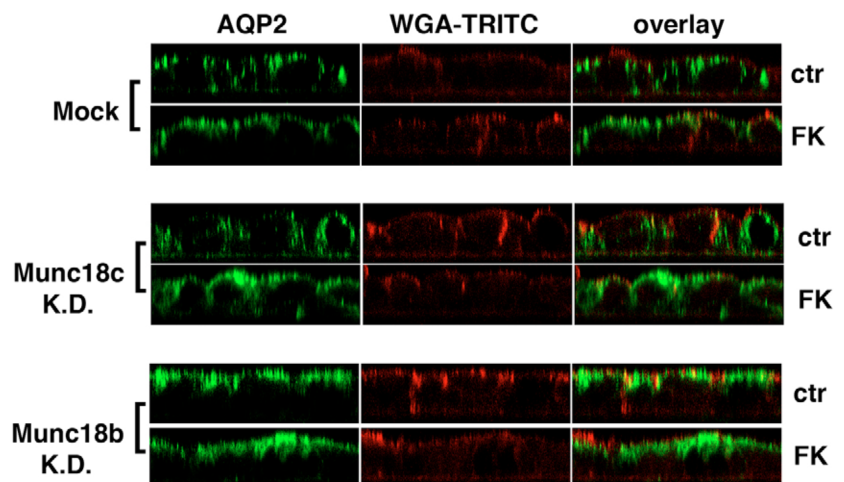

Fig. 7. CLSM analysis of AQP2 translocation in MCD4 cells silenced for Munc18b and Munc18c. AQP2 trafficking was analyzed in polarized MCD4 mock-transfected or after transfection with siRNA targeting Munc18b ad Munc18c. Cells were stained with antibodies against AQP2 and counterstained with WGA-TRITC (red). Confocal scans were taken in the *yz* plane. In mock transfected and in Munc18c-silenced cells AQP2 was found mainly associated with subapical vesicles and translocated towards the apical membrane after FK treatment. Munc18b knockdown produced a clear accumulation of AQP2 at the apical plasma membrane, even without FK stimulation.

demonstrate a partial compensatory role of VAMP3 in VAMP2 knockout mice during synaptic-vesicle fusion (Borisovska et al., 2005).

Regarding the mutual role of VAMP2 and VAMP3 in AQP2 exocytosis, we speculate that VAMP2 is sorted – together with AQP2 – on vesicles ready for regulated fusion, whereas VAMP3 resides in the endosomal compartment that is involved in the re-exocytosis of recycling endosomes. This is the case for GLUT4 translocation in 3T3-L1 and L6 cells (Randhawa et al., 2000). In a second and more possible scenario, VAMP2 and VAMP3 are sorted to the same AQP2-containing vesicle, and are both able to form productive SNARE complexes with plasma-membrane-associated SNAREs. The effect on AQP2 fusion of silencing either VAMP2 or VAMP3 is partially rescued by the presence of either VAMP3 or VAMP2, respectively. The presence of both VAMP2 and VAMP3 in immunoisolated AQP2 vesicles is in accordance with both of the above mentioned scenarios, but the finding that Stx3 precipitates together with VAMP2 and VAMP3 strongly supports the second option. Further studies need to be conducted to better identify the endosomal compartment(s) to which VAMP2 and VAMP3 are sorted in renal cells.

The second important finding presented here is the identification of apical Stx3 as the Qa-SNARE, which is functionally involved in the fusion of AQP2 to the apical membrane. In MCD4 cells we found Stx3 and Stx4 specifically expressed on the apical and basolateral plasma membrane, respectively. Stx3 apical expression has been demonstrated in kidney (Lehtonen et al., 1999b; Li et al., 2002), as well as in many other cell lines including MDCK (Low et al., 1996; Sharma et al., 2006) and CaCo-2 colon epithelial cells (Delgrossi et al., 1997; Galli et al., 1998; Riento et al., 1998) and in hepatocytes (Fujita, et al., 1998). Stx4 basolateral localization has been verified in kidney, MDCK cells (Li et al., 2002) and pancreatic acinar cells (Gaisano et al., 1996).

Three lines of evidence in this paper strongly support the indication of Stx3 as the membrane Qa-SNARE acting as acceptor of AQP2 vesicles in MCD4 cells: (1) Stx3 is exclusively expressed on the apical membrane; (2) Stx3 co-precipitates with VAMP2, VAMP3 and SNAP23; (3) treatment with RNAi of Stx3 dramatically affects fusion of AQP2 to the apical membrane. We found that a member of a third class of SNARE proteins, the Qbc-SNARE SNAP23, is a functional partner of VAMP2 and VAMP3 and Stx3, and involved in fusion of AQP2 vesicles to the apical plasma membrane. The possibility that SNAP23 forms a binary intermediate acceptor together with Stx3 is supported by co-immunoprecipitation experiments, which indicate that it can form a physical complex with Stx3 and that the amount of SNAP23 precipitating together with Stx3 is significantly increased after FK stimulation. The functional role of SNAP23 in AQP2 fusion is clearly unmasked after the RNAi experiments in MCD4 cells.

Taken together, the data of the direct functional analysis demonstrate the regulatory role of VAMP2, VAMP3, Stx3 and SNAP23 in the FK-stimulated translocation of AQP2 intracellular vesicles to the plasma membrane in renal collecting-duct cells. The presence of a SNARE complex formed by Stx3, VAMP3 and SNAP23, and by Stx3, VAMP2 and SNAP23 have already been reported in CaCo-2 cells by Galli et al. (Galli et al., 1998) and in dense-core vesicles (DCV) (Scales et al., 2000). Another novel finding is the demonstration of the role of Munc18b isoform, a functional partner of Stx3, as a negative regulator in the FK-stimulated exocytosis of AQP2 to the apical membrane.

Munc18b association with Stx3 at the apical membrane in the kidney has been shown previously in adult kidney and in developing kidney (Lehtonen et al., 1999a), and in MDCK cells (Sharma et al., 2006). Interestingly, both proteins are simultaneously upregulated during kidney development, which indicates that they function in concert to regulate membrane transport and/or polarity. MCD4 cells express the two non-neuronal isoforms of Munc18: Munc18b (the epithelial-specific isoform) and Munc18c (the ubiquitously expressed isoform). Both proteins were identified to localize at the apical plasma membrane by using CLSM. Since it is known that Munc18b preferentially interacts with Stx3 (Lehtonen et al., 1999b; Riento et al., 1996), its apical expression in MCD4 cells might reflect the association with Stx3 as already demonstrated in kidney (Lehtonen et al., 1999a) and in epithelial cells including CaCo-2 (Riento et al., 1998). Since Munc18c preferentially interacts with Stx2 and Stx4 (Grusovin et al., 2000) – both proteins localized at the basolateral membrane in the kidney cortical collecting-duct cells – its apical localization in MCD4 might reflect a low-affinity interaction with Stx3, with other syntaxins or with other attachment proteins.

Two important results favor the hypothesis that the displacement of Munc18b from Stx3 is part of a physiological cascade that leads to AQP2 fusion at the plasma membrane: (1) co-immunoprecipitation experiments performed in MCD4 cells indicate that a stable physical interaction occurs between Stx3 and Munc18b rather than with Munc18c; (2) FK stimulation clearly decreases the association between Stx3 and Munc18b, and increases concomitantly the association between Stx3 and SNAP23. The observation that reduced levels of Munc18b and, to a lesser extent, Munc18c per se induced the accumulation of AQP2 at the plasma membrane strongly suggests a negative role of Munc18b in regulating Stx3 availability to form SNARE complexes. It is well-known that, in renal cells, AQP2 exocytosis proceeds at a low rate even in the absence of cAMP-elevating agents (Katsura et al., 1996; Gustafson et al., 2000). In this condition, removal of a negative effector, preventing spontaneous fusion, would result in AQP2 accumulation at the plasma membrane.

A univocal role for Sec/Munc proteins in the regulation of membrane fusion is not well established. This is because of their dual role of being essential for exocytosis and to be able to prevent SNARE-complex formation by stabilizing syntaxins in their 'closed' conformation. It has been shown in yeast that fusion cannot proceed to SNARE-complex assembly in the absence of Sec/Munc proteins (Bryant and James, 2001; Sato et al., 2000). Recently, using an in-vitro fusion assay, it has been demonstrated that Munc18a interacts with neuronal and/or exocytic SNARE complexes and that it accelerates fusion (Shen et al., 2007). This role of Munc18a, in contrast to the one we propose here, could either be synapse-specific or might result from the simplification of the cellular milieu – which could exalt a particular function of Munc18. By contrast, a negative regulatory role for non-neuronal Munc18 isoforms has been proposed for GLUT4 exocytosis in adipocytes (Macaulay et al., 2002; Tamori et al., 1998; Thurmond et al., 1998), insulin exocytosis in β-cells (Zhang et al., 2000), exocytosis of human platelets (Houng et al., 2003), the apical delivery of influenza virus hemagglutinin (HA) in CaCo-2 cells (Riento et al., 2000) and H^+-ATPase exocytosis in rat IMCD (Nicoletta et al., 2004). More recently, it has clearly been shown that adipocytes derived from Munc18c$^{-/-}$ mice display an increased sensitivity to insulin-stimulated fusion of GLUT4 with the plasma membrane (Kanda et al., 2005). The generation of homozygotic knockout mice for

Munc18a and Munc18c have resulted in post-natal and early embryonic lethality (Kanda et al., 2005; Verhage et al., 2000), thus pointing to new and undiscovered roles of Munc18 proteins. So far, Munc18b-knockout mice have not been generated. If they are not lethal, the analysis of the of Munc18b$^{-/-}$ phenotype would be helpful in understanding the physiological role of this protein in AQP2 trafficking.

This study provides the first functional evidence that, (1) VAMP2 and VAMP3, Stx3 and SNAP23 are essential in the fusion of AQP2 vesicles to the apical membrane; (2) Munc18 acts as a negative modulator of SNARE complex formation, by limiting the amount of free Stx3 that can associate with SNAP23 and VAMP2/3 to participate in the fusion process. These observations are of particular physiological relevance because the studied SNAREs proteins were not overexpressed but reflected the cell-biological function of the endogenous proteins. This opens the possibility that Munc18b and Stx3 are candidate therapeutic targets in order to increase the availability of AQP2 water channels in the membrane of collecting-duct cells of those patients affected by nephrogenic diabetes insipidus (NDI).

Materials and Methods

Antibodies and reagents

Antibodies against mouse VAMP3, syntaxin4 and SNAP23 were purchased from Synaptic System. Antibodies against VAMP2, Syntaxin3 and Munc18b were from Abcam. Rabbit polyclonal antibodies against Munc18c were prepared as previously described (Thurmond et al., 1998). Affinity-purified rabbit polyclonal antibodies against human AQP2 were prepared as described elsewhere (Tamma et al., 2007). Anti ZO-1 antibodies were from Zymed.

siRNA (SiGENOME SMART pool) specifically targeting mouse VAMP2 (M-041975, GenBank accession number NM_009497), VAMP3 (M-057724, GenBank accession number NM_009498), Syntaxin3 (M-044346, GenBank accession number NM_011502), Syntaxin4 (M-062379, GenBank accession number NM_009294), SNAP23 (M-058871, GenBank accession number NM_009222) Munc18b (M-062780, GenBank accession number NM_011503) and Munc18c (M-044349, GenBank accession number NM_011504) were from Dharmacon. The sequences of the SMARTpool siRNA are proprietary.

Cell culture

Mouse cortical collecting-duct MCD4 cells, stably expressing human AQP2 were generated as described elsewhere (Iolascon et al., 2007) and maintained in DMEM/F12 1:1 supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 i.u./ml penicillin, 100 μg/ml streptomycin and 5 μM dexamethasone until sub-confluent. For all experiments, cells were treated with indomethacin 5×10^{-5} M over night in the culture medium to reduce the basal cAMP concentration. For RNAi and biotinylation experiments cells were plated on six-well 0.4-μm pore size cell culture inserts (Becton Dickinson Labware) at a density of 25×10^4 cells per filter and allowed to grow for 16 hours before the experiment.

Immunofluorescence

For immunofluorescence experiments, MCD4 cells were grown on porous filters and used 2 days after full confluence. For cells transfected with siRNA, cells were used 48 hours after transfection. Cells were treated with 10^{-4}M forskolin or not in the culture medium for 20 minutes. Monolayers were then fixed with 4% PFA in PBS for 20 minutes at room temperature and washed twice for 5 minutes in PBS. The apical plasma membrane was labeled by incubating cells with WGA-TRITC (Sigma) diluted 1:500 in PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 15 minutes at room temperature followed by additional three washes in PBS. Antigen retrieval was carried out to better expose protein epitopes. Briefly, monolayers were treated with 0.5% SDS in PBS for 5 minutes, then washed three times in PBS. Cells were blocked with 1% BSA in PBS then the primary antibodies were diluted in PBS-BSA and incubated for 2 hours at room temperature. Bound antibodies were detected with Alexa-Fluor-conjugated donkey anti-rabbit IgG antibodies (Invitrogen). All incubations were performed from both sides of the filters. Filters were excised from the support, mounted on microscope slides and viewed with a Leica confocal microscope TSC-SP2.

Cell fractionation and cell homogenization

Plasma membrane and intracellular vesicles from MCD4 cells were prepared using a standard method for cell fractionation from kidney (Marples et al., 1995). Cells were resuspended in homogenizing buffer (300 mM sucrose, 25 mM imidazole, 1 mM EDTA, 1 mM PMSF, protease cocktail inhibitor, pH 7.2) and homogenized with 5 strokes in a Potter-Elvehjem at 1,250 rpm. The homogenate was spun at 4,000 ***g*** for 15 min and the pellet discarded. The supernatant was spun at 17,000 ***g*** for 30 min to obtain a pellet enriched in plasma membranes. The resulting supernatant was centrifuged for 1 hour at 200,000 ***g*** to obtain an intracellular vesicle fraction. Fractions were assayed for protein content and stored at –80°C. MCD4 and mouse kidney homogenates were prepared in RIPA Buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% Deoxycholate, 5 mM EDTA).

Immunoisolation of AQP2-containing intracellular vesicles

Dynabeads M-500 (Dynal, Invitrogen) were coated with goat anti-rabbit IgG Fc specific (Biodesign), and then coupled to affinity-purified rabbit anti-AQP2 polyclonal antibodies. All steps were performed according to the manufacturer's protocol. Beads coated with goat anti-rabbit IgG alone were used as negative control for immunoisolation. Intracellular vesicles were prepared from cultured MCD4 cells as described before and incubated overnight at 4°C with Dynabeads (coupled or not with anti-AQP2 Ab) under rotation. Beads were washed and proteins eluted with NuPAGE LDS sample buffer (Invitrogen) containing 100 mM DTT, heated at 95°C for 10 minutes and resolved on 4-12% NuPAGE gels.

Gel electrophoresis and Immunoblotting

Cellular proteins (homogenates or subcellular fractions) were separated on 4-12% NuPAGE Bis-Tris gels under reducing conditions. Protein bands were electrophoretically transferred to ImmobilonP membranes (Millipore) for western blot analysis, blocked in TBS-Tween containing 3% BSA and incubated with primary antibodies. Immunoreactive bands were detected with secondary antibody conjugated to horseradish peroxidase (HRP). After each step, the membranes were washed with TBS-Tween. Membranes were developed with SuperSignal West Pico Chemiluminescent Substrate (Pierce) and were exposed to autoradiographic Kodak Biomax XAR film, (Sigma). Band intensities were quantitated by densitometric analysis using National Institutes of Health (NIH) ImageJ software.

Co-immunoprecipitation

MCD4 were cultured in 20-cm diameter Petri dishes as described before, prior to being treated 10^{-4} M FK or not with for 20 minutes at 37°C. Cells were washed twice in ice-cold PBS and cells scraped into 1 ml of ice-cold EBC lysis buffer [50 mM Tris pH 8.0, 120 mM NaCl, 0.5% NP-40, 0.2 mM sodium orthovanadate, 100 mM sodium fluoride, 2 mM phenylmethylsulphonyl fluoride (PMSF) and protease inhibitor cocktail]. Lysates were clarified by centrifugation at 13,000 ***g*** for 15 minutes at 4°C. Supernatants were pre-cleared with 100 μl of protein A-sepharose suspension (Sigma) for 1 hour under rotation at 4°C. Pre-cleared lysates were incubated with 15 μl of anti-Stx3 antibody or with the same amount of normal rabbit serum for 3 hours at 4°C under rotation. Immunocomplexes were bound using 100 μl of protein A-sepharose suspension for 2 hours at 4°C and washed five times in NETN buffer (20 mM Tris pH 8.0, 1 mM EDTA, 900 mM NaCl, 0.5% NP-40) and once in NETN buffer containing 100 mM NaCl. After complete removal of the washing solution, immunocomplexes were dissociated in NuPAGE LDS sample buffer with 100 mM DTT, heated at 95°C for 10 minutes and resolved on 4-12% NuPAGE gels.

After transfer to Immobilon P membrane, lanes were probed with antibodies against Stx3, VAMP2, VAMP3, SNAP23, Munc18b and Munc18c as described above.

RNAi

For RNAi, cells cultured in six-well-cell permeable inserts until 30% confluent, were treated twice on two consecutive days with 5 μl of Lipofectamine 2000 reagent (Invitrogen) only or with 100 nM siRNA targeting VAMP2, VAMP3, Syntaxin3, Syntaxin4, SNAP23, Munc18b and Munc18c. Cells were then cultured as described above, only that antibiotic-free medium was uses as culture medium. Cells were lysed 48 hours later for immunoblotting analysis or used for AQP2 translocation assay described below.

Apical surface biotinylation

Forty eight hours after transfection with or without siRNAs, cell monolayers were treated with 10^{-4} M FK or not for 10 minutes in the culture medium at 37°C.

Filters were then rapidly washed twice in ice-cold EBS buffer for biotinylation (10 mM triethanolamine pH 9.0, 150 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$) and the apical side was incubated with 650 μl of 2.5 mg/ml EZ-link Sulfo NHS-biotin (Pierce) in EBS buffer on ice for 30 minutes. Filters were washed twice in ice-cold PBS-CM and unbound biotin was quenched for 10 minutes in quenching buffer (50 mM NH_4Cl in PBS-CM) on ice. Cells were scraped from filters in 500 μl of lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% BSA, 1 mM PMSF, protease inhibitors cocktail), lysates were sonicated twice for 15 seconds and incubated at 37°C for 20 minutes. Insoluble material was pelleted at 13,000 ***g*** for 10 minutes and biotinylated proteins in the supernatants were precipitated for 16 hours with 50 μl of Immunopure immobilized-streptavidin bead suspension (Pierce) under rotation at 4°C. Beads from each condition were washed three times in complete lysis buffer and three times in lysis buffer without BSA. Biotinylated proteins were extracted in 30 μl of NuPAGE LDS Sample buffer with 100 mM DTT, heated at 95°C for 10 minutes and resolved on 4-12% NuPAGE gels.

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