Functional involvement of VAMP/synaptobrevin-2 in cAMP-stimulated aquaporin 2 translocation in renal collecting duct cells

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Summary

The involvement of soluble N-ethylmaleimide sensitive factor-attachment protein receptor (SNARE) proteins in the cAMP-induced exocytosis of aquaporin 2 (AQP2)-containing vesicles was investigated in AQP2-transfected renal CD8 cells. RT-PCR and western blot analysis confirmed the presence of the SNARE homologs VAMP/synaptobrevin-2, syntaxin-1, syntaxin-4 and SNAP-23 in CD8 cells. Tetanus neurotoxin (TeNT) was efficient in cleaving synaptobrevin-like protein both in vitro and in intact CD8 cells incubated with the toxin. TeNT treatment in intact CD8 cells completely abolished cAMP-stimulated AQP2 targeting to the plasma membrane, as assessed by quantification of cell-surface immunoreactivity to an anti-AQP2 antibody raised against a peptide reproducing the extracellular AQP2 C-loop.

These results represent the first evidence for the functional involvement of VAMP-2 in cAMP-induced AQP2 exocytosis in renal cells.

Key words: Aquaporin, SNARE, Tetanus toxin, Trafficking, Exocytosis

Introduction

In the kidney collecting duct, water reabsorption is regulated by exocytotic insertion and endocytotic retrieval of the water channel aquaporin 2 (AQP2). AQP2 resides in intracellular vesicles that fuse with the apical membrane of collecting duct principal cells in the presence of the antidiuretic hormone vasopressin (Knepper and Inoue, 1997; Klussmann et al., 2000). Prominent candidates for proteins that provide specificity to polarized sorting events are soluble adapter molecules, the N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. The specificity of this process is achieved by pairing SNAREs in carrier vesicles [v-SNAREs, recently reclassified as Q-SNAREs (Fasshauer et al., 1998)] with their cognate SNAREs in the target membrane (t-SNARE, now named R-SNAREs) (Aroeti et al., 1998; Rothman, 1994; Rothman and Wieland, 1996). Both Q-SNAREs and R-SNAREs have been identified in targeting processes from the Golgi to the plasma membrane in neurons and yeast (Rothman, 1994). It is likely that the exocytotic event that regulates translocation of AQP2 in renal cells utilizes a mechanism for exocytosis that is similar to that described in neuronal cells. Consistent with this hypothesis, previous studies documented the expression of SNARE proteins in kidney collecting duct cells (Jo et al., 1995; Liebenhoff and Rosenthal, 1995; Nielsen et al., 1995; Mandon et al., 1996; Inoue et al., 1998). However, to date, no studies have provided direct evidence for the functional involvement of SNARE proteins in the regulated exocytosis of the AQP2 water channel.

To investigate whether SNAREs participate in exocytosis of AQP2, we used AQP2-transfected CD8 cells (Valenti et al., 1996), which are an ideal model for studying the molecular basis of exocytosis in non-excitable tissue (Valenti et al., 1998; Valenti et al., 2000; Tamma et al., 2001), and we have determined the expression of SNAREs. In addition, we have investigated the effect of tetanus neurotoxin (TeNT) on cAMP-induced AQP2 translocation in intact CD8 cells. The present study provides clear evidence that the SNARE protein VAMP/synaptobrevin-2 (VAMP-2) is functionally involved in cAMP-stimulated AQP2 translocation in renal collecting duct cells.

Materials and Methods

Antibodies

Monoclonal antibodies directed against human VAMP-2 and rabbit polyclonal antibodies corresponding to residues 2-23 of rat or mouse syntaxin-4 were purchased from Chemicon International, Inc, Temecula, CA. The rabbit anti-VAMP-2 polyclonal antibody (residues 36-56) was purchased from StressGen Biotechnologies Corp. Rabbit polyclonal antibodies recognizing syntaxin-1A and 1B and rabbit polyclonal antibodies recognizing SNAP-25 were kind gifts from

Prof. Montecucco (Department of Biomedical Sciences, University of Padova, Italy). The polyclonal antibody raised against a C-terminal peptide of SNAP-23 was kindly provided by Dr Knepper, NHI, Bethesda, MD. Secondary goat anti-rabbit and anti-mouse IgG were conjugated to peroxidase. The specificity of AQP2 antiserum generated against a synthetic peptide corresponding to the AQP2 C-terminus has been described previously (Valenti et al., 1996).

Rabbit polyclonal antibodies specifically directed against the external C-loop of AQP2 were raised using a peptide (IRGDLAVNALSNSTT) reproducing the C-loop of the human AQP2.

Cell culture

The study was performed on AQP2-transfected renal CD8 cells (Valenti et al., 1996). This cell line was established by stably transfecting the RC.SV3 rabbit cortical collecting duct with cDNA encoding rat AQP2. This cell model system possesses the key properties of principal cells and has been useful to clarify some key events triggered by vasopressin action (Valenti et al., 1998; Valenti et al., 2000; Tamma et al., 2001). CD8 cells were grown at 37°C, as described previously, in a hormonally defined medium (Valenti et al., 1996). Confluent monolayers were used at days 3-5 after plating.

RNA isolation, RT-PCR and cDNA sequencing

Total RNA was extracted from confluent CD8 cells or rat brain, lung or kidney by the TRIzol extraction method (TRIzol reagent, Life Technologies, Gaithersburg, MD). The RNA was then used to amplify fragments of the cDNA of the SNARE isoforms syntaxin-1, -3, -4, VAMP-2 and SNAP-23 by degenerate RT-PCR employing the GeneAmp RNA PCR Core kit (Perkin-Elmer, Branchburg, NJ). The degenerate primers (Table 1) were designed on the basis of the SNARE nucleotide sequences available in the GenBank database (web site: www.ncbi.nlm.nih.gov/Entrez/nucleotide.html). A positive control was performed by using primers specific for *β-actin* cDNA, BAF (5′-CAGATCATGTTTGAGACCTT-3′) and BAR (5′-CGGAT-GTCMACGTCACACACTT-3′). PCRs were performed with the following program: (95°C, 3 minutes)×1 cycle, [94°C, 1 minute; 49 or 59°C (depending on the non-degenerate or degenerate primers used, respectively) for 1 minute; (72°C, 30-50 seconds)]×32 cycles, (72°C, 10 minutes)×1 cycle. The SNARE cDNAs amplified from the CD8 cells were cloned into the *Eco*RI/*Eco*RI site of the pCR2.1 vector (TA cloning kit, Invitrogen, San Diego, CA) following a TA strategy. The sequence of the cloned DNA fragments was assessed by sequencing. Sequence alignments were performed by using the Lasergene program (DNASTAR, UK).

Construction of the GFP/AQP2 expression plasmid and transfection

Total RNA extracted from rat kidney was employed to amplify the AQP2 cDNA by using the specific primers AQP2 RAT-FW 5′-ATGTGGGAACTCAGATCCATA-3′ and AQP2 RAT-REV 5′-TTCTTGAGGCTCACTGCACT-3′. The AQP2 cDNA was then cloned into the pcDNA3.1/NT-GFP-TOPO vector (GFP Fusion TOPO TA Expression kit, Invitrogen) in frame and at the 3′ end of the GFP coding region. The resulting plasmid, pcDNA3.1-AQP2, was then used to transfect the RC.SV3 rabbit cortical collecting duct cell line by employing the Lipofectin Reagent (Gibco BRL) as previously described (Valenti et al., 1996).

Subcellular fractionation

Cells grown to confluency on 25 cm^2 flask were washed in PBS, scraped with a rubber policeman and homogenized with a glass/Teflon homogenizer in ice-cold buffer containing 250 mM sucrose, 10 mM Tris pH 7.5, 1 mM PMSF, 1 μg/ml leupetin and pepstatin. For the preparation of the low-speed pellet, enriched in plasma membranes (LS), and the high-speed pellet, enriched in intracellular vesicles (HS), the cell suspension was centrifuged at 700 ***g*** for 10 minutes at 4°C. The supernatant was centrifuged at 17,000 ***g*** for 45 minutes at 4°C. The LS was recovered in PBS and the supernatant was spun at 200,000 ***g*** in a Beckman Rotor 60 2Ti for 60 minutes at 4°C. The final pellet (HS) was recovered in PBS. Cell membranes were stored at –20°C until used for immunoblotting studies. For LS and HS membrane preparations from rabbit brain or rabbit kidney, the organs were removed, cut into small slices and homogenized following the same procedure used for CD8 cells.

Western blotting

Membranes were solubilized in Laemmli buffer at 60°C for 10 minutes and subjected to SDS-polyacrylamide gel electrophoresis (13% or 15% polyacrylamide). Gels were transferred to Immobilon-P membrane (Millipore), blocked in blotting buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.4 and 1% Triton X-100) containing 5% non-fat dry milk for 1 hour. The membranes were incubated with the first antibody at the dilutions reported in the legends for 2 hours at room temperature in blotting buffer, washed in several changes of the same blotting buffer. For the detection of syntaxin-1, syntaxin-4, SNAP-25, SNAP-23 and AQP2, the membranes were incubated with goat anti-rabbit IgG conjugated to peroxidase (Sigma, 1:5000 dilution). For the detection of VAMP-2, membranes were incubated with goat anti-mouse IgG conjugated to peroxidase (Sigma, 1:5000 dilution). Immunoreactive proteins were revealed with ECL-plus chemiluminescence reaction (Amersham, Life Science).

Light and confocal microscope immunocytochemistry

Cells grown on glass coverslips were left in basal condition or stimulated with 10^{-4} M forskolin (FK), a cAMP- elevating agent, for 15 minutes at 37°C. Cells were then fixed in a fixative containing 2% paraformaldehyde, 10 mM sodium periodate and 75 mM lysine (PLP) for 20 minutes at room temperature. In one set of experiments, cells were permeabilized with 0.1% Triton X-100 for 10 minutes, whereas, in parallel experiments, the permeabilzation step was omitted (see Results section). After blocking in 0.1% gelatin in PBS for 5 minutes, cells were incubated at room temperature for 2 hours either with the anti-AQP2 antibody raised against the peptide reproducing the C-terminus (AQP2 C-term, 1:100 dilution) or with the anti-AQP2 antibody raised against the peptide reproducing the loop C (AQP2 C-loop, 1:50 dilution).

After washing for 3×5 minutes with 0.1% gelatin, cells were incubated for 1 hour with FITC-conjugated secondary antibodies and then sequentially washed twice for 1 minute in 2.7% NaCl (high-salt PBS) and twice in regular PBS. Coverslips were mounted in 50% glycerol in 0.2 M Tris-HCl, pH 8.0 containing 2.5% *n*-propyl gallate as an anti-quenching agent. The slides were examined by confocal microscopy (MRC-1024 Bio-Rad equipped with a Krypton/Argon mixed gas laser). A specific software (Imaris 2.7, Bitplane, CH) was used for acquisition and processing of confocal images.

Double labeling of AQP2 and VAMP-2 was performed in cells transiently transfected with GFP-tagged AQP2. Briefly, GFP-tagged AQP2-transfected cells grown on coverslips, were fixed in ice-cold methanol for 5 minutes and incubated with the polyclonal anti VAMP-2 antibody (1:100 dilution). Cells were then stained with a goat anti-rabbit Cy3-conjugated secondary antibody (1:200 dilution).

To monitor the internalization of TeNT-FITC (see below), CD8 cells were incubated with the fluorescent toxin for 1 hour. Cells were then fixed with a solution containing 4% paraformaldehyde in PBS for 20 minutes at room temperature and examined with a Leica photomicroscope equipped for epifluorescence.

Digital images were obtained by a cooled CCD camera interfaced to the microscope (Princeton Instruments, NJ).

Table 1. SNARE isoforms expressed in CD8 cells

SNARE isoform	Specific primers		Homologous sequence	
	Sense primer (5′ to 3′)	Anti-sense primer (5′ to 3′)	Closest homologous sequence	% identity
Syntaxin-1(A,B)	GACAT**H**AA**R**AA**R**A**SR**GC**M**AA	A**R**CATGTC**Y**TC**Y**AG**Y**TCYTC	Rat syntaxin-1A mRNA	91%
Syntaxin-3(A,B,C)	A**R**GA**Y**GACCT**W**GA**R**CAGCTC	CG**W**CCCTCAATCTC**R**CTGAG	*Mouse Syntaxin-3(A,B,C) mRNA*	92%
Syntaxin-4	AGTTGGAGAAACAGCAGGTC	GACACAAACACCTC**R**CT**Y**TG	*Human Syntaxin-4 mRNA*	91%
VAMP-2	TAACAGGAGACTGCAGCAGA	GAT**R**ATGAGGATGATGGCGC	Human VAMP-2 mRNA	96%
SNAP-23(A,B)	GGTTTAGCCATTGAGTCTCA	CTGCCCACTTGAGTCAGGTT	*Human SNAP-23A mRNA*	91%

Degeneration: **H**=A+T+C; **M**=A+C+G; **R**=A+G; **S**=G+C; **W**=A+T; **Y**=C+T.

Tetanus toxin purification

The single-chain toxin was purified from cultures of *Clostridium tetani* (Harvard strain) by the extraction procedure of Ozutsumi et al. (Ozutsumi et al., 1985). The crude toxin solution was chromatographed on DEAE-cellulose (Whatman) and Ultrogel ACA-34 (LKB), followed by high-performance size exclusion liquid chromatography analysis on a TSK G4000SW column (LKB) equilibrated with 0.1 M sodium phosphate (pH 6.8). TeNT concentration was determined spectrophotometrically at 280 nm with an $\varepsilon_{1mg/ml}$ of 1.55. The di-chain form of TeNT was obtained by nicking single-chain toxin with tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Serva) at 25°C for 60 minutes with a toxin-to-protease ratio of 1,000:1 (wt/wt). Proteolysis was terminated by addition of soybean trypsin inhibitor at a final protease-to-inhibitor ratio of 1:4 (wt/wt). TeNT was kept at 4°C, or after being frozen in liquid nitrogen, was stored at –80°C at a protein concentration of 2 to 10 mg/ml in 10 mM sodium 4-(2-hydroxyethyl)-piperazine-1-ethansulfonate (pH 7.4). TeNT was conjugated to FITC (Pierce) by following the supplier's recommendations, and the conjugate (TeNT-FITC) was purified by chromatography on a Sephadex G-25 column.

Treatments with tetanus neurotoxin

To investigate the effect of TeNT on VAMP-2, the toxin was employed either in vitro on crude membrane samples or in vivo in intact CD8 cells. For the in vitro experiments, TeNT was incubated with 10 mM DTT for 2 hours at room temperature before use. Membrane fractions from rabbit brain (15 μg) and CD8 cells (15 μg) were treated with TeNT (500 nM) for 1 hour at 37°C, and the reaction was stopped by the addition of 1% SDS. Samples were then resolved by SDS-PAGE and blotted with appropriate antibodies.

Treatment of intact CD8 cells with TeNT

CD8 cells were grown to confluency in 10 mm Petri dishes. Cells were incubated in the presence or in the absence of whole TeNT (100 nM, for 3 hours at 37°C in the medium). Cells were then washed in PBS Ca^{2+} and Mg^{2+}, scraped and centrifuged at 11,000 ***g*** for 10 minutes. The pellet was resuspended in 30 μl distilled water and passed through a 29 G needle syringe to break the cells. The cell suspension was solubilized in Laemmli buffer and loaded into 15% polyacrylamide gel. Proteins were transferred and subjected to western blotting using monoclonal antibodies (1:100 dilution) against human VAMP-2.

Quantitation of AQP2 cell-surface immunoreactivity

Cells were grown to confluence in 24 multiwells (Falcon, NJ, USA). For each experimental condition, six wells, corresponding to approximately 4×10^6 cells in total, were tested. In experiments employing TeNT, cells were preincubated with TeNT (100 nM, whole molecule) for 3 hours at 37°C in the culture medium. Cells were then washed with PBS and either left under control conditions or stimulated with 10^{-4} M forskolin for 15 minutes at 37°C in PBS Ca^{2+}, Mg^{2+}. In parallel, cells were treated under the same experimental conditions without TeNT pretreatment. Cells were brought to 4°C and immediately fixed in freshly made fixative containing 2% paraformaldehyde, 10 mM sodium periodate and 75 mM lysine (PLP) at 4°C for 20 minutes. Cells were washed in ice-cold PBS and saturated for 15 minutes with 0.1% gelatin in PBS (PBS-gelatin). Subsequently cells were incubated with AQP2 C-loop antibodies (1:300 dilution in PBS-gelatin) for 1.5 hours at 4°C, washed three times in PBS-gelatin and incubated with goat anti-rabbit IgG peroxidase-conjugated for 1 hour at 4°C. After three washes in ice-cold PBS, cells were incubated with a solution containing 3.69 mM o-phenylenediamine, in phosphate-citrate buffer (pH 5.0) in the presence of 0.012% H_2O_2.

The solution was incubated for 15 minutes at room temperature, cells were immediately transferred to 4°C and the colored solution removed. The samples were read in a spectrophotometer at 450 nm. Cells in each well were solubilized in 200 μl formic acid, and the protein content was determined with the Bradford protein assay (Bradford, 1976). The peroxidase activity was expressed as OD/mg proteins.

Results

RT-PCR amplification of the SNARE cDNAs

Degenerate RT-PCR studies were carried out to evaluate the expression of SNARE isoforms in cultured CD8 cells. The RT-PCR experiments were performed with total RNA extracted from confluent CD8 cells and using degenerated primers on the basis of conserved regions of known SNARE nucleotide sequences (Table 1). The specificity of the cDNAs amplified by RT-PCR was confirmed by cloning and sequencing the PCR fragment. As showed in Fig. 1 and Table 1, the CD8 cells were found to express syntaxin-1A (GenBank accessing number: AF262029), syntaxin-3 A,B,C (AF262951), syntaxin-4 (AF262952), VAMP/synaptobrevin-2 (VAMP-2) (AF262953) and SNAP-23A (AF262954), sharing the highest homology with the rat syntaxin-1A (91%) (AF217191.1), mouse syntaxin-3 (A,B,C) (92% respectively D29797.1, D29798.1, D29799.1), human syntaxin-4 (91%) (X85784), human VAMP-2 (96%) (AJ225044.1) and human SNAP-23A (91%) (Y09567.1), respectively.

Expression and distribution of SNAREs in AQP2-transfected CD8 cells

To analyze the expression of the detected SNAREs proteins, western blot experiments were carried out using specific antibodies. Membrane samples isolated from CD8 cells or from rabbit brain and rabbit kidney used as positive controls were immunoblotted with anti-VAMP-2, syntaxin-1A, syntaxin-4, SNAP-23 and SNAP-25 antibodies. CD8 cells

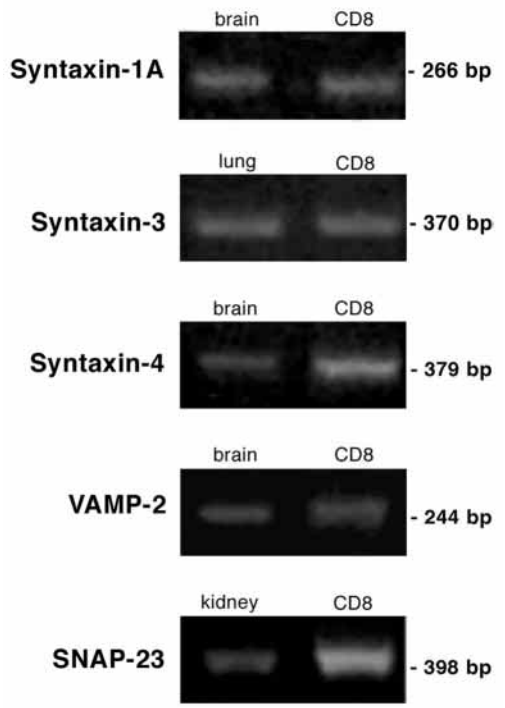

Fig. 1. Degenerate RT-PCR amplification of *SNARE* cDNAs expressed by CD8 cells. Total RNA from confluent CD8 cells was subjected to reverse transcription followed by PCR amplification using degenerate primers for the coding region of known SNARE isoforms (see Materials and Methods for experimental details). Bands of 266, 370, 379, 244 and 398 bp corresponding to syntaxin-1A, syntaxin-3, syntaxin-4, VAMP-2 and SNAP-23, respectively, were amplified. As a positive control, parallel RT-PCR experiments with total RNA extracted from the rat brain, lung or kidney were performed. The results shown are representative of at least three separate experiments.

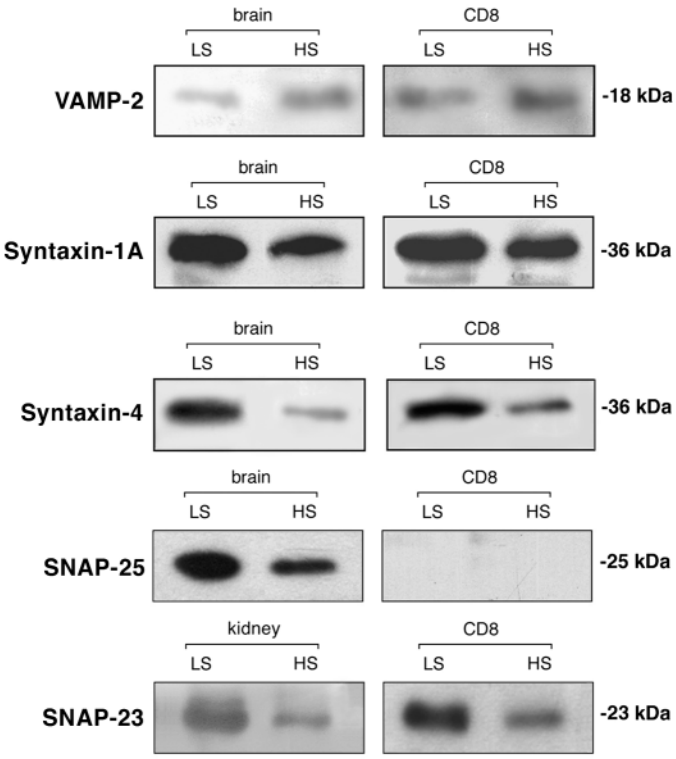

Fig. 2. An immunoblot of membrane fractions from different tissues or CD8 cells. Each lane was loaded with 60 μg of protein. Membrane fractions enriched in the plasma membrane (LS, 17,000 ***g*** pellet) or in intracellular vesicles (HS, 200,000 ***g*** pellet) were probed with VAMP-2 (1:100 dilution) or syntaxin-1A (1:300) or syntaxin-4 (1:300) or SNAP-25 (1:500) or SNAP-23 (1:300) antibodies. Immunoreactive bands were revealed with chemiluminescence ECL-plus (Amersham, USA). The results shown are representative of at least three separate experiments.

were fractionated to separate a plasma-membrane-enriched fraction (LS) and a membrane fraction enriched in intracellular vesicles (HS). VAMP-2 stained a 18 kDa band in both brain and CD8 cells membrane fractions, with a stronger signal in intracellular vesicle membranes (HS), a fraction in which AQP2 is enriched in control cells (Fig. 2). Similar results were obtained in membrane fractions obtained from the rabbit brain.

By contrast, syntaxin-1 and syntaxin-4 antibodies labeled a distinct 36 kDa band corresponding to the expected molecular mass of syntaxins, displaying a stronger signal in a plasma membrane fraction (LS) both in CD8 and rabbit brain. This suggests that syntaxin-1A and syntaxin-4 are mainly present in the plasma membrane of CD8 cells (Fig. 2).

Our results are in agreement with those of others (Nielsen et al., 1995; Mandon et al., 1996), who found VAMP-2 in the kidney inner medulla and syntaxin-4 enriched in a membrane fraction from inner medullary collecting duct cells. SNAP-25 was not detected in CD8 cells, whereas it was highly expressed in rabbit brain (Fig. 2). SNAP-25 is in fact present principally in the brain, although Shukla et al. reported that SNAP-25-associated Hrs-2 protein colocalizes with AQP2 in rat kidney collecting duct principal cells (Shukla et al., 2001). On the other hand, the SNAP-23, a homologous SNAP-25 protein that binds to multiple syntaxins and synaptobrevins, is ubiquitously expressed. SNAP-23 was detected in CD8 cells as well as in rabbit kidney and found enriched in the low-speed pellet from both membrane samples (Fig. 2).

Cleavage of VAMP-2 by tetanus neurotoxin

To directly address the issue of the functional involvement of SNAREs in AQP2 targeting, we took advantage of the use of clostridial neurotoxins that specifically cleave target SNAREs. This susceptibility afforded a specific strategy to probe the function of VAMP-2 in AQP2 traffic.

TeNTs specifically hydrolyze rat VAMP-2 in synaptic vesicles but not VAMP-1 (Link et al., 1992). To test whether TeNT was efficient in cleaving in vitro the VAMP-2-like protein expressed in CD8 cells, a membrane fraction enriched for intracellular vesicles was incubated with the toxin (500 nM) for 1 hour. As a control, membranes from the rabbit brain were run at the same time. Membrane samples were subjected to western blotting using anti-VAMP-2 antibodies. As shown in Fig. 3 (in vitro cleavage, left panel), toxin treatment of both membrane preparations from CD8 cells and rabbit brain revealed complete cleavage of VAMP-2. This result further supports the conclusion that the VAMP/synaptobrevin-like protein expressed in CD8 cells is a VAMP-2-like protein. The cleavage fragment of about 12 kDa was not detectable under

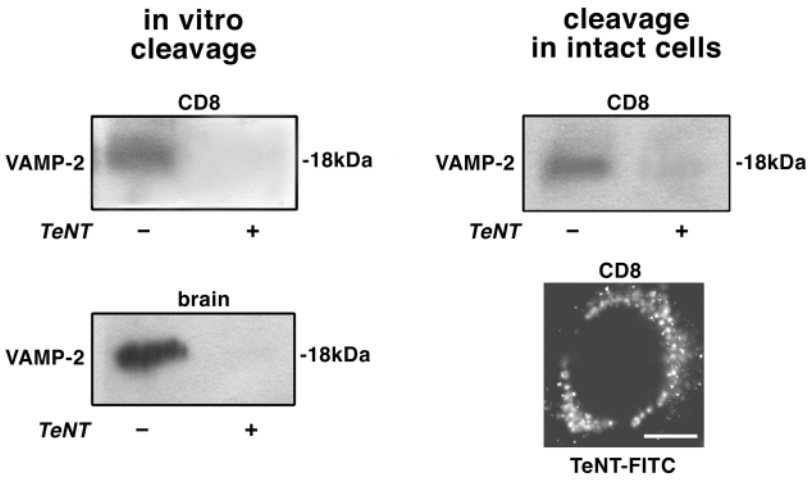

Fig. 3. Cleavage of VAMP-2 by the clostridial neurotoxins TeNT: in vitro cleavage. Membrane fractions enriched in intracellular vesicles from CD8 cells and rabbit brain membranes were incubated with TeNT 500 nM for 1 hour at 37°C. TeNT was previously activated by incubation with 10 mM dithiothreitol (DTT) for 2 hours at 37°C. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL-plus). Cleavage of VAMP-2 in intact CD8 cells. CD8 cells were grown to confluency in 10 mm Petri dishes. Cells were incubated in the presence or in the absence of whole TeNT (100 nM, for 3 hours at 37°C in the medium). Proteins were transferred and subjected to western blotting using monoclonal antibodies (1:100 dilution) against human VAMP-2. The results shown are representative of at least three separate experiments. Internalization of TeNT-FITC was visualized by immunofluorescence analysis. Intact cells were exposed to TeNT-FITC, fixed and examined at the fluorescence microscope. TeNT-FITC was internalized in intracellular structures. Bar, 8 μm.

our experimental conditions, probably because of the reduced ability of the fragment to bind the nitrocellulose membrane during blotting as described previously (Link et al., 1992).

As a next step, we investigated whether TeNT was able to cleave VAMP-2 in intact CD8 cells. We first checked whether TeNT can be internalized in intact CD8 cells. To this end, fluorescein isothiocyanate (FITC) was conjugated to TeNT and purified by chromatography on a Sephadex G-25 column as described previously (Matteoli et al., 1996). Intact cells were exposed to TeNT-FITC, fixed and examined by fluorescence microscopy. As shown in Fig. 3 (cleavage in intact cells, right panel), TeNT-FITC was internalized in intracellular structures (TeNT-FITC) as assessed by fluorescence detection. Western blots of solubilized CD8 cells exposed to TeNT in the medium for 3 hours revealed that the internalized toxin was able to cleave the endogenously expressed VAMP-2.

Analysis by western blotting with AQP2 antibodies revealed that cells pretreated with TeNT for 3 hours had the same amount of AQP2 as untreated control cells, indicating that the toxin did not affect the expression of AQP2 (data not shown).

Characterization of the anti-AQP2 C-loop antibody

We next examined the effect of TeNT treatment on AQP2 distribution in resting CD8 cells and in cells that had been treated with the cAMP elevating agent forskolin (FK). To determine the amount of AQP2 inserted in the apical plasma membrane, we generated an antibody against a peptide reproducing the external C-loop of human AQP2 (IRGDLAVNALSNSTT), and the cell-surface expression of AQP2 was monitored by determining the protein immunoreactivity. The antibody recognized both the glycosylated and non-glycosylated AQP2 from both rat kidney and CD8 cells, with a stronger expression in a membrane fraction enriched in intracellular vesicles (Fig. 4A). The specificity of the immune serum was assessed by preincubation of antiserum with a 100-fold molar excess of immunizing peptide (Fig. 4A). To further confirm the recognition of an extracellular epitope by the AQP2 C-loop antibody, non-permeabilized FK-stimulated CD8 cells were stained with the AQP2 C-loop antibody or with the conventional AQP2 C-terminus antibody recognizing an epitope located in the cytosolic C-terminus and examined by confocal microscopy. Under these experimental conditions (Fig. 4B, –Triton X-100), immunofluorescence staining demonstrated that only the AQP2 C-loop antibody stained the cell surface of FK-stimulated CD8 cells, whereas no staining was observed with the AQP2 C-terminal antibody (Fig. 4B and xz reconstruction in the inset). These data are fully consistent with the efficacy of the AQP2 C-loop antibody in recognizing an external epitope of the AQP2 protein. By contrast, in permeabilized control cells, both antibodies stained intracellular vesicles, giving a similar staining of the AQP2-bearing vesicles (Fig. 4B, +Triton X-100).

Determination of cell-surface AQP2 immunoreactivity

The anti AQP2 C-loop antibody was therefore employed to monitor the AQP2 density on the plasma membrane in cells grown to confluency in ELISA multiwells. In non-permeabilized cells, the antibody is expected to cross-react only with AQP2 inserted into the plasma membrane (Fig. 5A). After stimulation of untreated cells with the cAMP-elevating agent forskolin, the immunodetectable AQP2 on the cell surface increased by approximately two-fold compared with that present in the plasma membrane in control cells (Fig. 5B, –TeNT). By contrast, TeNT pretreatment completely abolished FK-stimulated AQP2, targeting the apical plasma membrane, as assessed by quantification of cell surface immunoreactivity (Fig. 5B, +TeNT). In control cells, TeNT treatment was found to have no effect on the AQP2 density found at the cell surface.

Moreover double labeling of AQP2 and VAMP-2 in cells AQP2 transiently transfected with GFP-tagged AQP2 showed a partial colocalization of VAMP-2 in AQP2-bearing vesicles (Fig. 5C).

Overall, these results indicate that VAMP-2 is functionally involved in cAMP-induced AQP2 targeting to the plasma membrane.

Discussion

The rate of water reabsorption in kidney collecting ducts is

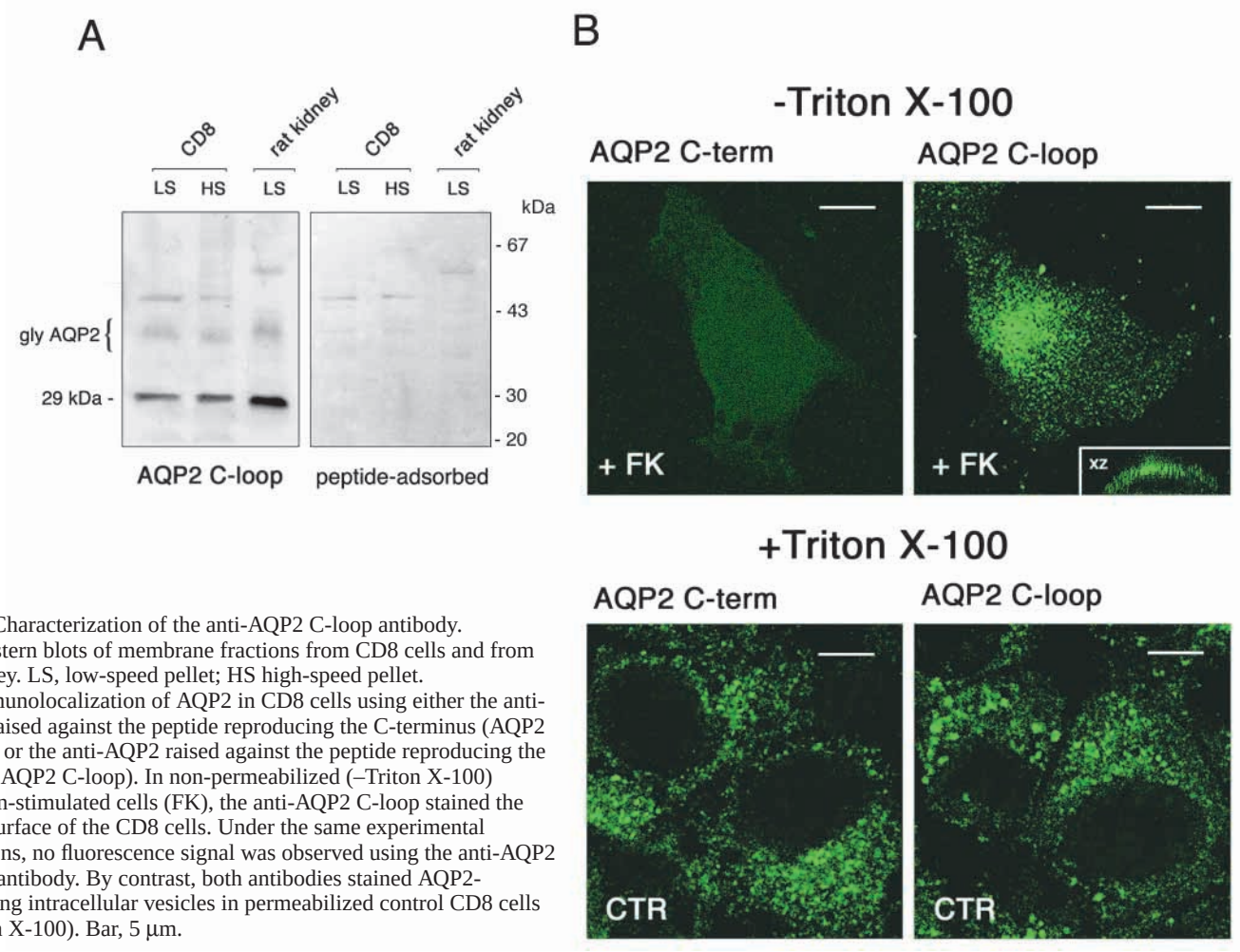

Fig. 4. Characterization of the anti-AQP2 C-loop antibody. (A) Western blots of membrane fractions from CD8 cells and from rat kidney. LS, low-speed pellet; HS high-speed pellet. (B) Immunolocalization of AQP2 in CD8 cells using either the anti-AQP2 raised against the peptide reproducing the C-terminus (AQP2 C-term) or the anti-AQP2 raised against the peptide reproducing the loop C (AQP2 C-loop). In non-permeabilized (–Triton X-100) forskolin-stimulated cells (FK), the anti-AQP2 C-loop stained the apical surface of the CD8 cells. Under the same experimental conditions, no fluorescence signal was observed using the anti-AQP2 C-term antibody. By contrast, both antibodies stained AQP2-containing intracellular vesicles in permeabilized control CD8 cells (+Triton X-100). Bar, 5 μm.

regulated by vasopressin. This hormone controls transcellular water transport by regulating the redistribution of AQP2-bearing vesicles to the apical membrane of collecting duct principal cells (Knepper and Inoue, 1997; Klussmann et al., 2000). We utilized the collecting duct cell line CD8 stably transfected with the vasopressin-sensitive water channel AQP2 to investigate the functional involvement of SNARE proteins in the process of regulated insertion of the AQP2 water channel.

In the present study, evidence is presented that directly supports the hypothesis that the v-SNARE protein VAMP-2 is involved in the targeting of AQP2 vesicles to the apical plasma membrane. Forskolin-induced translocation of AQP2 to the apical membrane was abolished by TeNT in intact CD8 cells (Fig. 5). This toxin proteolytically cleaves VAMP-2 at a single site (Link et al., 1992). It has been suggested that VAMP-2 plays a major role in the mechanism of vesicle trafficking (Sollner et al., 1993). This evidence is mainly formed on the basis of the ability of TeNT and of botulinum neurotoxins to cleave VAMP-2 at different sites (Schiavo et al., 1995), which results in the inhibition of neurotransmitter release in neuronal and neuroendocrine cells (Hunt et al., 1994). The regulatory role of synaptobrevin isoforms has also been proved in the regulated exocytosis of GLUT4 in adipocytes, in exocytosis of histamine in enterochromaffin-like cells, in sperm acrosome reaction and in H^+-ATPase trafficking in the inner medullary collecting duct (Banerjee et al., 2001; Banerjee et al., 1999; Foster and Klip, 2000; Hohne-Zell et al., 1997; Martin et al., 1998; Schulz et al., 1997). Thus, SNARE proteins might fulfil a universal role in vesicle-membrane fusion in neuronal and non-neuronal cells.

In renal cells, by quantitative double immunolabeling, VAMP-2 has been shown to colocalize with AQP2-containing vesicles (Nielsen et al., 1995), supporting a role for VAMP-2 in vasopressin-regulated vesicular trafficking. In the same study, the authors demonstrated that TeNT caused a complete cleavage of VAMP-2 in the crude membrane fraction enriched for intracellular vesicles from kidney inner medulla (Nielsen et al., 1995). Jo and colleagues (Jo et al., 1995) reported that purified papillary AQP2-containing endosomes from rat kidney possess VAMP-2. The authors demonstrated that endosomes fuse, in vitro, by means of an ATP-dependent process that is significantly inhibited when endosomes are preincubated with either anti-VAMP-2 antibody or TeNT.

Despite the fact that these observations strongly support a functional involvement of VAMP-2 in AQP2-regulated redistribution, direct evidence for this role in intact renal cells has so far been lacking. We attempted to demonstrate the role of VAMP-2 in polarized sorting of AQP2 by determining the effect of clostridial TeNT on cAMP-induced translocation of AQP2 in intact renal CD8 cells. Central to this study was the demonstration that TeNT can enter the cells and is able to

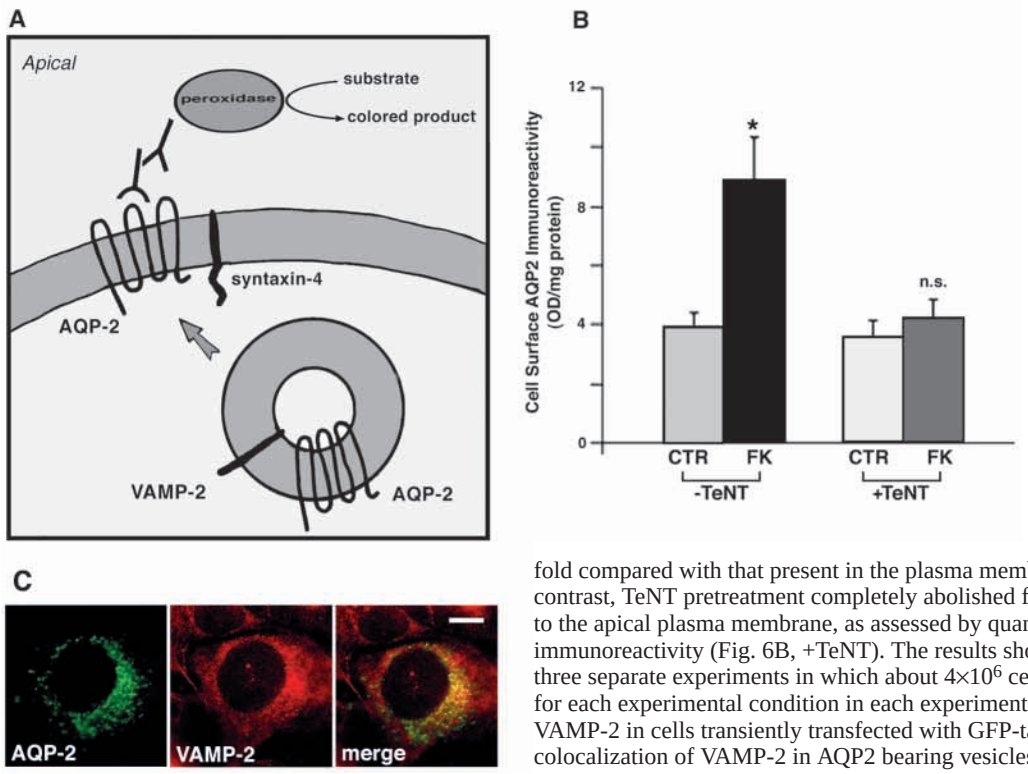

Fig. 5. Determination of cell-surface AQP2 immunoreactivity. The anti-AQP2 C-loop antibody was employed to monitor the AQP2 density on the plasma membrane in CD8 cells. (A) In non-permeabilized cells, the antibody is expected to cross-react only with AQP2 inserted into the plasma membrane. (B) After stimulation of untreated cells with forskolin, the immunodetectable AQP2 on the cell surface increased by approximately two-fold compared with that present in the plasma membrane in control cells (–TeNT). By contrast, TeNT pretreatment completely abolished forskolin-stimulated AQP2 targeting to the apical plasma membrane, as assessed by quantification of cell-surface immunoreactivity (Fig. 6B, +TeNT). The results shown represent the means±s.e.m. of three separate experiments in which about 4×10^6 cells (six separate wells) were tested for each experimental condition in each experiment. (C) Double labeling of AQP2 and VAMP-2 in cells transiently transfected with GFP-tagged AQP2 showed a partial colocalization of VAMP-2 in AQP2 bearing vesicles. Bar, 5 μm.

cleave the endogenously expressed VAMP-2. TeNT treatment in intact CD8 cells completely abolished cAMP-stimulated AQP2 targeting of the plasma membrane, as assessed by quantification of the cell-surface immunoreactivity of the anti-AQP2 antibody raised against a peptide reproducing the extracellular AQP2 C-loop. These results represent the first evidence for the functional involvement of VAMP-2 in cAMP-induced AQP2 redistribution in renal cells. In contrast to the effect of the toxin on cAMP-induced translocation of AQP2 to the apical membrane, the amount of cell-surface AQP2 in resting cells is not reduced by exposure to toxin (Fig. 5). This observation would suggest that the constitutive delivery of AQP2 may be VAMP-2 independent.

In neurons, VAMP-2 forms a complex with two plasma-membrane-associated SNAREs: syntaxin and SNAP-25. These proteins bind together in a parallel manner (Hanson et al., 1997) to form a four-helix bundle, with two helices contributed by SNAP-25 and one each by VAMP and syntaxin (Sutton et al., 1998). The formation of the SNARE complex is by itself sufficient to bring the two interacting membranes close enough to fuse (Weber et al., 1998).

Among the known syntaxin isoforms, only syntaxin-1 and syntaxin-4 bind to VAMP-2 with high affinity (Calakos et al., 1994; Pevsner et al., 1994). Syntaxin-4 has been localized in the apical plasma membrane of collecting duct principal cells (Mandon et al., 1996), suggesting that it may represent the counterpart protein interacting with VAMP-2 found localized in AQP2-containing vesicles (Jo et al., 1995; Nielsen et al., 1995). In this study, the presence of VAMP-2, syntaxin-1A and syntaxin-4 have been demonstrated by RT-PCR and western blotting in CD8 cells. VAMP-2 was found enriched in intracellular vesicles, whereas both syntaxin-1A and syntaxin-4 were found enriched in a plasma membrane fraction. Further studies will address whether the v-SNARE VAMP-2 interacts with syntaxin-1A or syntaxin-4 to mediate AQP2 targeting.

SNAP-25 has been implicated at a late step in fusion (Banerjee et al., 1996; Mehta et al., 1996; Rossi et al., 1997). In inner medullary collecting duct cells, treatment with botulinum toxin E, which cleaves rat SNAP-23, reduced the amount of H^+-ATPase translocated to the apical membrane by about 52%, demonstrating that SNAP-23 has a critical role in the regulation of H^+-ATPase exocytosis (Banerjee et al., 2001). We speculate that SNAP-23 detected in a plasma-membrane-enriched fraction of CD8 cells acts similarly in renal cells. Further investigation is needed to evaluate the role of the specific SNAREs in the dynamic of complex formation.

In summary, the data reported in this study provide clear evidence that the v-SNARE VAMP-2 is directly involved in the docking and fusion of AQP2-containing vesicles with the plasma membrane in intact AQP2-transfected CD8 cells and provide the basis for understanding the mechanism and regulation of AQP2 trafficking in response to a cAMP-elevating agent.

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References

Aroeti, B., Okhrimenko, H., Reich, V. and Orzech, E. (1998). Polarized trafficking of plasma membrane proteins: emerging roles for coats, SNAREs, GTPases and their link to the cytoskeleton. *Biochim. Biophys. Acta* **1376**, 57-90.

Banerjee, A., Barry, V. A., DasGupta, B. R. and Martin, T. F. (1996). N-Ethylmaleimide-sensitive factor acts at a prefusion ATP-dependent step in Ca^{2+}-activated exocytosis. *J. Biol. Chem.* **271**, 20223-20226.

Banerjee, A., Shih, T., Alexander, E. A. and Schwartz, J. H. (1999). SNARE proteins regulate H(+)-ATPase redistribution to the apical membrane in rat renal inner medullary collecting duct cells. *J. Biol. Chem.* **274**, 26518-26522.

Banerjee, A., Li, G., Alexander, E. A. and Schwartz, J. H. (2001). Role of SNAP-23 in trafficking of H+-ATPase in cultured inner medullary collecting duct cells. *Am. J. Physiol. Cell Physiol.* **280**, C775-C781.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Calakos, N., Bennett, M. K., Peterson, K. E. and Scheller, R. H. (1994). Protein-protein interactions contributing to the specificity of intracellular vesicular trafficking. *Science* **263**, 1146-1149.

Fasshauer, D., Sutton, R. B., Brunger, A. T. and Jahn, R. (1998). Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. *Proc. Natl. Acad. Sci. USA* **95**, 15781-15786.

Foster, L. J. and Klip, A. (2000). Mechanism and regulation of GLUT-4 vesicle fusion in muscle and fat cells. *Am. J. Physiol. Cell Physiol.* **279**, C877-C890.

Hanson, P. I., Roth, R., Morisaki, H., Jahn, R. and Heuser, J. E. (1997). Structure and conformational changes in NSF and its membrane receptor complexes visualized by quick-freeze/deep-etch electron microscopy. *Cell* **90**, 523-535.

Hohne-Zell, B., Galler, A., Schepp, W., Gratzl, M. and Prinz, C. (1997). Functional importance of synaptobrevin and SNAP-25 during exocytosis of histamine by rat gastric enterochromaffin-like cells. *Endocrinology* **138**, 5518-5526.

Hunt, J. M., Bommert, K., Charlton, M. P., Kistner, A., Habermann, E., Augustine, G. J. and Betz, H. (1994). A post-docking role for synaptobrevin in synaptic vesicle fusion. *Neuron* **12**, 1269-1279.

Inoue, T., Nielsen, S., Mandon, B., Terris, J., Kishore, B. K. and Knepper, M. A. (1998). SNAP-23 in rat kidney: colocalization with aquaporin-2 in collecting duct vesicles. *Am. J. Physiol.* **275**, F752-F760.

Jo, I., Harris, H. W., Amendt-Raduege, A. M., Majewski, R. R. and Hammond, T. G. (1995). Rat kidney papilla contains abundant synaptobrevin protein that participates in the fusion of antidiuretic hormone-regulated water channel-containing endosomes in vitro. *Proc. Natl. Acad. Sci. USA* **92**, 1876-1880.

Klussmann, E., Maric, K. and Rosenthal, W. (2000). The mechanisms of aquaporin control in the renal collecting duct. *Rev. Physiol. Biochem. Pharmacol.* **141**, 33-95.

Knepper, M. A. and Inoue, T. (1997). Regulation of aquaporin-2 water channel trafficking by vasopressin. *Curr. Opin. Cell Biol.* **9**, 560-564.

Liebenhoff, U. and Rosenthal, W. (1995). Identification of Rab3-, Rab5a- and synaptobrevin II-like proteins in a preparation of rat kidney vesicles containing the vasopressin-regulated water channel. *FEBS Lett.* **365**, 209-213.

Link, E., Edelmann, L., Chou, J. H., Binz, T., Yamasaki, S., Eisel, U., Baumert, M., Sudhof, T. C., Niemann, H. and Jahn, R. (1992). Tetanus toxin action: inhibition of neurotransmitter release linked to synaptobrevin proteolysis. *Biochem. Biophys. Res. Commun.* **189**, 1017-1023.

Mandon, B., Chou, C. L., Nielsen, S. and Knepper, M. A. (1996). Syntaxin-4 is localized to the apical plasma membrane of rat renal collecting duct cells: possible role in aquaporin-2 trafficking. *J. Clin. Invest.* **98**, 906-913.

Martin, L. B., Shewan, A., Millar, C. A., Gould, G. W. and James, D. E. (1998). Vesicle-associated membrane protein 2 plays a specific role in the insulin-dependent trafficking of the facilitative glucose transporter GLUT4 in 3T3-L1 adipocytes. *J. Biol. Chem.* **273**, 1444-1452.

Matteoli, M., Verderio, C., Rossetto, O., Iezzi, N., Coco, S., Schiavo, G. and Montecucco, C. (1996). Synaptic vesicle endocytosis mediates the entry of tetanus neurotoxin into hippocampal neurons. *Proc. Natl. Acad. Sci. USA* **93**, 13310-13315.

Mehta, P. P., Battenberg, E. and Wilson, M. C. (1996). SNAP-25 and synaptotagmin involvement in the final Ca(2+)-dependent triggering of neurotransmitter exocytosis. *Proc. Natl. Acad. Sci. USA* **93**, 10471-10476.

Nielsen, S., Marples, D., Birn, H., Mohtashami, M., Dalby, N. O., Trimble, M. and Knepper, M. (1995). Expression of VAMP-2-like protein in kidney collecting duct intracellular vesicles. Colocalization with Aquaporin-2 water channels. *J. Clin. Invest.* **96**, 1834-1844.

Ozutsumi, K., Sugimoto, N. and Matsuda, M. (1985). Rapid, simplified method for production and purification of tetanus toxin. *Appl. Environ. Microbiol.* **49**, 939-943.

Pevsner, J., Hsu, S. C., Braun, J. E., Calakos, N., Ting, A. E., Bennett, M. K. and Scheller, R. H. (1994). Specificity and regulation of a synaptic vesicle docking complex. *Neuron* **13**, 353-361.

Rossi, G., Salminen, A., Rice, L. M., Brunger, A. T. and Brennwald, P. (1997). Analysis of a yeast SNARE complex reveals remarkable similarity to the neuronal SNARE complex and a novel function for the C terminus of the SNAP-25 homolog, Sec9. *J. Biol. Chem.* **272**, 16610-16617.

Rothman, J. E. (1994). Mechanisms of intracellular protein transport. *Nature* **372**, 55-63.

Rothman, J. E. and Wieland, F. T. (1996). Protein sorting by transport vesicles. *Science* **272**, 227-234.

Schiavo, G., Rossetto, O., Tonello, F. and Montecucco, C. (1995). Intracellular targets and metalloprotease activity of tetanus and botulism neurotoxins. *Curr. Top. Microbiol. Immunol.* **195**, 257-274.

Schulz, J. R., Wessel, G. M. and Vacquier, V. D. (1997). The exocytosis regulatory proteins syntaxin and VAMP are shed from sea urchin sperm during the acrosome reaction. *Dev. Biol.* **181**, 80-87.

Shukla, A., Hager, H., Corydon, T. J., Bean, A. J., Dahl, R., Vajda, Z., Li, H., Hoffmann, H. J. and Nielsen, S. (2001). SNAP-25-associated Hrs-2 protein colocalizes with AQP2 in rat kidney collecting duct principal cells. *Am. J. Physiol. Renal. Physiol.* **281**, F546-F556.

Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J. E. (1993). SNAP receptors implicated in vesicle targeting and fusion. *Nature* **362**, 318-324.

Sutton, R. B., Fasshauer, D., Jahn, R. and Brunger, A. T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 Å resolution. *Nature* **395**, 347-353.

Tamma, G., Klussmann, E., Maric, K., Aktories, K., Svelto, M., Rosenthal, W. and Valenti, G. (2001). The small GTP-binding protein Rho inhibits cAMP-induced translocation of aquaporin-2 into the apical membrane of renal cells. *Am. J. Physiol. Renal. Physiol.* **8**, 8.

Valenti, G., Frigeri, A., Ronco, P. M., D'Ettorre, C. and Svelto, M. (1996). Expression and functional analysis of water channels in a stably AQP2-transfected human collecting duct cell line. *J. Biol. Chem.* **271**, 24365-24370.

Valenti, G., Procino, G., Liebenhoff, U., Frigeri, A., Benedetti, P. A., Ahnert-Hilger, G., Nurnberg, B., Svelto, M. and Rosenthal, W. (1998). A heterotrimeric G protein of the Gi family is required for cAMP- triggered trafficking of aquaporin 2 in kidney epithelial cells. *J. Biol. Chem.* **273**, 22627-22634.

Valenti, G., Procino, G., Carmosino, M., Frigeri, A., Mannucci, R., Nicoletti, I. and Svelto, M. (2000). The phosphatase inhibitor okadaic acid induces AQP2 translocation independently from AQP2 phosphorylation in renal collecting duct cells. *J. Cell Sci.* **113**, 1985-1992.

Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T. H. and Rothman, J. E. (1998). SNAREpins: minimal machinery for membrane fusion. *Cell* **92**, 759-772.