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Expression and Trafficking of the γ Subunit of Na,K-ATPase in Hypertonically Challenged IMCD3 Cells

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The γ subunit (FXYD2) of Na,K-ATPase is an important regulator of the sodium pump. In this investigation we have analysed the trafficking of γ to the plasma membrane in cultures of inner medullary collecting duct cells (IMCD3) following acute hypertonic challenge and brefeldin A (BFA) treatment. Following hypertonic challenging for 24 hr immunofluorescence labeling revealed initial co-localization of the γ subunit and 58K Golgi protein in the cytoplasm, but no co-localization of **α**1 and Golgi protein. Exposure of the challenged cells to BFA prevented the subsequent incorporation of γ into the basolateral plasma membrane. The γ subunit instead remained in cytoplasmic vesicles while cell proliferation and cell viability decreased simultaneously. Following removal of BFA from the hypertonic medium the IMCD3 cells recovered with distinct expression of γ in the basolateral membrane. The **α**1 subunit was only marginally influenced by BFA. The results demonstrate that the γ subunit trafficks to the plasma membrane via the Golgi apparatus, despite the absence of a signal sequence. The results also suggest that the γ and **α** subunits do not traffic together to the plasma membrane, and that the γ and **α** subunit have different turnover rates during these experimental conditions.

Key words: FXYD2, hypertonicity, brefeldin A, Golgi apparatus, confocal microscopy

I. Introduction

The Na,K-ATPase is made up of α and β subunits and in renal tissue also a small, single-span ca 7 kD γ subunit (FXYD2) [33]. All three subunits together form the enzyme complex in most parts of the nephron, including the principal cells in the collecting ducts at the tip of the papilla (IMCD3 cells) [24, 30, 32, 35]. However, the γ subunit is absent in immortalized murine cells originating from IMCD3 cells cultured under isotonic conditions, but becomes strongly expressed in the basolateral plasma membrane by acute hypertonicity [7, 8, 29]. The cellular pathway of γ to the cell membrane has not been clarified, and it is not known whether γ is transported to the basolateral membrane alone or together with αβ heterodimers. The protein domains responsible for the polarized sorting of transport proteins have been intensely investigated [e.g. 12, 27], but as recently pointed out [6] surprisingly little is still known about the targeting and trafficking of the Na,K-ATPase to the basolateral surface. Both the α and β subunits are assembled at the level of the ER [1, 17, 18, 25], but if the α subunit is expressed alone it is retained and degraded in the endoplasmic reticulum, while the β subunit is able to traffick alone to the plasma membrane [10, 16]. It is likely that the γ subunit becomes synthesized and transported to the basolateral membrane along the same route as the mature α and β subunits, but it has also been expressed alone on the apical surface of mouse blastocysts [20]. Furthermore the γ subunit has no N-terminal signal sequence [24], in contrast to the five other FXYD proteins [11, 13, 15].

The aim of this paper was to obtain information about the trafficking of γ in IMCD cells following hypertonic challenging specifically as related to the α subunit. To interfere with the structure and function of the Golgi complex we ap-

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plied brefeldin A (BFA), which is known to influence the protein secretory pathways generally in eukaryotes [14, 21, 34, 36]. The results suggest that the γ subunit transits alone, separate from α1, through the Golgi apparatus and then trafficks to the basolateral membrane to become assembled with the Na,K-ATPase αβ-heterodimer.

II. Materials and Methods

Cell cultures

The 300 mOsm/kgH_2O medium was prepared by mixing D-MEM and F-12 nutrient solutions at 1:1 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Immortalized murine IMCD3 cells, kindly provided by Dr. C. J. Rivard (University of Colorado Health Science Center, Denver, CO), were propagated in the 300 mOsm/kgH_2O medium and acutely challenged with a 550 mOsm/kgH_2O medium to induce γ synthesis by addition of 5 M NaCl according to Capasso *et al.* [7, 8]. Media were routinely changed every 24 hr in each experiment.

Reagents and antibodies

Stock solution of Brefeldin A (Sigma-Aldrich Denmark, Vallensbæk Strand, Denmark), was made up as 10 mM in methanol and stored at −20°C. Polyclonal rabbit antibodies against the COOH terminus of the γ subunit of Na,K-ATPase and corresponding purified peptide were kindly provided by Dr. Steven Karlish (The Weizmann Institute of Science, Rehovot, Israel). Monoclonal antibodies against the 58K Golgi protein were obtained from Sigma-Aldrich (Sigma-Aldrich Denmark A/S, Vallensbæk Strand, Denmark). The 58K protein antibody recognizes an epitope on a 58KDa protein (formiminotransferase cyclodeaminase) located on the peripheral Golgi membrane [4]. Monoclonal GM 130 antibodies, which recognize a 130 kDa Golgi matrix protein associated with the *cis*-compartment of the Golgi apparatus, were obtained from BD Biosciences Laboratories (Brødby, Denmark). To-Pro-3 (Molecular Probes, Invitrogen A/S, Taastrup, Denmark) was used as a nuclear counterstain.

Experimental protocols

Protocol 1

Experimental cell cultures were exposed to 1 μM (0.28 μg/ml) or 5 μM brefeldin A (BFA) (1.4 μg/ml) in hypertonic 550 mOsm/kgH_2O medium. Control cultures were grown in hypertonic or isotonic media without BFA. Some cultures were allowed to continue to grow in hypertonic medium after BFA treatment to follow recovery of the IMCD3 cells.

Protocol 2

Cell cultures were first challenged with hypertonic 550 mOsm/kgH_2O medium and subsequently exposed to 1 μM or 5 μM BFA solution in hypertonic medium. Control cultures were grown either in hypertonic or iso-osmotic media without BFA for equivalent times, and some cultures were allowed to continue to grow in hypertonic medium without BFA to follow recovery of the IMCD3 cells.

Cell counting and cell proliferation

For cell counting 0.5 ml of 0.4% trypan blue, 0.3 ml of culture medium and 0.1 ml of cell suspension were mixed in an eppendorf tube. After 5 min the hemocytometer counting chambers were loaded and viable cells (unstained) were counted from five squares in each chamber. Average numbers from three wells were used for cell proliferation graphs. From the IMCD3 cell suspension 10,000 cells were seeded in each well on two 24-well plates (Corning, Inc., NY, USA) with one ml of 300 mOsm/kgH_2O medium. After 24 hr cells from 3 wells were split, counted and this time point taken as zero. Cells on one plate continued to grow in 300 mOsm/kgH_2O medium, and cells on the other plate were challenging with the 550 mOsm/kgH_2O medium. Cell numbers from 3 wells from each plate were counted every 24 hr up to 168 hr.

Cell viability

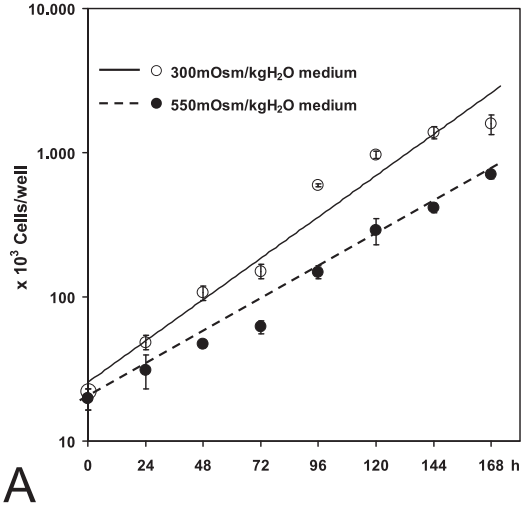
Initially 10,000 or 25,000 cells were seeded per well on 24-well-cell culture plate. Viability of IMCD3 cells was measured using CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay (Promega, Ramcon, Birkerød, Denmark). The absorbance of the colored reaction product, formazan, was measured at 490 nm and is directly proportional to the number of living cells in the culture. Cell viabilities from isotonic 300 mOsm/kgH_2O medium and cultures acutely challenged with 550 mOsm/kgH_2O were compared with the corresponding values of cell cultures treated with BFA.

Immunofluorescence microscopy and confocal laser scanning microscopy

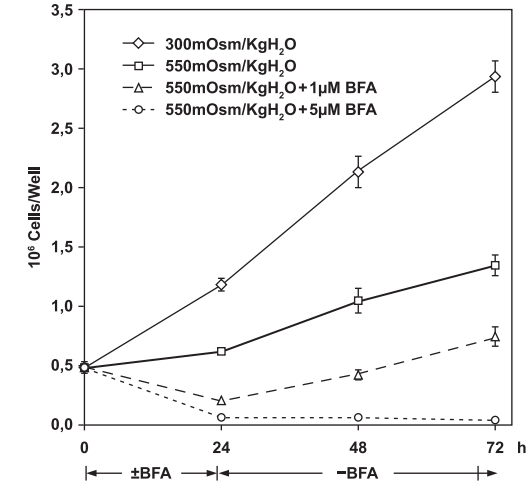
Cells were grown on cover glasses in 24-well plates until subconfluent and then treated with hypertonic medium with or without BFA for 24 or 48 hr. Cells were then rinsed with PBS with an osmolality corresponding to the osmolality of the growth medium before fixation with 4% paraformaldehyde (PFA) in isotonic or hypertonic PBS for 10 min. Permeabilization, blocking of unspecific binding sites and incubation with primary and secondary antibodies were performed as described previously [29]. Cell cultures prepared for immunofluorescence microscopy were analyzed by confocal laser scanning microscopy as previously described [29].

Data analysis

Statistical analyses of differences between curves were performed using stratified Wilcoxon test (van Eltern test) and for cell viability Mann-Whitney non-parametric test. Data were expressed as means±SE. Differences were considered significant for values of $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***).



A



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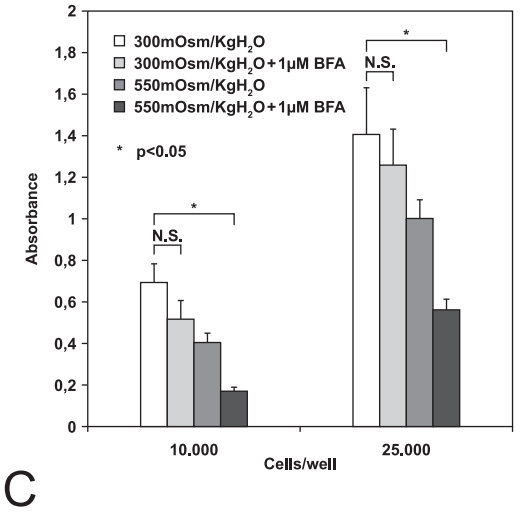

C

III. Results

Hypertonicity and BFA decrease cell proliferation and viability

IMCD3 (inner medullary collecting duct) cell proliferation for 168 hr was approximately exponential in isotonic medium (300 mOsm/kgH_2O), and following acute challenge to hypertonic medium (550 mOsm/kgH_2O), (Fig. 1A). However, the proliferation rate of challenged cells in hypertonic medium was significantly slower than the proliferation rate of IMCD3 cells in isotonic medium.

In the hypertonic medium in the presence of 1 μM BFA (*Protocol 1*) the number of IMCD3 cells decreased significantly ($p<0.001$) during the first 24 hr, but removal of BFA from the hypertonic medium (the recovery period) induced slow cell proliferation ($p<0.001$) (Fig. 1B). Treatment with 5 μM BFA caused the cell numbers to decrease during the whole period illustrating cell death also after removal of the drug (Fig. 1B). The effect of BFA on cell viability based using the non-radioactive cell viability test was the same with 10,000 and 25,000 cells and cell viability was significantly decreased ($p<0.05$) with 1 μM BFA in the hypertonic medium (Fig. 1C).

Brefeldin A inhibits transport of γ to the plasma membrane (Protocol 1)

The Golgi proteins, 58K and GM130, were strongly expressed in all IMCD3 cells growing in 300 mOsm/kgH_2O medium (Fig. 2A and B), but the γ subunit was totally absent (Fig. 2B). GM130 was expressed as several small vesicles

Fig. 1. **A:** IMCD3 cell proliferation is retarded in hypertonic media. Cells were seeded in two 24-well plates in 300 mOsm/kgH_2O medium at a concentration of 10,000 cells/well. After subconfluences, 3 wells in each plate were counted at the starting point (0 hr) and daily up to 168 hr. Cells on one plate continued to grow in 300 mOsm/kgH_2O medium, while cells on the other plate were challenged with hypertonic 550 mOsm/kgH_2O medium, which significantly retarded cell proliferation. **B:** IMCD3 cell proliferation is retarded with BFA. IMCD3 cells were seeded on two 24-well plates at a concentration of 200,000 cells/well. When the cells were subconfluent, the cell numbers were counted and this time was defined as the starting point (0 hr). The wells were then divided into 4 groups with different media: 300 mOsm/kgH_2O, 550 mOsm/kgH_2O, 550 mOsm/kgH_2O+1 μM BFA, 550 mOsm/kgH_2O+ 5 μM BFA. Cell numbers were determined after 24 hr and BFA-media were changed to BFA-free media for another 48 hr. All pairwise comparisons between the curves are significantly different ($p<0.001$) and illustrate that BFA causes distinct decreases in cell proliferation. **C:** Cell viability decreases after treatment with BFA. IMCD3 cells were seeded on two 24-well plates at a concentration of 10,000 or 25,000 cells/well. When the cells were subconfluent, 3 wells were split, the number of cells counted, and this time determined as the starting point. The IMCD3 cell viability based on the non-radioactive cell viability test decreased following BFA treatment both in 300 and 550 mOsm/kgH_2O media. Graphs in all figures show mean values±SE.

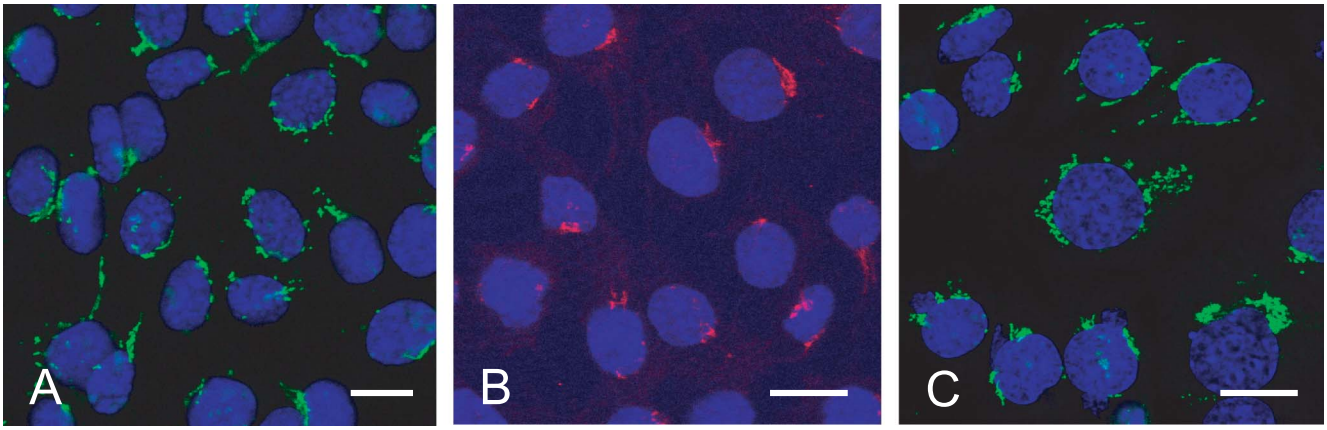

Fig. 2. Localization of Golgi proteins in IMCD3 cells. Immunolocalization of Golgi proteins GM130 (**A, C**) and Golgi 58k (**B**) in IMCD3 cells, cultured in isotonic medium, and acutely challenged with 550 mOsm/kgH_2O medium (**C**). In 300 mOsm/kgH_2O medium both the GM130 protein (green) (**A**) and 58k protein (red) (**B**) are strongly expressed in the Golgi apparatus located close to the nucleus. Nuclei are stained with To-Pro-3. Bar=20 μm.

and short cisternae (Fig. 2A), while 58K Golgi protein appeared mainly as larger but fewer cisternal and vesicular complexes (Fig. 2B). The expression of GM130 in IMCD3 cells acutely challenged in the hypertonic medium for 24 hr was similar or sometimes even stronger than in IMCD3 cells grown in 300 mOsm medium (Fig. 2C, cf. Fig. 2A).

The role of the Golgi apparatus in the transport of the γ subunit to the plasma membrane was studied in IMCD3 cells treated with BFA according to protocols 1 and 2. The γ subunit (green) was strongly expressed in the plasma membrane of groups of hypertonically challenged IMCD3 control cells (Fig. 3A), but only faintly expressed in some cells if the medium contained BFA (Fig. 3B). However, if BFA was then excluded from the medium and the cells recovered for 48 hr in hypertonic medium only, γ expression was distinctly enhanced. Thus γ was observed in larger groups of cells than after challenging in medium without BFA (compare Fig. 5E and 5G). High magnification images showed that the γ label (green) was present both in the plasma membrane and in the Golgi region of some cells (Fig. 3D, arrows), while 58K Golgi protein (red) was present in all cells (Fig. 3E). Double immunolabeling for γ and 58K Golgi protein revealed strong co-localization in the perinuclear cytoplasm in merged images (arrows in Fig. 3F). BFA treatment decreased γ labeling of the plasma membrane, as well as 58K Golgi protein (Fig. 3G–I). Double labeling with γ (Fig. 3J) and 58K Golgi protein (Fig. 3K) showed return of both proteins to the Golgi regions after recovery for 48 hr (Fig. 3L, arrows). Treatment with 5 μM BFA for 24 hr together with hypertonic challenging caused extensive cell death and the remaining cells showed disassembly of the Golgi apparatus and absence of the γ subunit in the plasma membrane (results not shown).

The immunolabeling of the α1 subunit after hypertonic challenging for 24 hr was only observed in the plasma membrane but not in the cytoplasm (Fig. 4A). It also remained in the plasma membrane after BFA treatment (Fig. 4B). There was no apparent co-localization of α1 with the 58K Golgi protein (Fig. 4C–F).

Localization of γ and α after hypertonic challenge and subsequent BFA treatment (Protocol 2)

The number of γ labeled cells was increased in groups of cells in cultures challenged with 550 mOsm/kgH_2O medi-

Fig. 3. Simultaneous acute hypertonic challenge and BFA treatment redistributes Na,K-ATPase γ subunit and Golgi proteins in IMCD3 cells. Over-view images (**A–C**) and high magnification images (**F, I** and **L**) of double-immunolocalized 58K Golgi protein and the γ subunit of Na,K-ATPase in the cytoplasm of IMCD3 cells acutely challenged for 24 hr with 550 mOsm/kgH_2O medium (**A** and **F**), simultaneously treated with 1 μM brefeldin A (BFA) (**B** and **I**), and subsequently again in 550 mOsm/kgH_2O without BFA (**C** and **L**). The γ subunit is strongly expressed in the plasma membrane in clusters of challenged cells (green) (**A**), very little or not at all expressed in BFA treated cells (**B**) but again intensely expressed in large clusters of cells during a recovery period in hypertonic medium without BFA treatment (**C**). This is further demonstrated in high magnification single color and overlay images: A stripe of cells with γ label (green) in the plasma membrane as well as in the cytoplasm of the same IMCD cells (arrows, **D**). 58K Golgi protein labeling (red) is present in all cells. Golgi areas in the γ-positive cells marked with arrows (**E**). Co-localization (yellow) in the γ-positive cells in a merged image (arrows, **F**). In the BFA treated cells γ is lacking in the plasma membrane but is present differently in the cytoplasm of some cells (arrows, **G**). The expression of 58K Golgi protein is diminished and diffuse in the cytoplasm (**H**) and co-localizes with γ in overlays (arrows, **I**). The γ subunit is strongly expressed in the plasma membrane of cells recovered from the BFA treatment in the hypertonic medium for 48 hr and expressed also in the cytoplasm (**J**). These cytoplasmic areas are also labeled for Golgi 58K and show resemblance to the Golgi apparatus (**K**, red) and are co-localizing with the γ label (**L**, arrows). Nuclei are stained with To-Pro-3. Bars=40 μm (**A**), 15 μm (**B**). Magnification is the same in **A–C**, and **D–L**, respectively.

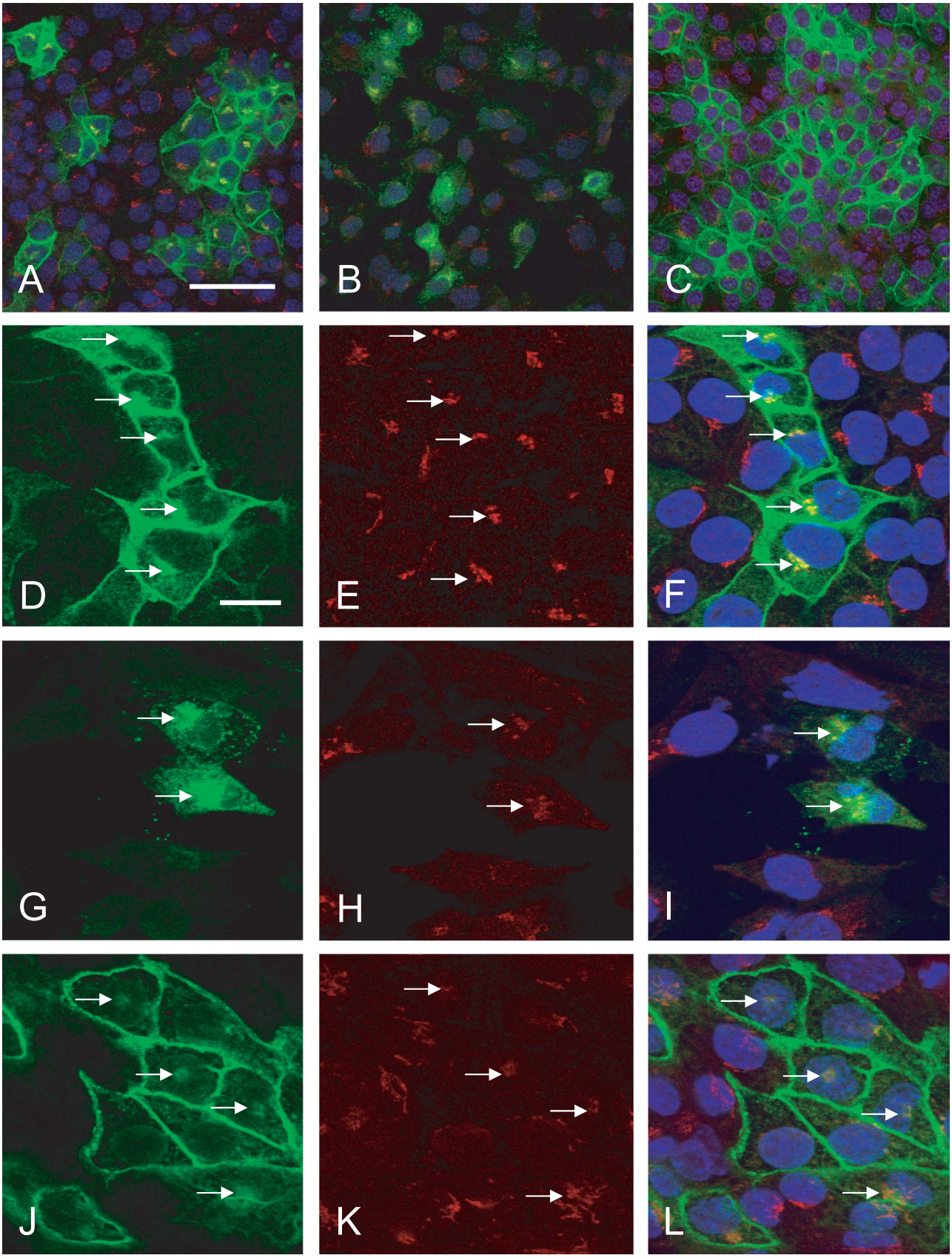

Fig. 3

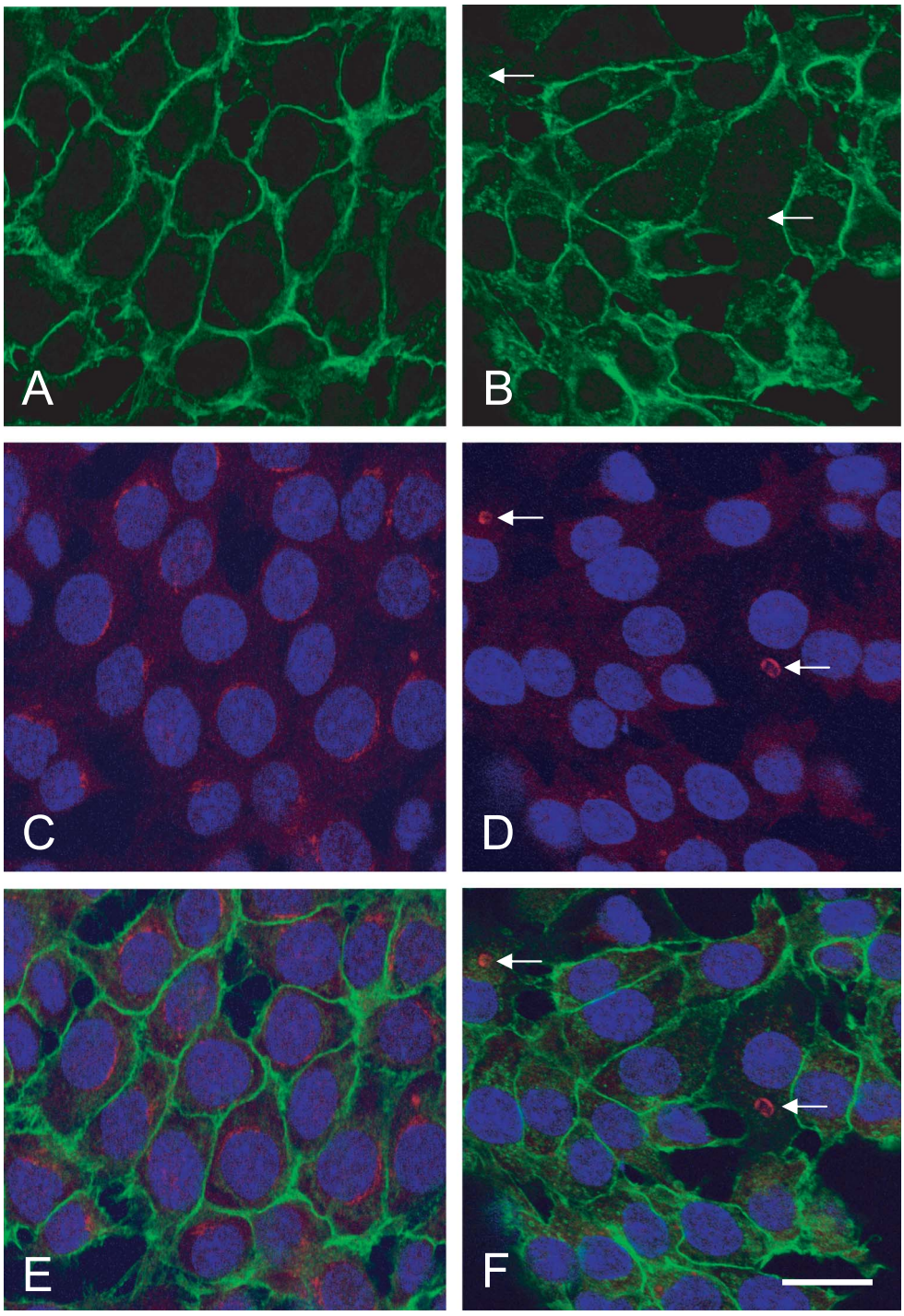

Fig. 4. Simultaneous acute hypertonic challenge and BFA treatment does not redistribute Na,K-ATPase α1 subunit. Hypertonically challenged (**A, C** and **E**) and simultaneously BFA-treated (**B, D** and **F**) IMCD3 cells with double-immunostaining for the α1 subunit of Na,K-ATPase (green, **A** and **B**) and the 58K Golgi protein (red, **C** and **D**) show no co-localization in the overlay images (**E** and **F**). Immunolabeling for the α1 subunit in the plasma membrane remains strong after BFA treatment (**B**). The 58K Golgi protein label is visible in all control cells (**C**) but only in a few experimental cells (arrows, **D**). Cell nuclei are stained with To-Pro-3. Same magnification in all images. Bar=15 μm.

um for 48 hr (Fig. 5A, B, cf. Fig. 3A). Cells challenged first for 24 hr and subsequently exposed to 1 μM BFA in hypertonic medium for 24 hr (Fig. 5C, D), showed, on the contrary, diffuse and vesicular cytoplasmic γ label but no label of the plasma membrane (Fig. 5, cf. A vs. C, and B vs. D). Some of the vesicles were arranged like pearls in a necklace in the periphery of the cells (Fig. 5D, arrows). Interestingly, the proportion of cells labeled for γ in cell cultures exposed

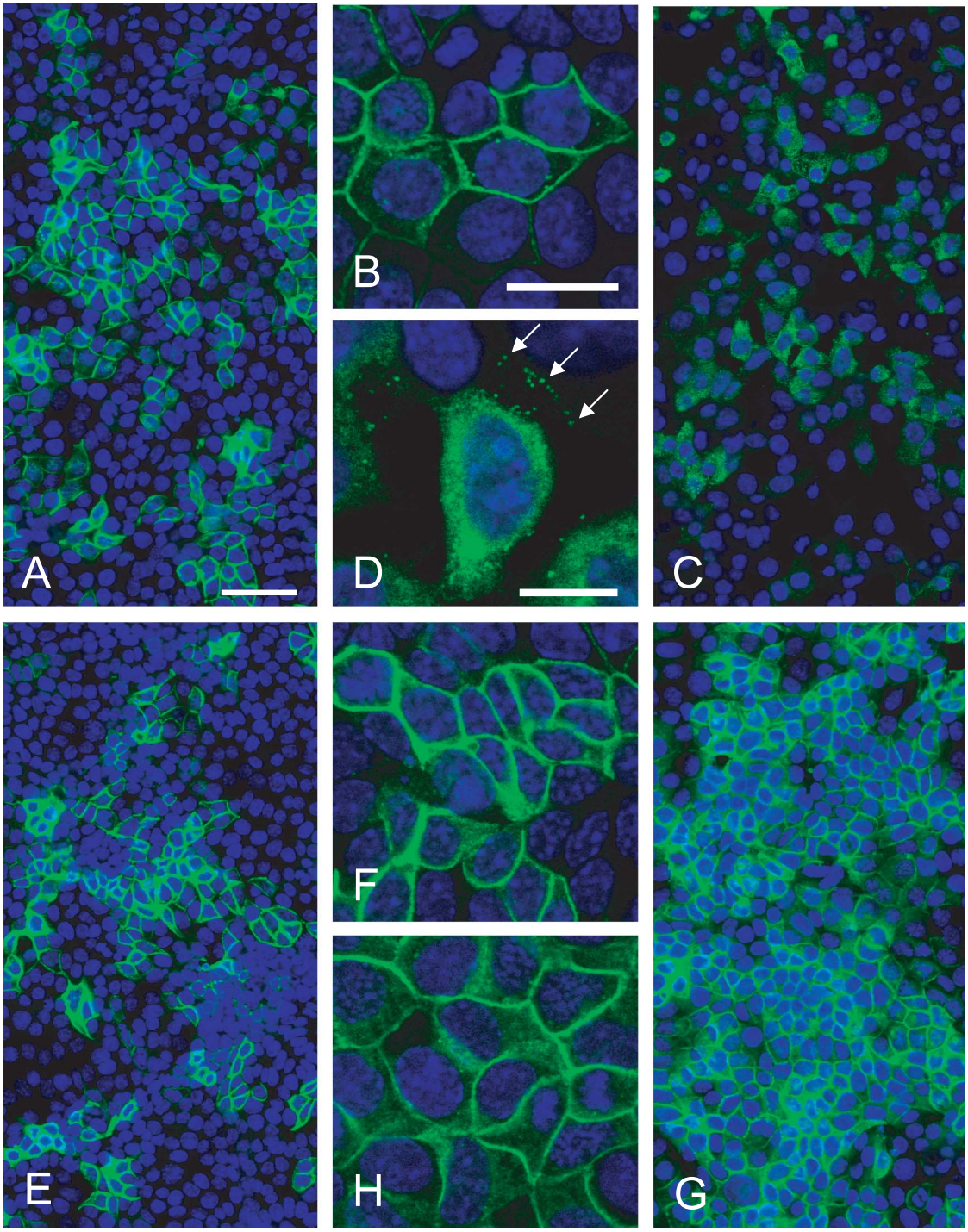

Fig. 5. Effect of acute hypertonic challenge and consecutive BFA treatment vs. hypertonic challenge without BFA on the location of Na,K-ATPase γ subunit. Immunolocalization of the γ subunit shows strong plasma membrane staining in IMCD3 cells acutely challenged in 550 mOsm/kgH_2O medium for 48 hr (control) (**A**) and at higher magnification (**B**). First hypertonic challenging (24 hr) and then 1 μM BFA in 550 mOsm/kgH_2O medium (24 hr) results in absence of γ from the plasma membrane but diffuse or vesicular labeling in the cytoplasm (**C** and **D**). In some cells small vesicles are located like pearls in string close to the plasma membrane (arrows, **D**). Continuous hypertonic challenging for 4 d without BFA does not induce more γ-stained cells than cultures challenged for two days (**E** cf. **A**) but indicates strong plasma membrane location (**F**). The ability of cells to recover after the 1 μM BFA treatment is demonstrated in **G** and **H**. Cells received the same BFA treatment as in **C** and **D**, but were then incubated in 550 mOsm/kgH_2O medium for 48 hr without BFA (**G**). The γ subunit is observed in more cells than in the cultures that have been in hypertonic medium only for four days (**E**). High magnification image (**H**) demonstrates that γ is present both in the plasma membranes and in the cytoplasm. Cell nuclei are stained blue with To-Pro-3. Magnification is the same in **A, C, E** and **G**; bar in **A**=40 μm. Magnification is the same in **B, F** and **H**; bar in **B**=20 μm. Bar in **D**=10 μm.

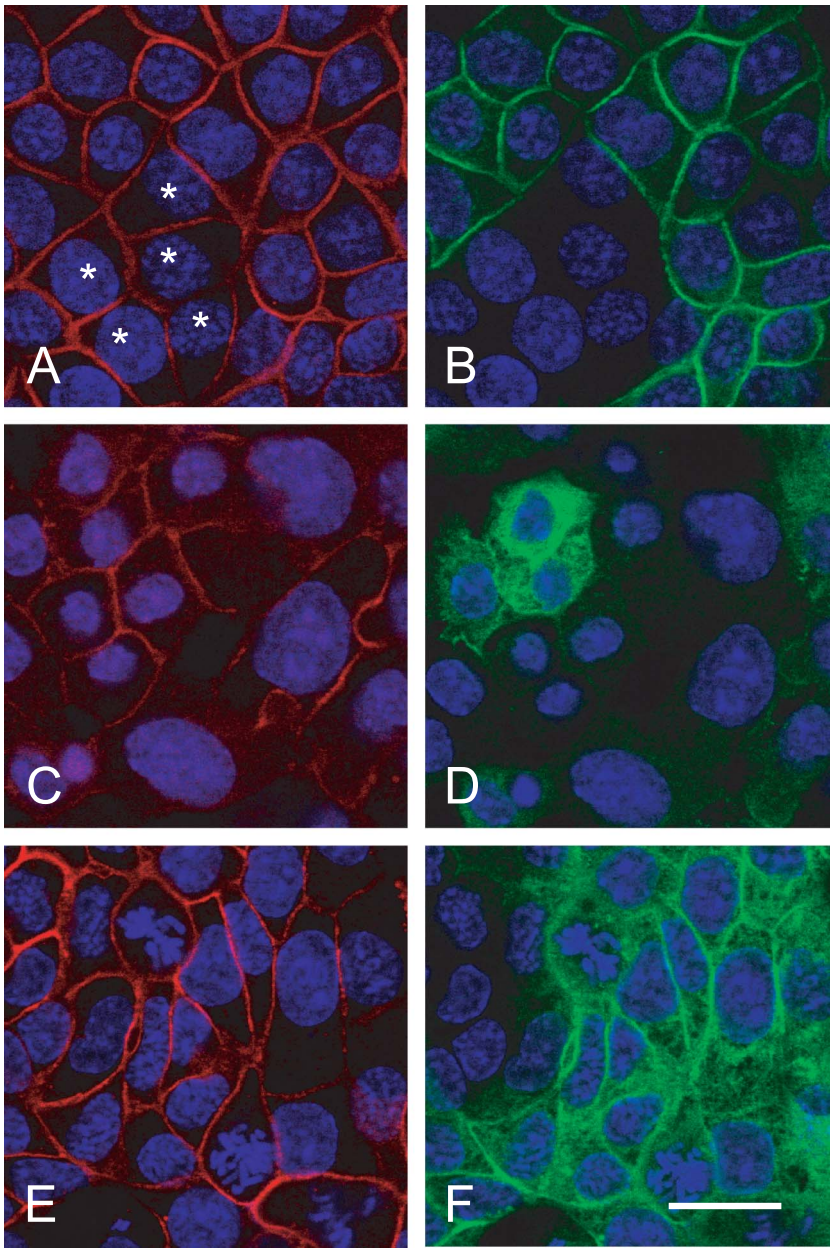

Fig. 6. Different responses of Na,K-ATPase α1 and γ subunits in hypertonic challenged IMCD3 cells with or without BFA treatment. Immunolocalization after acute hypertonic challenge of IMCD3 cells for 48 hr with the monoclonal antibody against the α1 subunit demonstrates uniform location of α1 in the plasma membranes of all cells, while only a portion of cells (γ-negative cells marked with stars in **A**) shows immunolocalization with polyclonal antibody against the Na,K-ATPase γ subunit (**B**). Hypertonic challenging for 24 hr and then BFA in a hypertonic medium for 24 hr results in distinct location of α1 (**C**) in the plasma membrane in most cells and additional weak diffuse location in the cytoplasm, while γ is almost exclusively located diffusely in the cytoplasm of some cells (**D**). IMCD3 cells in (**E**) and (**F**) immunostained for α1 and γ, respectively, were treated as in **C** and **D** but subsequently allowed to recover in 550 mOsm/kgH_2O medium without BFA for 48 hr. Similar location of α1 in the plasma membrane as in control (**E** cf. **A**), while γ is located both in the plasma membrane but still also diffusely in the cytoplasm (**F**). Cell nuclei are stained blue with To-Pro-3. Magnification is the same in all images. Bar=20 μm.

to hypertonic medium for 96 hr without BFA treatment (Fig. 5E, F) was not greater than in cell cultures grown in the same medium for 48 hr only (Fig. 5E vs. A) or in cell cultures that had been treated with BFA (Fig. 5G, H).

Double immunolabelings for the α1 and γ subunits showed that α1 was distinctly present in the plasma membrane of all hypertonically challenged IMCD3 cells (Fig. 6A), while γ was present only in some of the challenged cells (Fig. 6B, cells without γ marked with stars in A). After exposure of cell cultures to BFA for 24 hr in hypertonic medium the α1 remained in the plasma membrane although somewhat reduced (Fig. 6C). The γ subunit was

almost exclusively present in the cytoplasm (Fig. 6D). Following a recovery period for 48 hr without BFA in the hypertonic medium α1 was as before strongly expressed in the plasma membrane of all cells (Fig. 6E), while γ was present both in the plasma membrane and in the cytoplasm.

IV. Discussion

The present immunocytochemical observations suggest that γ is not transported together with α1 to the plasma membrane during hypertonic challenging of IMCD3 cells. Immunolabeling for α1 overlaps neither with γ nor with Golgi marker 58K protein at the time points, when γ is overlapping with the Golgi marker. This probably also excludes the possibility that γ trafficks together with αβ heterodimers after co-translational synthesis of α and β subunits in the ER [16, 25, 26].

Our previous immunofluorescence observations indicated that biosynthesis of γ after hypertonic challenging is slow and that the subunit is detectable in the cytoplasm of IMCD3 cells only after 6 hr and distinctly in the plasma membrane after 24 hr [29]. This is consistent with the observed doubling time of about 18 hr for the γ subunit of IMCD3 cells in 600 mOsm/kgH_2O medium reported by Klawitter *et al.* [22]. The half-time of γ expression is about 17 hr, when adapted cells are returned to isotonic conditions [7]. In comparison Na,K-ATPase reached the cell surface in cultured MDCK cells 50 min after synthesis [9], and the peak of both radioactive-labeled α and β subunits appeared in the plasma membrane-enriched sample from MDCK cells at 60 min suggesting that they moved as αβ heterodimers [26]. Hypertonicity induced increased Na,K-ATPase expression in MDCK cells as studied by mRNA levels of α and β subunits, pump activity in cell homogenates and elevated capacity for active K^+ influx [5]. Thus, it is likely that hypertonicity increased rather than decreased the rate of synthesis and transport of Na,K-ATPase. Also in IMCD3 cells α1 may be synthesized and transported to the cell surface more quickly than γ. Different synthesis and transport times to reach the cell surface are consistent with our immunocytochemical observations indicating that α and γ subunits are not transported together, and that γ is transported alone to the plasma membrane.

The different responses of the γ and α1 subunits to BFA observed in the present study show that α1 is more tolerant to BFA than the small γ subunit. Less sensitivity of the α1 subunit to BFA may mean that if trafficking after biosynthesis is inhibited by BFA, α1 will remain unchanged longer in the plasma membrane than γ, while the plasma membrane contents of γ will decrease more rapidly, perhaps due to the different stabilities and turnover rates of the proteins. On the other hand, slower clearance of α1 from the plasma membrane compared to that of γ, may parallel a lower degree of neosynthesis, which would explain the invisibility of α1 in the Golgi apparatus.

The over-all effect of BFA in the present study was a reduction of cell proliferation in hypertonically challenged IMCD cells. As demonstrated in previous studies on mammalian cells [3, 14, 23, 35; for reviews see 19, 21, 28], the effects of BFA vary in different cell types and to different extents. However, they generally include disassembly of the Golgi stacks into tubules and vesicles, and reversible inhibition of protein secretion at an early stage in the secretory pathway. In these changes dissociation of ADP-ribocylation-factor-1 (ARF1) and the coatomer protein complex-1 (COP-I) from Golgi membranes play an important role [2, 31]. The present results showed that simultaneous exposure of IMCD3 cells to hypertonicity and BFA had a stronger effect on the cells than if they were first hypertonically treated and then received BFA. In this case the γ synthesis continued and resulted in accumulation of γ in cytoplasmic vesicles, but γ did not incorporate into the plasma membrane, until BFA was removed from the medium.

As shown in several previous studies, the effects of BFA on cells are reversible. In the IMCD3 cells γ became expressed again after the removal of BFA from the hypertonic medium. This expression was even more enhanced than when the cells were continuously kept in the hypertonic medium (Fig. 5E versus 5G). The reason for the overexpression of γ following removal of BFA remains to be established.

V. Acknowledgements

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VI. Abbreviations

BFA, brefeldin A; IMCD, inner medullary collecting duct.

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