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Role of Microwave Heating in Antigen Retrieval in Cryosections of Formalin-Fixed Tissues

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1. Introduction

Immunocytochemistry is a common method for studying localization of proteins in the mammalian tissues and cultured cells. Tissues and cells to be used in immunocytochemical experiments are often fixed and preserved with 2%-10% *p*-formaldehyde (PFA, formaldehyde, formalin), embedded in paraffin or frozen, and sectioned. In histological research, PFA has been used as a fixative preferable to alcohols since the end of 19th century. It is praised for its effectiveness in a broad spectrum of concentrations and conditions without producing "overfixation". Moreover, in comparison with the alcohol-fixed tissues, the PFA-fixed tissues show minor tissue distortion and better staining with the "classical dyes", such as hematoxylin and aniline dyes. The effects of PFA fixation were shown to be pH-, temperature-, and protein type-dependent (Fox et al., 1985).

In contrast to excellent results of PFA fixation when used on tissues to be stained with the afore mentioned "classical dyes", in immunocytochemical studies the PFA-fixed tissues often do not yield acceptable results; fixation with PFA denatures the proteins while preserving antigenicity, but it can affect the tissue and cell integrity by damaging the structure and it can "mask/hide" the antibody binding sites (epitopes) (McNicol & Richmond, 1998). Sometimes the severity of this phenomenon affects the availability of epitopes, thus making it harder or even impossible for antibodies to react with their binding sites. Although the problem of epitope masking has been recognized a long time ago, it is still unclear how PFA changes proteins in tissues. In general, the epitope masking occurs due to PFA-induced cross-linking of the reactive sites on the same protein and/or among adjacent proteins *via* bridges that can be formed between methylene, amino, imino, aromatic, and other reactive groups (Fraenkel-Conrat et al., 1948, 1949). However, this masking does not happen at the same rate for all kinds of proteins; in tissues fixed for a prolonged time period, different proteins react differently, and the consecutive staining efficiency is protein-dependent. For example, in the absence of any unmasking technique, the proteins vimetin and desmin failed to be stained with the respective monoclonal antibodies after one day of tissue fixation, the lymphocyte antigens LN1, LN2, and LN3 were stained weakly with polyclonal antibodies in the cells fixed in formalin for three days, while thyroglobulin and carcinoembryonic antigen showed good staining with polyclonal

antibodies in the tissues even after 14 days of formalin fixation (Leong & Gilham, 1989). These phenomena can be only partially explained by the antigen affinity for an antibody, where monoclonal antibodies, being more specific in nature then the polyclonal antibodies, have fewer reactive binding sites (McNicol & Richmond, 1998). Some additional explanations of the epitope masking problem have been offered by Seshi et al., 2004, who used crafted peptides to mimic the antibody binding sites on the targeted proteins; the authors were able to divide peptides in three distinct groups based on their sensitivity to PFA: (a) PFA-sensitive peptides, that contained tyrosine and arginine, (b) peptides without tyrosine and arginine, which were refractory to PFA, and (c) tyrosine-containing peptides, which were sensitive to PFA only if an another, arginine-containing peptide was in the vicinity. Other data have shown that the PFA treatment can change peptides depending on their amino acid sequence (Metz et al., 2004), and that calcium and some other divalent cations could affect the epitope masking by forming tight complexes with proteins during fixation (Morgan et al., 1994). Recent studies have shown that not only the reactive sites (amino groups, tryptophan, tyrosine, lysine, etc…), but also their position in the three dimensional protein structure may be important for masking the epitopes during the PFA fixation (Toews et al., 2008). The resulting changes in protein conformation can make epitopes inaccessible to antibodies in immunocytochemical studies (McNicol & Richmond, 1998).

In order to overcome the problem of masked (hidden) epitopes caused by PFA fixation, one has to unmask/recover these epitopes by various protocols of antigen retrieval before applying specific antibodies. These protocols are normally used with sections of the paraffin-embedded tissues, and include heating at high temperatures, treatment with various alcohols, detergents or high pressure, or various combinations of these procedures. Antigen retrieval is commonly described as a ''high temperature heating method to recover the antigenicity of tissue sections that had been masked by formalin fixation'' (Shi et al., 2001), but in some cases, the hidden epitopes can be recovered only with the detergent treatment, without heating (Brown et al., 1996). Good antigen retrieval protocols can significantly increase the staining even in the archival tissues that have been stored in formaline for months (Hann et al., 2001).

Although the method of antigen retrieval has been in use for a few decades, possible mechanisms behind it are still unclear. A heat, combined with buffers of various pH values, seems to be a primary factor in most antigen retrieval protocols, and supposedly breaks down protein cross-links formed during fixation. In some cases, chelating agents can also help by breaking the fixation-generated calcium-protein complexes (Gown, 2004; Leong et al., 2007). In paraffin-embedded tissue sections, a microwave oven was found to be preferable to conventional oven as a heat source (Shi et al., 1991), whereas in a study by Cuevas et al., 1994, with 80 different antibodies, microwave heating of the paraffin sections significantly enhanced the staining with 41 antibodies, thus confirming microwave as an important tool in immunocytochemistry. In addition to conventional and microwave ovens, other sources of heat can be used, such as the pressure cooker (Norton et al., 1994) and hotplate for heating tissue sections (Hann et al., 2001) or whole tissues before sectioning (Ino, 2003). Besides heating, some antigen retrieval protocols include treatment with high vapour pressure, various detergents, buffers of different pH and concentration, and various metal ions, that can be used alone or in various combinations without or with heating (Shi et al., 2001).

A harsh antigen retrieval protocols, that include microwave heating and alcohol treatment, are common steps for unmasking epitopes in sections of the paraffin-embedded, PFA-fixed tissues (Cattoretti et al., 1993; Cuevas et al., 1994; Hoetelmans et al., 2002; Leong et al., 2002), whereas the use of such protocols with cryosections of the PFA-fixed tissues is less common. It is assumed that the availability of epitopes in tissue cryosections is a lesser problem than in the sections of paraffin-embedded tissues. However, the treatment of tissue cryosections with sodium dodecyl sulfate (SDS), without heating, has been efficiently used to enhance immunostaining with some antibodies (Brown et al., 1996; Sabolić et al., 1999), thus indicating that unmasking techniques may be beneficial for revealing the antibody binding epitopes also in cryosections. Furthermore, we have recently described that heating cryosections of the PFA-fixed rat and mouse organs in a microwave oven can be used to enhance immunostaining with specific antibodies for various transporters of organic anions (Bahn et al., 2005; Breljak et al., 2010; Brzica et al., 2009b; Ljubojević et al., 2004 & 2007; Yokoyama et al., 2008), glucose (Balen et al., 2008; Sabolić et al., 2006), and sulfate (Brzica et al., 2009a).

In order to demonstrate the importance of various unmasking protocols for immunocytochemical studies in tissue cryosections, we have used cryosections of the rat kidney and liver tissues that had been fixed with 4% PFA *in vivo*, treated them with various antigen retrieval protocols without and with the use of microwave heating, and tested for the intensity of immunostaining of several representative proteins known to reside in the cell membrane (cell adhesion molecule 105 (CAM105), megalin (GP$_{330}$), Na/K-ATPase, aquaporin 1 (AQP1)), cytoplasm (metallothionein), cell membrane and intracellular organelles (vacuolar H$^+$-ATPase (V-ATPase)), and cytoskeleton (actin, tubulin). Using the V-ATPase as an example, we have also tested effects of heating time and different power settings of the microwave oven on the intensity and distribution of antibody staining. The immunostaining was studied by the method of indirect immunofluorescence, where specific epitopes on the proteins were first labeled with the relevant primary polyclonal or monoclonal antibodies, followed by labeling with the fluorescent molecule-conjugated secondary antibodies and fluorescence microscopy.

2. Material and methods

2.1 Antibodies and other material

Primary antibodies used in these studies were monoclonal or polyclonal affinity purified antibodies raised against the holoproteins or defined, protein-specific peptide sequences. Some of them were purchased commercially, and some were non-commercial, but their use in immunocytochemistry has been described in our previous publications (down indicated in brackets). The commercial antibodies against the following proteins were used: Na/K-ATPase (monoclonal anti-peptide antibody against the α1 subunit of the human protein, Santa Cruz Biotechnology, CA, USA), metallothionein (monoclonal antibody against the polymerized MT1 and MT2 horse holoproteins; Dako North America, CA, USA), actin (monoclonal anti-peptide antibody; Millipore, MA, USA), and α-tubulin (monoclonal antibody against the see urchin filament; Sigma, MO, USA). The non-commercial antibodies against the following proteins were used: megalin (polyclonal antibody against the rat holoprotein; characterized in Abbate et al., 1994 and Sabolić et al., 2002), CAM105 (polyclonal antibody against the rat holoprotein; characterized in Sabolić et al., 1992a), AQP1; polyclonal antibody against the rat holoprotein; characterized in Sabolić et al., 1992b), and V-ATPase (polyclonal anti-peptide antibody against the 31 kDa ("E") subunit;

characterized in Herak-Kramberger et al., 2001). Cryosections were incubated in an optimal concentration of the antibody, as defined in preliminary experiments (data not shown), in a refrigerator over night (ON; 12-14 hours).

Secondary antibodies were the CY3-labeled goat anti-rabbit (GARCY3) or donkey anti-mouse IgG (DAMCY3), purchased from Jackson ImmunoResearch (West Grove, PA, USA), and FITC-labeled goat anti-rabbit (GARF) or goat anti-mouse (GAMF) IgG, purchased from Kirkergaard & Perry Laboratories (Gaithersburg, MD, USA). These antibodies were applied in the following concentrations: GARCY3 – 1.5 µg/mL , DAMCY3 – 1.25 µg/mL, GARF – 2.5 µg/mL, and GAMF – 5 µg/mL, at room temperature (RT) for 1 hour. All incubations with the antibodies were performed in the moist chamber.

The following noncommercial solutions have been prepared and used:

PBS (phosphate buffered saline (in mM): 137 NaCl, 2.7 KCl, 8 Na_2HPO_4, 2 K_2PO_4, pH 7.4)

PFA (4% w/v p-formaldehyde in PBS)

HS-PBS (high salt PBS; PBS containing a triple (411 mM) concentration of NaCl)

BSA (bovine serum albumin; 1% w/v in PBS)

Citrate buffer (10 mM citrate buffer, pH 3, pH 6, and pH 8)

Sucrose solution (30% w/v sucrose in PBS)

SDS (detergent sodium dodecyl-sulphate; 1% w/v in PBS)

T-X-100 (Triton-X-100; 0.1%, 0.5%, and 2% w/v in PBS)

Alcohols xylol and propanol were used undiluted (100%), whereas ethanol was used in concentrations of 60%, 70%, and 96%, prepared v/v in distilled water.

The ingredients in these solutions, and other chemicals used in this study were of the highest purity, and were purchased from Sigma (St. Louis, MO, USA). The SuperFrost-plus microscope slides were purchased from Thermo Scientific (Menzel-Gläser, Braunschweig, Germany). The fluorescence fading retardant (Vectashield) was purchased from Vector Laboratories Inc., CA, USA.

2.2 Animals, tissue fixation, cryosections

Adult male Wistar strain rats were anaesthetized and their circulatory system was perfused *in vivo* using the Masterflex pump (Cole-Parmer, IL, USA) *via* the left ventricle of the heart, first with aerated (95% O_2/5% CO_2) and temperature equilibrated (37 ^{0}C) PBS for 2-3 min to remove blood, and then with 150 mL of fixative (4% PFA) for 4-5 min. Kidneys and the largest liver lobe were removed, cut in ~1 mm thick slices, postfixed in the same fixative at 4 ^{0}C for 24 hours, and then washed with four abundant volumes of PBS to remove PFA. The tissues were then rinsed with, and stored refrigerated in PBS containing 0.02% NaN_3 until use. For cryosectioning, tissues were soaked in 30% sucrose solution at 4 ^{0}C overnight, embedded in OCT-medium (Tissue-Tek, Sakura, Japan), and frozen at –25 ^{0}C. Four µm-thick cryosections were sliced in a Leica CM 1850 cryostat (Leica instruments GmbH, Nussloch, Germany), collected on SuperFrost-plus microscope slides, dried at room temperature for 2-3 hours, and stored refrigerated until use.

2.3 Protocols of antigen retrieval

In order to reveal optimal protocols for unmasking the antibody-binding epitopes, tissue cryosections were treated with different protocols and then subjected to immunostaining procedure with specific antibodies. The immunostaining in the absence of any antigen retrieval (Protocol A) was compared with that following treatment with SDS (Protocol B),

following heating in a microwave oven in strongly acidic (pH 3), weakly acidic (pH 6), and alkaline (pH 8) citrate buffers (Protocols C, D, and E, respectively), and following treatment in alcohols and consecutive heating in a microwave oven in the same citrate buffers (Protocols F, G, and H, respectively). The incubations were performed either at room temperature (RT) or in a refrigerator (4 ^{0}C). Heating of cryosections was performed in a conventional microwave oven with the variable power settings (max. 800 W). Therefore, in these studies the following protocols were applied:

Protocol A, untreated cryosections (steps): Rehydration in PBS (15 min/RT) - incubation in BSA (30 min/RT) - incubation in primary antibody (refrigerator/ON) - rinsing in HS PBS (2x5 min/RT) - rinsing in PBS (2x5 min/RT) - incubation in secondary antibody (60 min/RT) - rinsing in HS PBS (2x5 min/RT) - rinsing in PBS (2x5 min/RT) – covering with Vectashield and the cover glass, and sealing lateral openings with nail polish.

Protocol B, SDS-treatment (steps): Rehydration in PBS (15 min/RT) - incubation in SDS (5 min/RT) – rinsing in PBS (4x5 min/RT) - incubation in BSA (30 min/RT) - incubation in primary antibody (refrigerator/ON) – other steps are the same as listed in protocol A.

Protocol C, microwave heating (steps): Rehydration in PBS (15 min/RT) – immersion in citrate buffer, pH 3, and heating in a microwave oven (4x5 min/800 W, with intermittent addition of the evaporated buffer; cryosections should be fully immersed in the buffer all the time) - cooling down of cryosections in the buffer (20 min/RT) – rinsing in PBS (3x5 min/RT) – incubation in 0,5% T-X-100 (15 min/RT) – incubation in 2% T-X-100 (30 min/RT), - rinsing in PBS (2x5 min/RT) – incubation in BSA (30 min/RT) - incubation in primary antibody (refrigerator/ON) - incubation in 0.1% T-X-100 (10 min/RT) – rinsing in PBS (2x5 min/RT) - incubation in secondary antibody (1 hour/RT) – incubation in 0.1% T-X-100 (10 min/RT) – rinsing in PBS (2x5 min/RT) covering with Vectashield and the cover glass, and sealing with nail polish.

Protocol D, microwave heating (steps): The same as protocol C, except the citrate buffer had pH 6.

Protocol E, microwave heating (steps): The same as protocol C, except the citrate buffer had pH 8.

Protocol F, alcohol treatment and microwave heating (steps): Immersion (without rehydration) and incubation in 100% xylol (30 min/RT) - incubation in 100% propanol (5 min/RT) – incubation in 96% ethanol (5 min/RT) – incubation in 70% ethanol (5 min/RT) – incubation in 60% ethanol (5 min/RT) – rinsing in distilled H$_2$O (5 min/RT) - rehydration in PBS (15 min/RT) - immersion in citrate buffer, pH 3, and heating in a microwave oven (4x5 min/800 W, with intermittent addition of the evaporated buffer; cryosections should be fully immersed in the buffer all the time) – the consecutive steps are the same as listed in protocol C.

Protocol G, alcohol-treatment and microwave heating (steps): The same as the protocol F, except the citrate buffer had pH 6.

Protocol H, alcohol-treatment and microwave heating (steps): The same as the protocol F, except the citrate buffer had pH 8.

In an additional set of experiments, the antigen retrieval-dependent immunostaining with the anti-V-ATPase antibody in cryosections of the PFA-fixed rat kidney cortex was used to test (a) the heating time-dependent effects at the fixed power, and (b) the power-dependent effects at the fixed heating time. Accordingly, to test the time-dependency, cryosections of the renal cortex were heated in a microwave oven at 800 W for 5 min, 2x5 min, 3x5 min and 4x5 min, using other conditions defined in the optimal protocol. To demonstrate the power-

dependent effects, cryosections of the renal cortex were heated in a microwave oven with 4x5 min cycles at 80 W, 160 W, 400 W, 560 W and 800 W, using other conditions defined in the optimal protocol. In both cases, the control cryosections were processed similarly, but without being heated.

The fluorescence was examined with an Opton III RS fluorescence microscope (Opton Feintechnik, Oberkochen, Germany) and photographed using the Spot RT slider camera and software (Diagnostic Instruments, Sterling Heights, MI, USA). Each set of images, related to the retrieval protocol for a specific antibody/antigen pair, was photographed under the same recording parameters. The images were imported into Adobe Photoshop 6.0 for processing, assembling, and labeling. In some cases, the CY3- or FITC-related fluorescence was converted into black and white mode using the same software.

3. Results

3.1 Antigen retrieval of epitopes specific for proteins located in cell membrane domains

In these experiments, cryosections of the PFA-fixed rat kidney or liver tissues underwent the above-listed unmasking protocols, followed by immunostaining of proteins known to be largely localized in the specific cell membrane domains of the nephron epithelium and hepatocytes: cell adhesion molecule CAM105, an ectoprotein expressed in the luminal (apical/brush-border (BBM)) membrane of the proximal tubules and the plasma membrane of peritubular capillaries (Sabolić et al., 1992a), megalin, a glycoprotein expressed in the BBM and subapical vesicles of the proximal tubules (Abbate et al., 1994; Kerjaschki & Farquhar, 1983; Sabolić et al., 2002), Na/K-ATPase, an integral membrane protein expressed in the contraluminal (basolateral (BLM)) cell membrane of the proximal and other tubules (Kashgarian et al., 1985; Sabolić et al., 1999) and in the hepatocyte sinusoidal membrane, and AQP1, a transmembrane protein expressed in both luminal and contraluminal membranes of the proximal tubules and thin descending limbs (Nielsen et al., 1993; Sabolić et al., 1992b).

3.1.1 CAM105

Previous studies showed localization of CAM105 in the BBM of S1 and S3, but not S2 segments of the rat kidney proximal tubules, and also in the plasma membrane of peritubular capillaries (Sabolić et al., 1992a). The present study was performed on cryosection of the kidney cortex, thus showing the protein expression in proximal tubule S1 segments and peritubular capillaries (Fig. 1). As shown in Fig. 1A, in the absence of any antigen retrieval conditions, a limited staining was observed in the BBM of S1 and in peritubular capillaries (arrowheads), but not in S2. In the SDS-treated cryosections (B), the staining distribution was similar to, but the intensity was weaker than that in (A). In cryosections treated with heating in a microwave oven (C-E), the staining intensity dependent on buffer pH, being weak at pH 3 (C), intermediate at pH 6 (D), and strong at pH 8 (E). Localization of the staining in the BBM of S1 and in the plasma membrane of peritubular capillaries, remained unchanged. Alcohol treatment plus microwave heating resulted in strong reduction of the staining intensity, to almost none at pH 3 (F) and pH 6 (G), while peritubular capillaries remained stained. However, at pH 8 (H), the staining in both location was present, but the intensity was weaker than in E. In summary, the CAM105-related staining of peritubular capillaries was present in all conditions, while the staining of BBM was dependent on the applied protocol. The best staining of CAM105 was achieved after using protocol E.
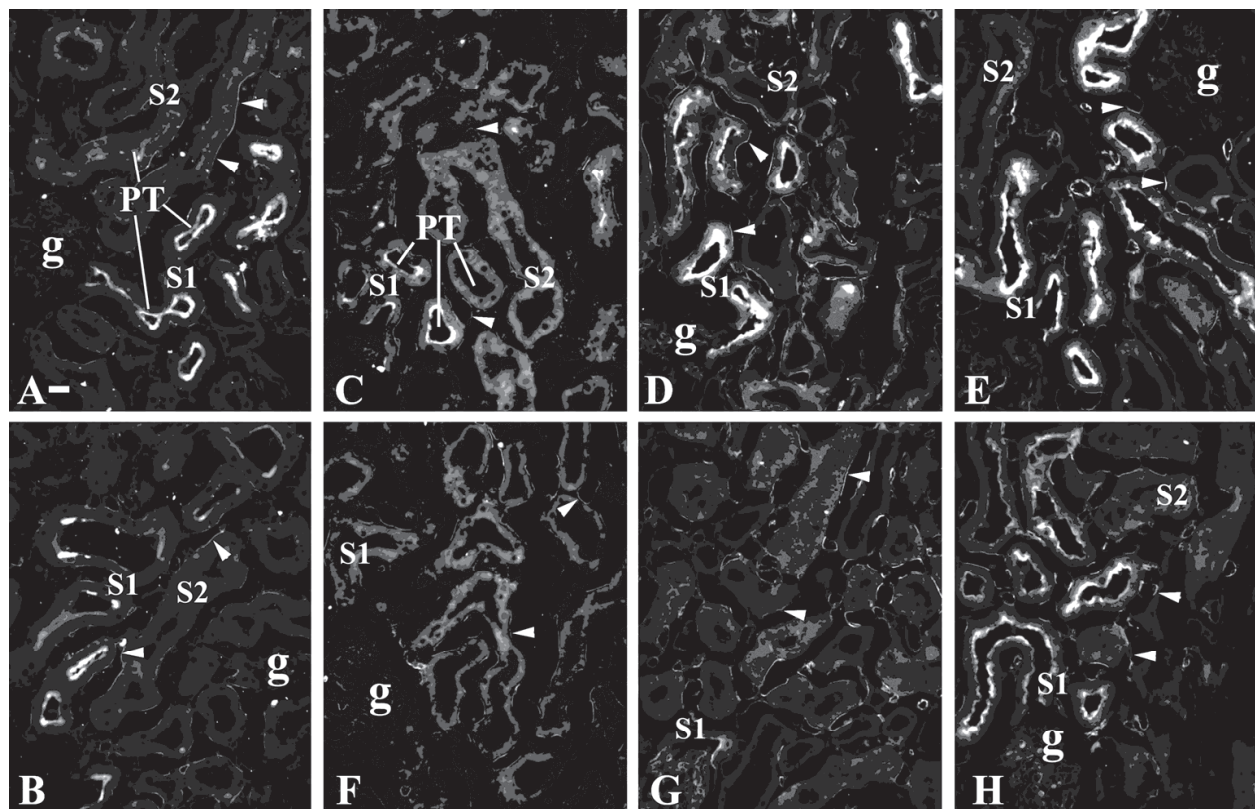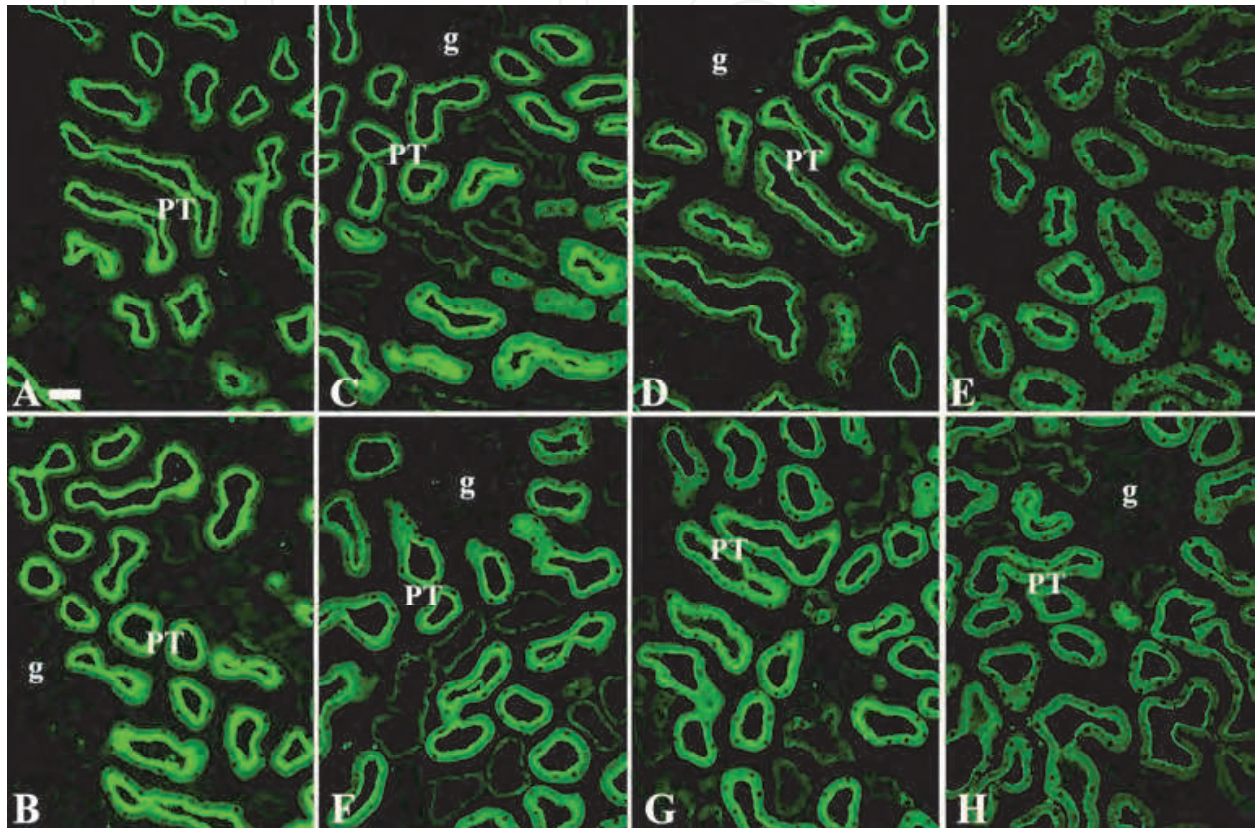
Fig. 1. Distribution and intensity of immunostaining of CAM105 in cryosections of the PFA-fixed rat kidney cortex tissue that underwent various antigen retrieval protocols listed in the section 2.3. g, glomeruli; PT, proximal tubules (S1, initial segment; S2, convoluted segment); arrowheads, peritubular capillaries. Bar, 20 µm.

3.1.2 Megalin

Immunostaining of megalin in the kidney cortex in cryosections treated with different antigen retrieval protocols is demonstrated in Fig. 2, A-H. A relatively strong staining of the proximal tubule apical domain (BBM and subapical vesicles) was present already in untreated cryosection (A), while SDS-treatment resulted in an additional intensity (B). Protocols with the microwave heating (C-E) showed the pH-dependent staining intensity with a sequence: pH 3 > pH 6 > pH 8, where the staining pattern and intensity at pH 3 (C) were similar to those in control conditions (c.f. A). Protocols with the alcohol treatment followed by heating (F-H) caused overall weaker staining when compared to protocols C-E, while displaying the same pH-dependency. Therefore, megalin immunostaining was not improved with heating cryosections at any pH without or with alcohol pretreatment, and the protocol with SDS-treatment alone (B) appears to be the best antigen-revealing method for megalin epitopes in the proximal tubules.

3.1.3 Na/K-ATPase

Fig. 3 (A-H) shows effects of different unmasking protocols on immunostaining of Na/K-ATPase in cryosections of the rat kidney cortex. In control conditions, Na/K-ATPase was stained weakly in the BLM of proximal tubules and slightly stronger in distal tubules and cortical collecting ducts (A). Following the SDS-treatment, an enhanced staining was observed in the distal tubules and collecting ducts, but the staining in proximal tubules remained

unchanged (B). Protocols with the microwave heating at pH 3 (C) and pH 6 (D) led to an enhancement in staining in all tubule profiles, particularly in the proximal and distal tubules (pH 3 < pH 6), whereas at pH 8 (E), the staining was weak in all tubules. In alcohol+heating-treated cryosections (F-H), the overall staining intensity was weaker than, but the staining pattern was similar to that in C-E. Therefore, protocol D represents the optimal antigen revealing approach for immunostaining of Na/K-ATPase along the rat nephron.

Fig. 2. Distribution and intensity of immunostaining of megalin in cryosections of the PFA-fixed rat kidney cortex tissue that underwent various antigen retrieval protocols listed in the section 2.3. g, glomeruli; PT, proximal tubules. Bar, 20 μm.

In order to check if the same protocol is valid for unmasking the epitopes of Na/K-ATPase in hepatocytes, cryosections of the PFA-fixed liver tissue underwent treatment with various protocols (Fig. 4, A-H). In control cryosections (A), and in cryosections treated with SDS (B), no significant staining of Na/K-ATPase was observed. In the microwave heating-treated cryosections (C-E), protocols C and E were only slightly unmasking (C < E), whereas the protocol D was better, exhibiting a fair staining of the hepatocyte sinusoidal membrane. Following alcohol treatment+microwave heating (F-H), protocols F and H were also weak, whereas protocol G was strongly unmasking, showing the strongest staining intensity in the hepatocyte sinusoidal membrane (arrows), whereas the bile canaliculi remained unstained (arrowheads). Therefore, unlike the situation in kidneys, where the best antigen revealing conditions were in protocol D, the best conditions for unmasking Na/K-ATPase in the liver cells were in protocol G. This experiment indicates that the unmasking conditions of a common antigen in tissue cryosection of different organs can be different.
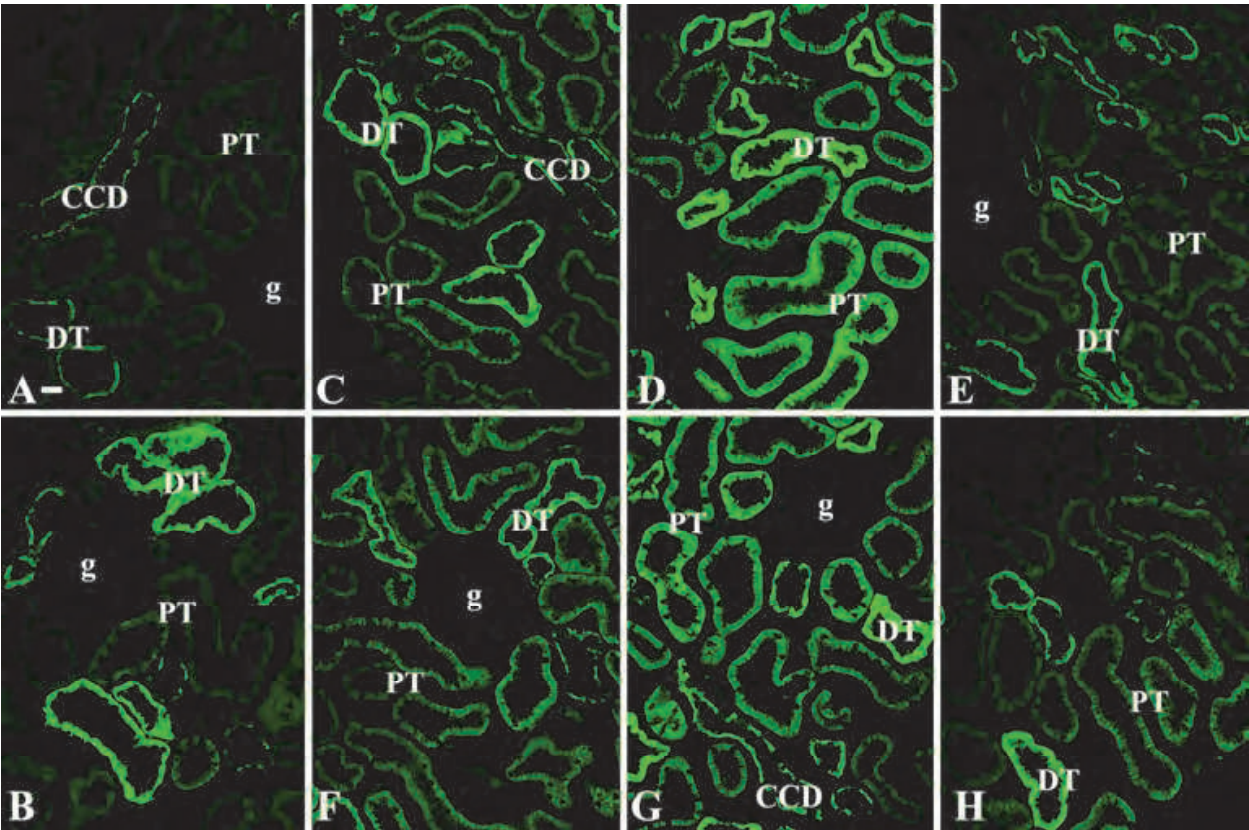
Fig. 3. Distribution and intensity of immunostaining of Na/K-ATPase in cryosections of the PFA-fixed rat kidney cortex tissue that underwent various antigen retrieval protocols listed in the section 2.3. g, glomeruli; PT, proximal tubules; DT, distal tubules; CCD, cortical collecting ducts. Bar, 20 µm.
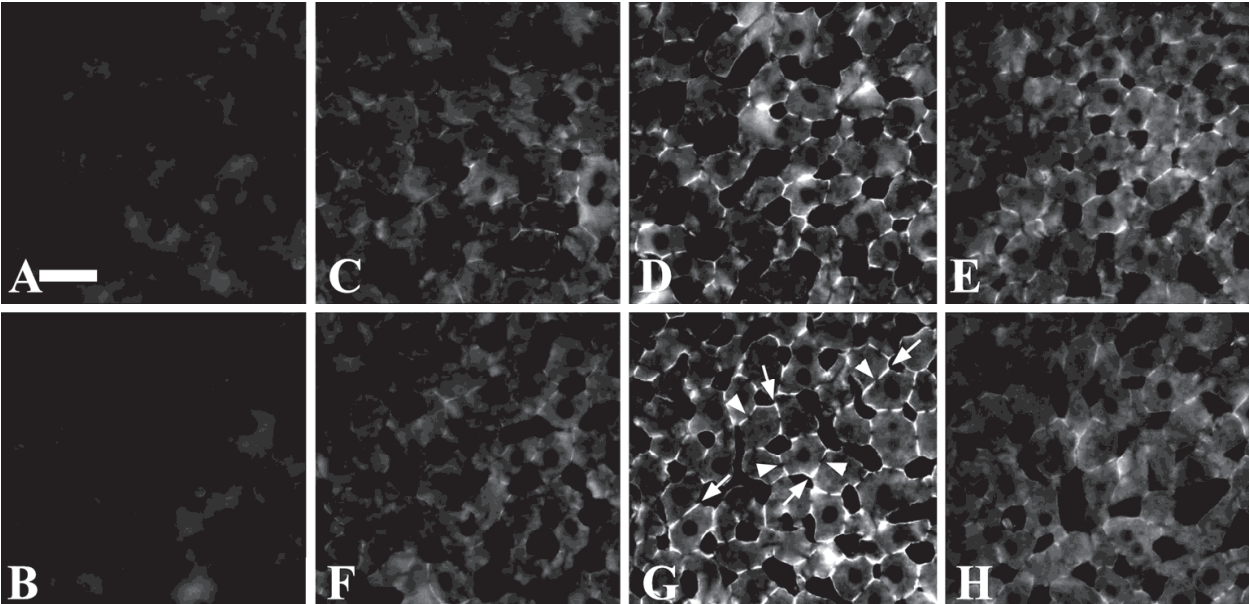

Fig. 4. Distribution and intensity of immunostaining of Na/K-ATPase in cryosections of the PFA-fixed rat liver tissue that underwent various antigen retrieval protocols listed in the section 2.3. Arrows, the stained sinusoidal membrane; arrowheads, unstained bile canaliculi. Bar, 20 µm.

3.1.4 AQP1

AQP1 was previously localized to the luminal and contraluminal cell membrane domains of the proximal tubules and thin descending limbs (Nielsen et al., 1993; Sabolić et al., 1992b). Fig. 5. shows effects of various antigen retrieval protocols on immunostaining of AQP1 in cryosections of the rat kidney cortex, where only the proximal tubules are expected to be positive for this water channel. In cryosection treated with protocol A, a fair staining of AQP1 was observed in both BBM (arrowheads) and BLM (arrows) in the proximal tubules (A), whereas in the SDS-treated cryosections, the overall staining was much weaker (B). Following microwave heating (C-E), the staining was pH-dependent, being in both membrane domains strongest at pH 3 (C), weaker and present largely in the BBM at pH 6 (D), and negligible in both membrane domains at pH 8 (E). Treatment of cryosections with alcohols+microwave heating (F-H) resulted in distinct staining pattern; at pH 3, the overall staining was relatively weak, and present largely in the BBM (F), at pH 6, the staining was hardy visible in both membrane domains (G), whereas at pH 8, the staining in the BLM was stronger than in the BBM (H). Therefore, this experiment indicates that protocol C is the best for unmasking the AQP1 epitopes in both BBM and BLM of the proximal tubules, protocols D and F preferably unmask these epitopes in the BBM, whereas protocol H preferably unmasks the same epitopes in the BLM.
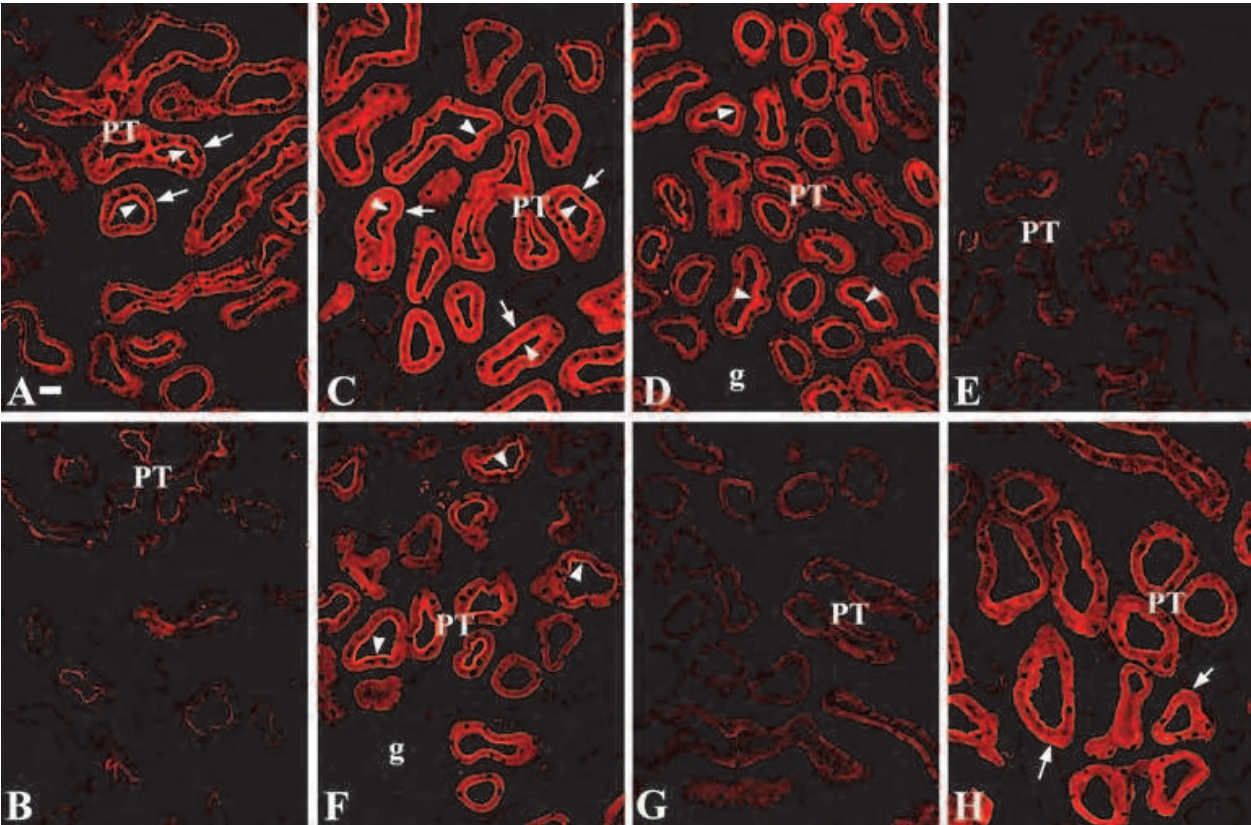

Fig. 5. Distribution and intensity of immunostaining of AQP1 in cryosections of the PFA-fixed rat kidney cortex tissue that underwent various antigen retrieval protocols listed in the section 2.3. g, glomeruli; PT, proximal tubules; arrows, BLM; arrowheads, BBM. Bar, 20 μm.

3.1.5 V-ATPase

V-ATPase is localized in the BBM, subapical vesicles, and in various intracellular acidic organelles in the proximal tubule cells and in other cell types along the rat nephron

(Brown et al., 1988). As shown in Fig. 6, a negligible staining of V-ATPase was recorded in control (A) and in the SDS-treated (B) cryosections of the kidney cortex. However, the microwave heating at pH 3 caused a strong unmasking effect, resulting in bright staining of the proximal tubule apical domain and intercalated cells in the cortical collecting ducts (C).
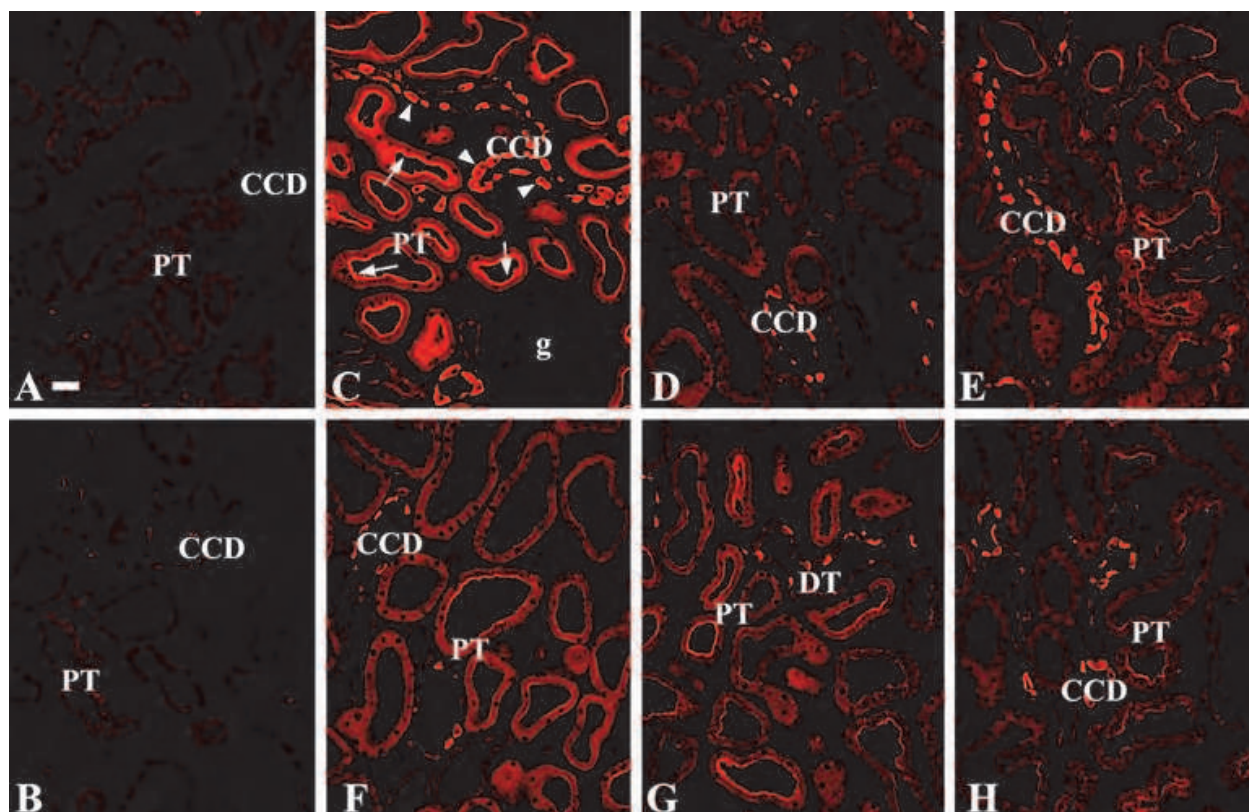

Fig. 6. Distribution and intensity of immunostaining of V-ATPase in cryosections of the PFA-fixed rat kidney cortex that underwent various antigen retrieval protocols listed in the section 2.3. g, glomeruli; PT, proximal tubules; DT, distal tubules; CCD, cortical collecting ducts; arrows, apical cell domain (BBM and subapical organelles); arrowheads, intercalated cells in CCD. Bar, 20 μm.

In cryosections treated with protocols D and E, the overall staining was much weaker than in those treated with protocol C, but intercalated cells in the collecting ducts were generally stained better than the proximal tubules. Compared with the staining after protocol C, treatment of cryosections with alcohols combined with microwave heating (F-H) resulted in overall weaker staining in all tubule profiles and in all conditions, with the pattern pH 3 $\leq$ pH 6 $\leq$ pH 8. Therefore, protocol C appeared to be an optimal unmasking approach for immunocytochemical presentation of V-ATPase in cryosections of the PFA-fixed kidney tissue.

3.2 Antigen retrieval of epitopes specific for cytoplasmic protein metallothionein

In the rat kidney, metallothionein is expressed in the cytoplasm of various cells localized along the nephron. In the kidney cortex, a variable expression of this protein has been detected in the proximal tubule cells (Sabolić et al., 2010, and unpublished data).
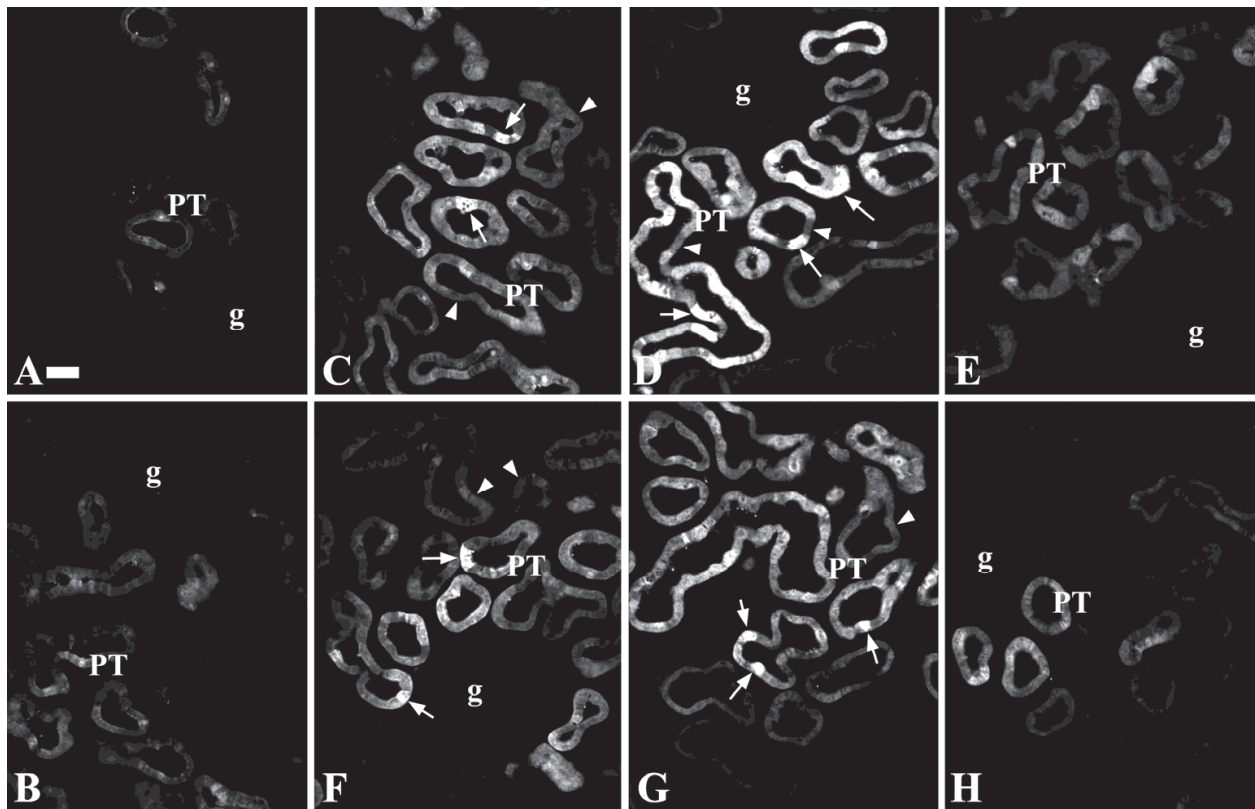
Fig. 7. Distribution and intensity of immunostaining of metallothionein in cryosections of the PFA-fixed rat kidney cortex that underwent various antigen retrieval protocols listed in the section 2.3. g, glomeruli; PT, proximal tubules; arrows, strongly-stained cells; arrowheads, weakly-stained cells. Bar, 20 μm.

In cryosections processed with protocol A, only a weak staining was observed in the cell cytoplasm of some proximal tubules (A). The SDS-treatment increased the number of metallothionein–positive tubules, and slightly increased the staining intensity (B). Following heating in the microwave oven at pH 3, the staining was further increased, exhibiting the cells with a variable staining intensity from weak (arrowheads) to strong (arrows) (C). The intensity and the heterogeneity in the staining intensity was additionally enhanced in cryosections treated with the microwave heating at pH 6 (D), but significantly diminished after heating at pH 8 (E). In cryosections treated with protocols F-H, in all these conditions the overall staining, particularly that in H, was weaker than in D. In conclusion, protocol D appears to be optimal for immunolocalization of metallothionein in cryosections of the PFA-fixed rat renal cortex.

3.3 Antigen retrieval of epitopes specific for cytoskeletal proteins
3.3.1 Actin
Fig. 8, A-H shows effects of various protocols of antigen retrieval on actin-related immunostaining in cryosections of the PFA-fixed rat kidney cortex. Control (A) and the SDS-treated cryosections (B) showed no significant staining of actin in any cortical structure. Following heating in the microwave oven (C-E), the staining intensity gradually increased with increasing pH (pH 3 < pH 6 < pH 8) in the glomeruli, proximal tubule BBM, and in individual cells (probably intercalated cells) of the cortical collecting duct.
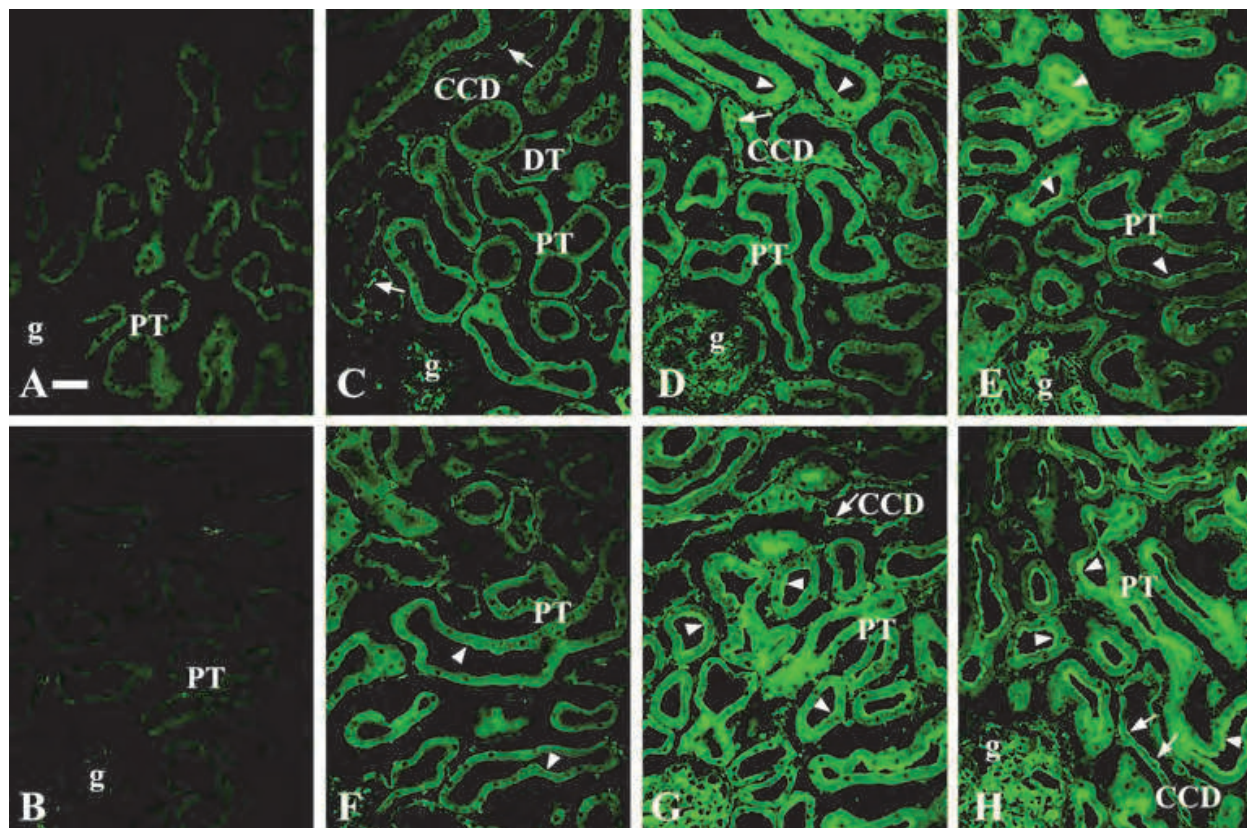
Fig. 8. Distribution and intensity of immunostaining of actin in cryosections of the PFA-fixed rat kidney cortex that underwent various antigen retrieval protocols listed in the section 2.3. g, glomeruli; PT, proximal tubules; DT, distal tubules; CCD, cortical collecting duct; arrows, the actin-positive individual (probably intercalated) cells in CCD; arrowheads, the actin-positive BBM in proximal tubules. Bar, 20 μm.

Cryosections treated with protocols that included treatment with alcohols and microwave heating (F-H) exhibited the same, pH-dependent staining pattern, but the staining intensity was higher in all conditions. Therefore, pretreatment with alcohols, followed by microwave heating in alkaline condition, as present in protocol H, enabled the strongest immunostaining of actin in cryosections of the PFA-fixed rat kidney cortex.

3.3.2 α-Tubulin

Effects of different antigen retrieval protocols on immunostaining with the anti-α-tubulin antibody was performed in cryosections of the PFA-fixed rat kidney outer stripe (Fig. 9, A-H). As shown in our previous publication (Sabolić et al., 2002), in the proximal tubule cells α-tubulin forms the apicobasally-oriented bundles of microtubules, which are particularly rich in the cells of proximal tubule straight segments (S3) in the outer stripe zone. A weak staining of α-tubulin in the S3 segments was evident in cryosections that had been processed with protocol A. The staining was somewhat stronger in the SDS-treated cryosections (B). Heating in the microwave oven did not significantly affect the staining intensity at pH 3 (C), heating at pH 6 resulted in a bright staining of microtubule bundles (D), whereas after heating at pH 8 (E), the staining was weaker than in D.
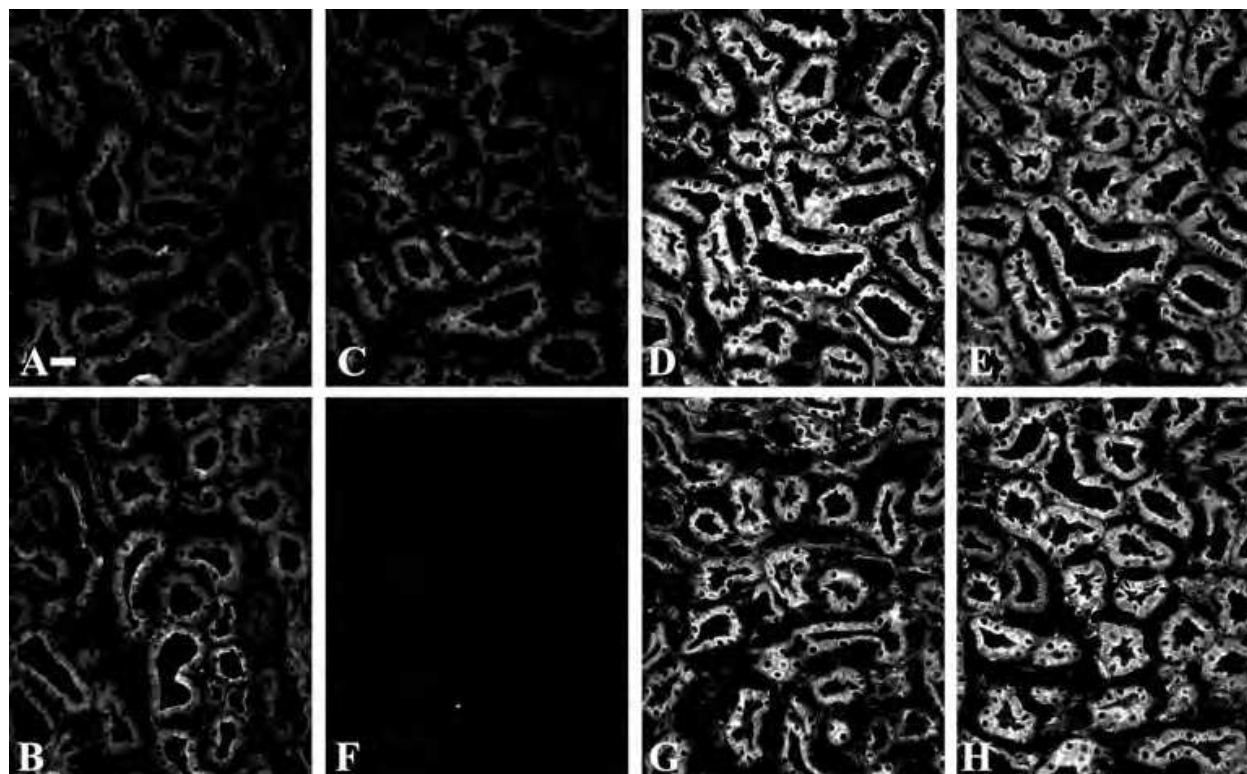
Fig. 9. Immunostaining of α-tubulin in cryosections of the PFA-fixed rat kidney outer stripe following various antigen retrieval protocols listed in the section 2.3. The staining is localized in intracellular bundles of microtubules largely in the proximal tubule S3 segments. Bar, 20 µm.

Alcohol treatment combined with heating at pH 3 unmasked virtually nothing (F), whereas similar protocols at pH 6 (G) and pH 8 (H) resulted in a relatively strong and similar staining, yet weaker then that elicited with protocol D. Therefore, protocol D was found to be optimal for unmasking α-tubulin epitopes in cryosections of the PFA-fixed rat kidney tissue.

Various antigen retrieval protocols were also applied on cryosections of the PFA-fixed liver tissue (Fig. 10, A-H) in order to compare the staining pattern with that in the kidney (c.f., Fig. 9). A very weak staining was observed in control conditions (A), and the SDS-treatment resulted in only limited enhancement of staining (B). After heating cryosections in a microwave oven at pH 3, no significant staining was obtained (C). However, heating at pH 6 resulted in strong staining of microtubules in hepatocytes (D), whereas at pH 8 (E), the staining was weaker than at pH 6. Similar pattern, but at the lower level of staining intensity was observed in cryosections that had been treated with protocols F-H. Therefore, unlike the situation with Na/K-ATPase (c.f., Fig. 3 and Fig. 4), where the optimal unmasking protocols in cryosection of the kidney and liver tissues were different, the optimal protocols for unmasking α-tubulin in cryosections of both organs were similar.

3.4 Effects of heating power for a fixed time and of time-dependent exposure to fixed heating power

The preceding experiments showed that for most antigens to be localized by their antibodies, the optimal unmasking effect was achieved with one of the protocols that

included heating of cryosections in a microwave oven at the power of 800 W for 20 min. Megalin was the only antigen that did not need heating for optimal presentation. These data thus indicate an indispensable role of microwave heating in unmasking epitopes for immunocytochemical presentation of a variety of antigens in cryosections of the PFA-fixed tissues. In the following two experiments we tested the importance of (a) microwave heating power at the fixed time, and (b) duration of the microwave heating at the fixed power, for unmasking efficiency using V-ATPase as the representative antigen and general conditions in protocol C. The conditions in this protocol were found to be optimal for unmasking epitopes of V-ATPase in cryosection of the PFA-fixed rat kidney cortex (c.f. Fig. 6).
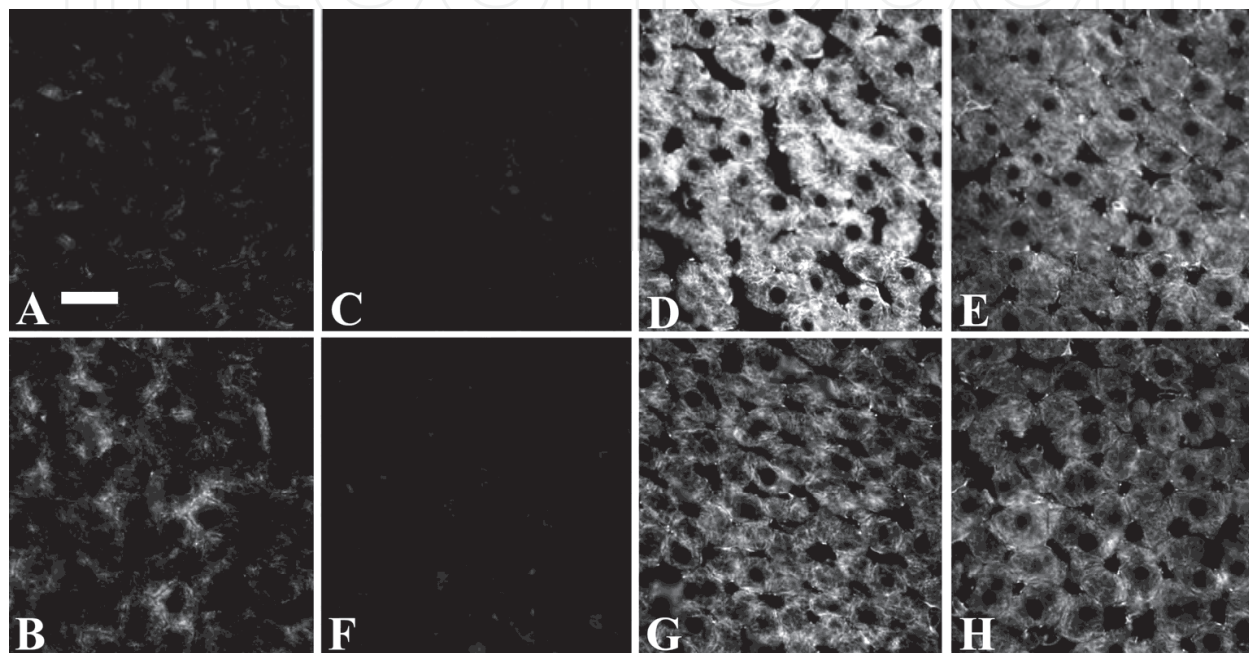

Fig. 10. Immunostaining of α-tubulin in cryosections of the PFA-fixed rat liver following various antigen retrieval protocols listed in the section 2.3. Bar, 20 μm.

Fig. 11. shows the effects of microwave heating of cryosections in citrate buffer, pH 3, at different power settings (0-800 W) for 20 min on the V-ATPase immunostaining. Incubation of cryosections for 20 min without heating resulted in a very weak staining of the cells in collecting ducts (a). After heating at 80 W (b) and 160 W (c), the staining intensity of the collecting duct cells was slightly increased, while other tubule profiles remained unstained. At 400 W (d), the staining intensity of the individual collecting duct cells was stronger, and a weak staining of the proximal tubule BBM was also observed. The overall staining in both collecting duct cells and proximal tubule BBM further increased at 560 W (e), and the brightest staining was recorded after heating at 800 W (f).

Effects of heating duration (0-20 min) at the fixed power (800 W) are shown in Fig. 12. Heating was applied in cycles, 5 min each. An incubation of cryosections at room temperature (heating duration 0 min) resulted in weak staining of collecting ducts (a). Heating for 5 min (one cycle) only slightly increased the staining intensity of the cells in collecting ducts (b). After heating for 10 min (two cycles), a limited staining was observed in the proximal tubule BBM, while the staining intensity of the collecting duct cells further increased (c). Three cycles of heating (15 min) revealed a strong staining in both nephron segments (d), whereas four heating cycles (20 min) resulted in an additional, but slight

increase in the staining intensity (e). From the data in these two experiments we conclude that both the heating power and duration of heating in a microwave oven are crucial for an optimal retrieval of hidden antibody-binding epitopes in cryosections of the PFA-fixed tissues.
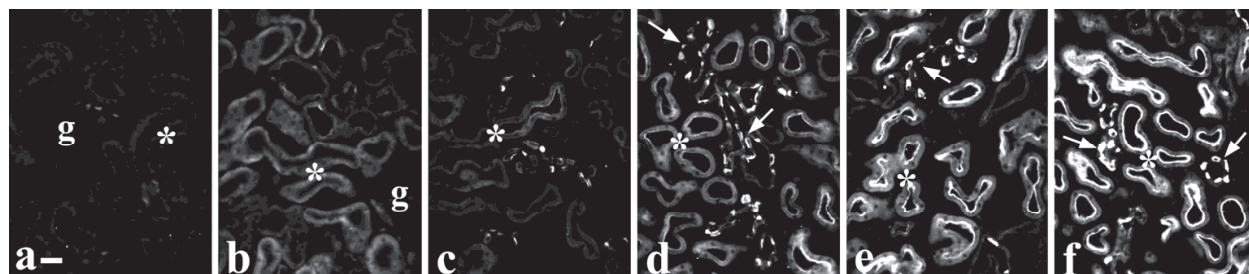

Fig. 11. Effect of power setting of microwave oven on the staining pattern and intensity of V-ATPase in cryosections of the PFA-fixed rat kidney cortex. (a) Without heating, and heating at (b) 80 W, (c) 160 W, (d) 400 W, (e) 560 W, and (f) 800 W for 20 min, other conditions being in protocol C. g, glomeruli; asterisks, proximal tubules; arrows, cortical collecting ducts. Bar, 20 μm.
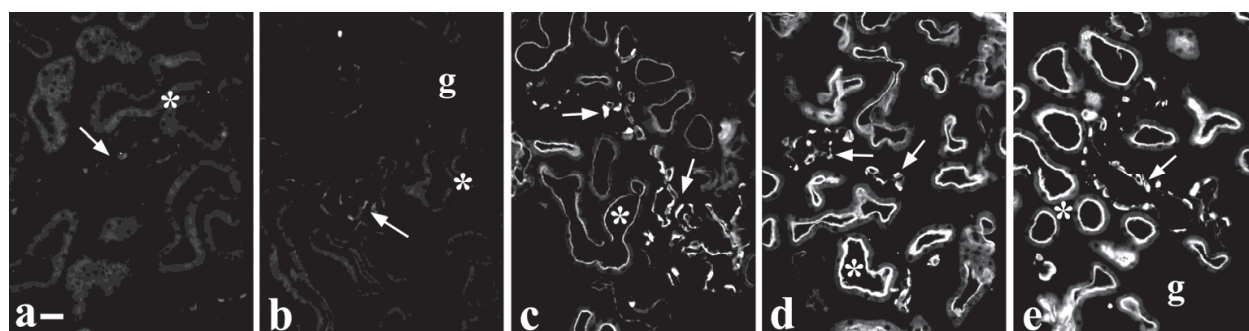

Fig. 12. Effect of duration of microwave heating at the fixed power setting (800 W) on the staining pattern and intensity of V-ATPase in cryosections of the PFA-fixed rat kidney cortex. (a) Without heating, and heating for (b) 5 min, (c) 10 min, (d) 15 min, and (e) 20 min, other conditions being in protocol C. g, glomeruli; asterisks, proximal tubules; arrows, cortical collecting ducts. Bar, 20 μm.

4. Discussion

A good immunocytochemistry is largely based on experience collected during the experimental search for optimal conditions of presenting specific antibody-binding epitopes. Our experience indicates that these conditions can not be predicted with certainty for any antibody and tissue or cells, and various protocols of antigen retrieval should be tested with each new antibody and tissue before starting detailed immunocytochemical studies.

The SDS-treatment of cryosections of the PFA-fixed tissues and cultured cells was previously demonstrated as a beneficial technique for unmasking epitopes of several membrane-bound proteins (Alper et al., 1997; Brown et al., 1996; Sabolić et al., 1999). However, using similar cryosections of the rat and mouse tissues, we have recently applied a few harsh antigen retrieval protocols that are commonly used to unmask hidden epitopes in the PFA-fixed, paraffin-embedded sections, and efficiently demonstrated the expression of various transporters in the respective organs (Bahn et al., 2005; Balen et al., 2008; Breljak

et al., 2010; Brzica et al., 2009a & 2009b; Ljubojević et al., 2004 & 2007; Yokoyama et al., 2008). In these studies, in preliminary experiments we have always tested a battery of antigen-revealing protocols with each antibody and tissue cryosections until one, a protocol that resulted in the highest immunostaining intensity, was defined and further used. In most cases, the SDS-treatement had only a limited potency in unmasking the tested antigens, and one of the revealing protocols with microwave heating without or with alcohol pretreatment usually proved to be more efficient in revealing cellular localization of the protein-specific epitopes and the intensity of their staining. In the present study, we describe our experience in using various antigen revealing protocols, and the importance of microwave heating in these protocols in order to compare their efficiency in unmasking epitopes on the proteins that reside in specific cellular locations. The major data from these experiments are summarized in Table 1.

As shown in Table 1, except for megalin in the proximal tubule BBM, whose staining was slightly enhanced only following treatment of cryosections with SDS (protocol B), all other antigens were better stained after being treated with one of the protocols that included microwave heating, without (protocols C-E) or with pretreatment with alcohols (protocols F-H). Each tested antigen needed one distinct unmasking protocol to be optimally exposed, with an unpredictable preference for the buffer pH and presence of alcohols.

| Antigen | Cellular localization | SDS treatment | Microwave heating | | | | | |
| | | | - Alcohol treatment | | | +Alcohol treatment | | |
			pH 3	pH 6	pH 8	pH 3	pH 6	pH 8
CAM105	PT – BBM	-1	-1	+2	**+3**	-2	-2	+2
Megalin	PT - BBM & SAV	**+1**	0	-1	-2	-1	-1	-2
Na/K-ATPase								
Kidney	BLM	+1	+1	**+3**	+1	+1	+2	+1
Liver	SM	0	+1	+2	+1	0/+1	**+3**	0/+1
AQP1	BBM & BLM	-2	**+2**	0/-1	-3	0/-1	-3	0/-1
V-ATPase	BBM & SAV & IO	0	**+3**	+1	+1	+1	+1	+1
Metallothionein	PT – Cytoplasm	+1	+2	**+3**	+1	+2	+2	+1
Actin	Cytoskeleton	0/-1	+1	+1	+2	+1	+2	**+3**
Tubulin								
Kidney	Cytoskeleton	+1	0	**+3**	+2	-3	+2	+2
Liver	Cytoskeleton	+1	-1	**+3**	+2	-1	+2	+1/+2

Table 1. Relative efficiency of various antigen retrieval protocols B-H in unmasking hidden epitopes in cryosections of the PFA-fixed tissues, as observed in the present study. The efficiency was based on visual estimation of the immunostaining intensity, relative to that obtained for the respective antigen processed with protocol A. PT, proximal tubule; BBM, brush-border membrane; SAV, subapical vesicles; BLM, basolateral membrane; SM, sinusoidal membrane; IO, intracellular organelles. (-) Relative decrease in staining intensity, (0) similar staining intensity, and (+) relative increase in staining intensity, when compared to the intensity in untreated (control) cryosections. Conditions that resulted in relatively strongest unmasking effect and staining intensity were indicated in bold.

The beneficial effect of the buffer pH combined with heating in unmasking hidden antigens has been previously well documented with paraffin sections (Evers & Uylings, 1994; Grossfeld et al., 1996; Shi et al., 1995; Shi et al., 1997). Furthermore, also unlike megalin, whose epitopes were temperature sensitive and were poorly exposed and

stained following microwave heating, all other antigens required heating in a microwave oven either without or with alcohol pretreatment for best unmasking effects. Since the benefits of microwave heating for antigen retrieval in paraffin sections have been described (Shi et al., 1991), various time-dependent and power-related protocols have been used to unmask the hidden epitopes for different antibodies. Most of them played with duration of heating at the boiling point of the buffers at the maximum power settings (Evers & Uylings, 1994; Hoetelmans et al., 2002; Munakata & Hendricks, 1993; Shi et al., 1991). Our data obtained with an optimal protocol for unmasking the V-ATPase epitopes following time-dependent and power-dependent heating in a microwave oven indicate the importance of such studies for defining optimal antigen retrieval conditions also with cryosections.

Our data indicate that the optimal protocols for unmasking specific epitopes can be different in cryosections of different tissues. Thus, the optimal conditions for revealing the Na/K-ATPase epitopes in cryosections of the kidney cortex were found in protocol D (microwave heating in buffer pH 6, without alcohol pretreatment), whereas the same epitopes in cryosections of the liver tissue were best exposed in using protocol F (microwave heating in buffer pH 6, with alcohol pretreatment). Similar data were recently found in the studies of sulfate anion transporter sat-1 in cryosections of the rat kidney and liver with a monoclonal antibody; the SDS-treatment was best in unmasking the sat-1 epitopes in cryosections of the kidney cortex, whereas in cryosections of the liver tissue, protocol D (microwave heating in buffer pH 6) was needed for optimal immunostaining (Brzica et al., 2009a). However, some epitopes were optimally retrieved in different tissue with the same protocols, as shown for tubulin epitopes, which were best exposed with the same protocol D in cryosections of both kidney and liver tissues. All these data support the previously reported findings that in *in vivo* conditions, the microenvironment in each tissue can play a role in antigen masking in addition to reactive sites of the protein and/or their three dimensional structures (Toews et al., 2008).

5. Conclusion

Based on the data obtained in the preceding experiments, we conclude that: (a) the antigen retrieval protocols, that are usually used with sections of the formalin-fixed, paraffin-embedded tissues, can be efficiently used with cryosections of the PFA-fixed tissues, (b) in order to unmask the hidden epitopes in tissue cryosection, in most, but not all cases the retrieval protocols that include microwave heating give the best unmasking effect and immunostaining intensity, (c) the unmasking effect with a defined protocol is epitope-related and dependent on the buffer pH, d) the unmasking efficiency is dependent on heating power (more power – better unmasking effect) and heating time in a microwave oven (longer heating – better unmasking effect); however, the heating power of 800 W, applied for 20 min, is enough to obtain the maximal unmasking effect and immunostaining intensity with a variety of antibodies, and (d) cryosections of different organs can respond differently to the antigen retrieval conditions required for the same epitope. The later observation indicates the necessity of using various antigen revealing protocols with cryosections of each tissue separately and for each pair of antigen-antibody before starting the immunocytochemical studies rutinely. This way, not only these studies can yield in an optimal staining intensity and distribution, but can also significantly diminish the amount of primary antibodies.

6. Acknowledgements

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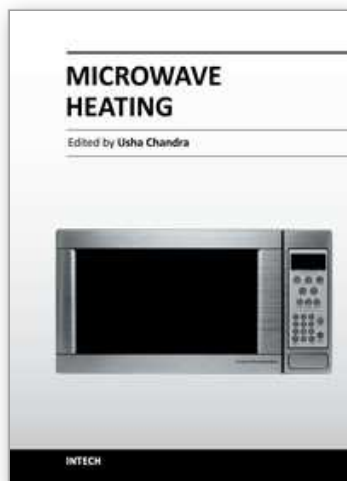
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