Enhanced ultrastructural localization of acid phosphatase using microwave irradiation during enzyme incubation

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SUMMARY

Microwave irradiation in enzyme cytochemistry is not only advantageous for shortening procedures, but will also improve the sensitivity of histochemical detection since short periods and less fixative are required to obtain good preservation. Moreover, shorter incubation procedures will reduce the diffusion and loss of enzyme activity. Here we compared conventional incubations in a shaking waterbath at 37°C, with incubations carried out in a microwave oven. Lysosomal and extralysosomal acid phosphatase were identified using microwave irradiation. Microwave irradiation elicited enhanced reactivity of the chemicals present in the tissue, leading to the detection of minuscule concentrations of enzyme activity, which was most pronounced in the detection of extralysosomal enzyme activity. A major advantage of the microwave is the accelerated formation of reaction product. Furthermore, enzymatic preincubation or chemical treatment of the tissues is no longer necessary.

By shortening the incubation time, the danger of artificial precipitations and diffusion of the final reaction product are reduced. The sensitivity and reproducibility of the enzymatic reaction are increased. Consequently, microwave irradiation during enzyme incubation leads to a highly specific localization of acid phosphatase.

Key Words: Acid phosphatase – Microwave – Cerium

INTRODUCTION

Microwaves have been used for fixation in light and electron microscopy and biochemical analyses, as well as for the preservation of intra- and extracellular antigens in immunohisto- and cytochemistry (Leong and Sormunen, 1998). Microwave irradiation has been used to accelerate staining procedures, for the demineralization of bones, and for tissue treatments such as dehydration, clearing and paraffin infiltration, epoxy resin polymerisation, and *in-situ* hybridization. In the publications of Login and Dvorak (1988, 1994), Kok and Boon (1990, 1992), Hopwood and Milne (1991), Ainley and Ironside (1994), Marani and Horobin (1994), Mizuhira and Hasegawa (1996) and Leong and Sormunen (1998), detailed information is given about the use of microwave ovens. For the visualization of phosphatase activities Hulstaert and coworkers (1989) made use of the microwave oven to convert cerium phosphate into lead phosphate. However, only Marani et al. (1990) and Rassner et al. (1997) evaluated microscopical details after enzyme incubation during microwave irradiation.

Enzyme histochemical localization at the ultrastructural level is often hampered by the conflicting use of glutaraldehyde for morphological preservation and its concomittant conflicting interference with the histochemical enzyme detection procedure. Furthermore, due to the slow diffusion of reaction products and reaction processes in conventional histochemical reactions, sensitivity and identification are not opti-

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mal. These conditions can be at least partially overcome by use of microwave irradiation, since the diffusion and reactivity of the chemicals present in tissues are increased (Kok and Boon, 1990).

In the present work we used enzyme incubations during microwave irradiation and compared the results with conventional incubations carried out in a shaking waterbath. The results of the microwave procedure were not only obtained more rapidly, but also gave stronger and more widespread and discrete activity. The enhanced sensitivity thus obtained substantially improves the detection of enzyme reaction products and will enable the localization of minute amounts of activity.

Materials and Methods

a) Calibration of the microwave

In this study a EMS-820 (Electron Microscopy Sciences, Fort Washington) device was used. Calibration was performed according to standard procedures. Exact calibration of the microwave oven is essential for the detection of hot and cold spots. By doing so, overheating of the samples or variations in temperature can be avoided. Exact calibration under incubation conditions guarantees a high reproducibility of the incubation method. In short, the procedure was as follows. Reaction vials were placed in the microwave oven on a transparent sheet on which the coordinates of a chess-board pattern were indicated. The temperature of the AcPase medium in each vial was measured during the incubation procedure. In this way, the exact localization of hot and cold spots could be determined.

b) Fixation

Male Wistar rats weighing 250-300 g were anaesthetised with sodium pentobarbital 6% i.p. (0.1 ml/ 100 g body weight). Kidneys were perfusion-fixed as described before (Rüdiger et al., 1998). The fixative consisted of 2% formaldehyde and 0.07% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4.

The kidneys were removed and cut into small blocks of approx. $5mm^3$. Subsequently, the blocks were postfixed for 15 minutes at 25 °C in the same fixative under microwave irradiation (350 W) in order to stabilize the tissue. Sections (50 (m) were cut on a vibratome and collected in 6.8% sucrose in 0.1 M cacodylate buffer, pH 7.4, cooled in an ice bath.

c) Cytochemical incubation procedures and con - trols

Enzyme incubations were carried out with and without microwave irradiation. One series of vibratome sections was incubated for 20 minutes at 37°C in the complete medium in a shaking waterbath. This series was compared to a series incubated for 20 minutes at 37°C under microwave irradiation in an EMS-820 precision-pulsed laboratory microwave oven at 350 W. The vibratome sections were incubated in vials containing 6 ml of incubation medium under continuous agitation with air bubbles. The 10 ml vials were placed in the microwave oven according to the calibration procedure at places with the same temperature rise. Temperature was kept constant at 37°C for enzyme cytochemical incubations by a temperature sensor and kept in the same position throughout the experiments. A 400 ml beaker with tap water was placed in one of the rear oven corners to control the amount of energy transferred to the sample (water load).

The incubation medium consisted of 0.1 M sodium acetate buffer (pH 5.6), 15% sucrose, 50 mM tricine, 5 mM levamisole (to inhibit alkaline phosphatase), 10 mM ouabain (to inhibit ouabain-sensitive K-pNPPase), 3 mM $CeCl_3$ and 1 mM pNPP. Control sections were incubated in medium without substrate or in the presence of ammonium molybdate to inhibit AcPase or 1 mM V_20_5 to inhibit adenosine polyphosphatases in the complete incubation medium. Further processing for transmission electron microscopy was carried out according to routine procedures (Rüdiger et al., 1998).

Results

Vibratome sections incubated in the microwave oven were compared with those incubated without microwave irradiation in a shaking waterbath, both at 37±1°C. The difference in reaction product was best appreciated in parts of the glomerular arrangement, especially the endothelial lining, which exhibited a rather weak activity without microwave treatment (Figs. 1a, 2a), whereas the glomerular basement membrane and the aligned podocytes showed strong reactivity in the cases when microwaves were applied (Figs. 1b, 2b). After incubations with the microwave, the basement membrane of the glomeruli and the membrane of the capillary endothelium lying beneath the basement membrane were clearly AcPase-positive (Fig. 2a). After incubations without the use of the microwave, the intensity of AcPase reactivity was markedly lower (Fig. 3a) but was most pronounced in extra-lysosomal structures, such as the border of the nuclear envelope and the basal infoldings (Fig. 3b). Lysosomes and parts of the Golgi apparatus were equally intense as regards density (Fig. 3a, b).

Glomerular structures incubated without microwave irradiation showed less AcPase-activi-

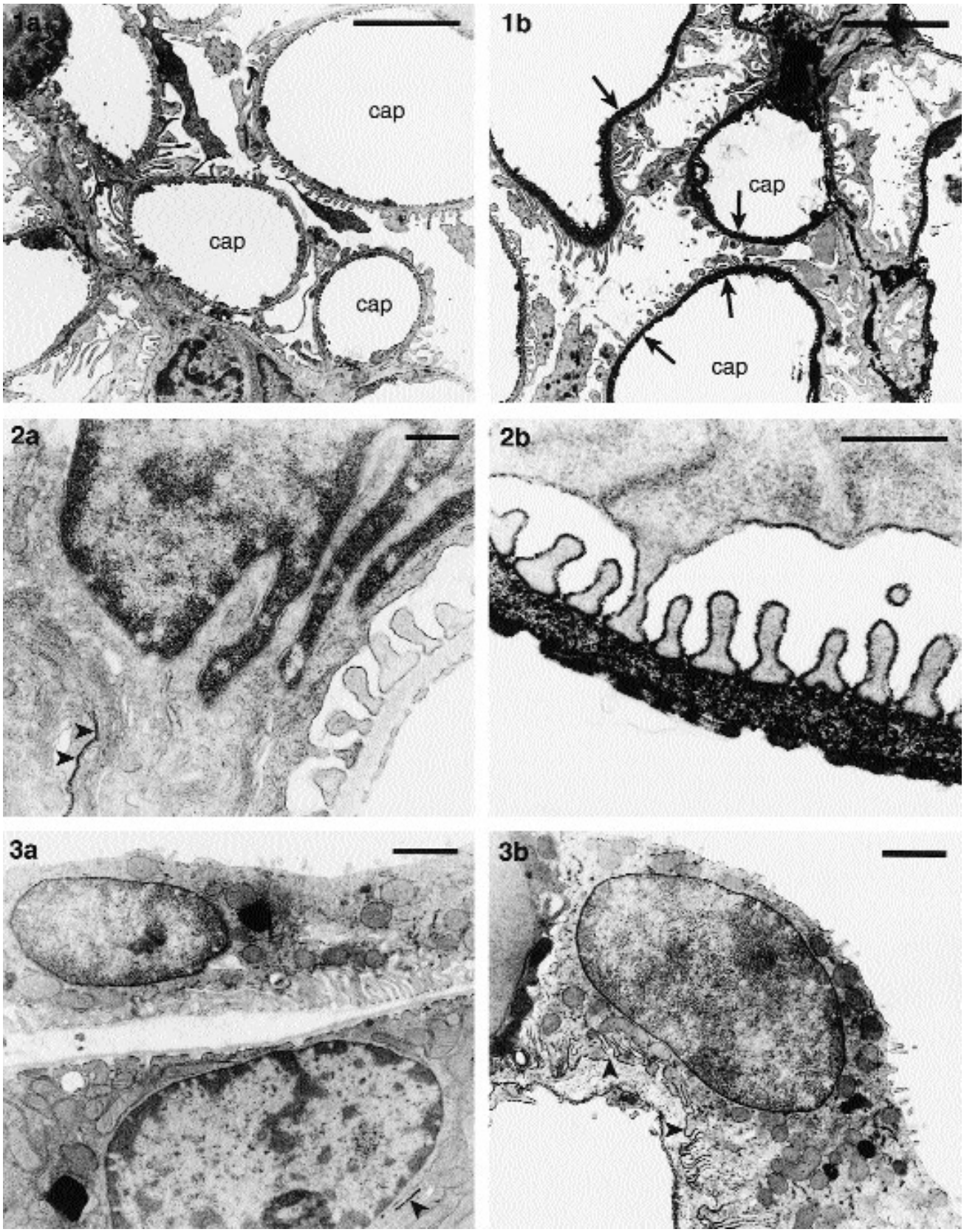

Figure 1 a, b.- Electron micrographs of glomerular profiles in rat kidney cortex incubated for acid phosphatase reactivity. In **1a**, no microwave irradiation was used, in **1b**, microwave irradiation was used. The difference in intensity of the reactions produced is most prominent in the capillaries (cap), where the glomerular basement membrane is strongly positive for acid phosphatase (arrows). Scale bar: 1 µm.

Figure 2 a, b. - Electron microscopy of the glomerular basement membrane and adjacent epithelial cell. In **2a**, no microwave was used and acid phosphatase activity can be seen in the Golgi apparatus (arrowheads), whereas the podocytes and glomerular basement membrane are only weakly stained. In **2b**, the intensity of the cytochemical reaction is strongly increased after microwave irradiation. Scale bar: 0,1 µm.

Figure 3 a, b.- Electron micrographs of distal tubular cells. In **3a**, no microwave was used: lysosomes, endoplasmic reticulum and a single cistern of the Golgi apparatus (arrowhead) are acid phosphatase positive. In **3b**, microwave irradiation was applied, which resulted in a remarkable increase in activity at the nuclear envelope, basal membrane infoldings (arrowheads). Scale bar: 0,5 µm.

ty than those incubated under microwave irradiation. Fig. 2b shows the dense distribution of acid phosphatase in the GBM and the AcPase-positive membranes of podocytes and endothelial cells; this distinction was completely absent in the non-microwave treated incubations (Fig. 2a).

DISCUSSION

Postfixation in the microwave allows a milder fixation of the tissue in order to maintain acid phosphatase activity. Since glutaraldehyde strongly inhibits enzyme activity, here the glutaraldehyde concentration was reduced to 0,07%. Microwave irradiation increases diffusion of the fixative and improves binding to the substrate during incubation (Noyan et al., 2000). This has been proved more reliably than by leaving the tissue in the fixation solution post perfusion for minutes or hours. With postfixation in the microwave oven, the morphology of the material is superior to immersion-fixed tissue or only perfusion-fixed tissue and still *expresses* enzyme activity. This is probably due to the fact that microwaves have a stabilizing effect on the ultrastructure of the tissue (Kok and Boon, 1992; Login and Dvorak, 1994).

Visualization of extralysosomal acid phosphatase with the use of microwave irradiation (Fig. 1b, 2b and 3b) is better than visualization in a shaking waterbath (Fig. 1a, 2a and 3a). After incubation for 20 minutes with microwave irradiation (Fig. 1b, 2b and 3b), the precipitation of cerium was equally or even stronger than after 45 minutes preincubation and 20 minutes incubation without microwave irradiation (not shown) although both incubation media were performed at 37±1°C. Therefore, preincubation for the enzyme cytochemical localization of AcPase as recommended by Hulstaert et al. (1989) is not necessary.

It is not known what exactly causes the effects of microwave irradiation. On one hand thermal effects may be involved (Hopwood et al., 1988; Leonard and Shepardson 1994). In a somewhat small sample, a relatively homogenous temperature has been reported during microwave irradiation (Gokhale and Khan, 1992). Homogeneous warming of the biological material leads to increased diffusion and an intense reactivity of the chemicals present in the tissue (Kok and Boon, 1990; Mizuhira et al., 1990). This may be due to an increase in the kinetic energy of the molecules (Brinn, 1983). On the other hand, non-thermal effects of microwave irradiation seem to play a role in the formation of an enzymatic reaction product. Vibratome sections incubated under microwave irradiation (Fig. 1b, 2b and 3b) showed stronger acid phosphatase activity than those incubated in a shaking waterbath (Fig. 1a, 2a and 3a). Different effects of microwave energy and conventional heat on the activity of an enzyme have also been reported by La Cara et al. (1999). Furthermore, Pashovkina and Akoev (2000, 2001) found that alkaline phosphatase activity depends on modulation of both the frequency and intensity of electromagnetic radiation.

It is likely that the microwaves enhance the enzymatic precipitation processes energetically. The increase in the reactivity of chemicals may be caused by the movements of polar molecules under microwave irradiation (Hopwood and Milne, 1991). This could lead to more reaction product being developed in a shorter time.

In sum, through microwave irradiation during fixation and enzyme incubation a strict localization of acid phosphatase with good preservation of the ultrastructure was achieved. Since preincubation of the samples is not necessary, the danger of artefacts and diffusion of the final reaction product is further reduced. With increased sensitivity of the enzymatic reaction, a high reproducibility of the cytochemical method can be obtained.

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