Ultracytochemical Localization of Alkaline Phosphatase in Swine Gonadotropic Cells

Takahiro YAMAGUCHI

Laboratory of Functional Morphology, Department of Animal Science, Faculty of Agriculture, Tohoku University, Aoba-ku, Sendai 981, Japan

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ABSTRACT. In swine gonadotropic (SGT) cells, the alkaline phosphatase (APase) activity was ultracytochemically demonstrated on the outer surface of the plasma membrane and in some vesicles in the cytoplasm. The plasma membrane-associated APase activity was sporadically detected on the outer surface and was intense at the site adjacent to the next SGT cells or follicular cells. APase-positive vesicles occurred around the Golgi apparatus and in the periphery of SGT cells. Some of the vesicles positive for APase activity attached to the plasma membrane. These results suggest that APase is synthesized in SGT cells and moves to the plasma membrane. —**KEY WORDS:** alkaline phosphatase, swine gonadotropic cell, ultracytochemistry.

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Alkaline phosphatase (APase) is found in various mammalian tissues [3, 6, 18, 19, 21] and its isozymic forms are classified in accordance with the predominant tissue distribution [17, 23]. In the adenohypophyseal cells of mammals, information on the presence and localization of APase activity obtained with classical metal-impregnation techniques is still fragmented and controversial [20, 22]. We have reported that an application of the azo dye method and the lead citrate method gives us better findings for the enzyme localization in the adenohypophyseal cells of swine and dog [29, 30]. In swine adenohypophyseal cells, when the co-localization of APase and luteinizing hormone (LH) or follicle-stimulating hormone (FSH) was examined, the cells positive for APase activity were immunostained with the antibodies against the both hormones and fine structures of APase-positive cells were consistent with those of swine gonadotropic (SGT) cells [31]. Therefore, the adenohypophyseal cells can be identified as SGT cells. However, the distribution of APase activity in SGT cells has not been elucidated ultracytochemically.

The present study was undertaken to determine the localization of APase in subcellular compartments of SGT cells in detail and to better understand the relationship between the diversity of APase activity and the functional state of the cell.

MATERIALS AND METHODS

Animals: Six crossbred female swine (1/4 Landrace, 1/4 Large White, 1/2 Duroc) aging 6 to 7 months were used. The animals were kept at normal conditions and received commercial diets and water *ad libitum*.

Simultaneous detection of APase activity and LH: The adenohypophyses were freshly removed, fixed in neutral formol calcium for 8 hr at 4°C and washed in cold saline for 24 hr [31]. The tissue was then frozen in a mixture of acetone and dry ice, and stored at –70°C. The sections were cut at 4 µm with a cryostat and mounted on glass slides. APase activity was visualized by the lead citrate

method of Mayahara *et al.* [16]. After the enzyme reaction, the sections were re-fixed in neutral formol calcium for 2 hr and immunostained for LH by the immunogold-silver staining method [11] using anti-porcine LH serum and anti-human LHβ serum (ucb Bioproduct, S. A., Belgium). The specificity of the immunostaining was tested by substitution of normal rabbit serum or the absorbed antiserum for the antiserum as previously described [31].

Ultracytochemistry of APase: The adenohypophyses were sliced into longitudinal strips of approximately 3 mm width with razor blades and fixed in a mixture of 1% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 hr at 4°C. The tissue slices were rinsed overnight in cacodylate buffer containing 8% sucrose and sectioned at 30 µm with a Vibratome (Oxford Lab., San Mateo, Calif., U.S.A.). The visualization of APase activity was performed by the lead citrate method as described above. Control sections were incubated in the absence of the substrate. After the reaction, the sections were post-fixed in 1% osmium tetroxide, dehydrated and embedded as described elsewhere [31]. Ultrathin sections were stained with uranyl acetate and lead citrate. Electron microphotographs were taken with a JEOL-100B electron microscope (JEOL, Tokyo, Japan).

RESULTS

The combination of APase reaction and LH-immunoreactivity showed that APase-positive cells contained LH. APase activity was clearly detected on the plasma membrane as lead precipitates and LH-immunoreactivity was detected in the cytoplasm as silver precipitates (Fig. 1). In those cells showing an intense activity on the plasma membrane, the LH-immunoreactivity was generally weak. Reversely, weak APase activity was occasionally accompanied by strong LH-immunoreactivity (Fig. 1). The results were confirmed by control sections for APase activity and/or LH-immunoreactivity.

Fig. 1. Simultaneous detection of APase activity and LH immunoreactivity. APase-positive cells are immunostained with anti-LH β serum (AH, AL). Intensively positive cells for APase activity contain a small number of immunological silver precipitates (AL). Occasionally, strongly immunostaining cells do not show APase activity (NH). Arrows show APase activity on the plasma membrane. $\times 1,400$.

In SGT cells, the reaction precipitates of APase were detected on the outer surface of the plasma membrane and in some vesicles in the cytoplasm (Fig. 2). The precipitates sometimes scattered in intercellular spaces (Fig. 3). Control sections in substrate-free media showed no lead precipitates.

The precipitates showing a positive APase activity were distributed sporadically on the outer surface of the plasma membrane (Fig. 4). Such precipitation on the membrane was generally much more evident than that in the cytoplasm. Much more intense activity was found on the plasma membrane facing adjacent SGT cells or follicular cells (Figs. 2, 3, 5). Those SGT cells showing an intense plasma membrane-associated APase activity were generally large and were characterized by several morphological aspects in such a way that a less number of granules accumulated in the cell periphery, that the endoplasmic reticulum was well developed and mainly located in the cell periphery, and that a clear zone with a small number of cell organelles was frequently observed in the perinuclear region (Fig. 5).

The reaction products of APase were also observed in variously sized vesicles of SGT cells. APase-positive vesicles around the Golgi apparatus were regarded as multivesicular bodies by their morphological characteristics (Fig. 6a). Small vesicles strongly positive for APase were frequently observed in the perinuclear zone (Fig. 2). On the other hand, medium-sized vesicles positive for APase activity were located closely under the plasma membrane and some of them were frequently opened into the intercellular space (Fig. 6b). However, dense bodies did not show any APase activity (Fig. 5). No activity was seen in the endoplasmic reticulum and Golgi apparatus (Figs. 2, 3, 6a).

Fig. 2. APase localization in SGT cells. The reaction products are visible on the outer surface of the plasma membrane (single arrowheads) and in the perinuclear vesicle (double arrowhead). MT: mitochondria, ER: endoplasmic reticulum. $\times 21,000$.

Fig. 3. APase activity on the plasma membrane of SGT cells. An intensively positive reaction occurs on the plasma membrane which is contacted with adjacent SGT cells or follicular cells (F) (arrowheads). The APase-positive precipitates also scatter in the intracellular spaces (arrows). GO: Golgi apparatus. $\times 5,700$.

Fig. 4. Detail of APase localization on the plasma membrane. The reaction products are sporadically located on the outer surface of the plasma membrane (arrows). $\times 54,000$.

Fig. 5. A profile of an SGT-cell positive for APase activity in the zona tuberalis. The cell is less granulated and possesses developed endoplasmic reticulum in the periphery. Dense bodies are negative for APase activity (arrows). The cell contains a characteristic clear zone (CZ). $\times 5,800$.

Fig. 6. Intracellular APase activity of an SGT cell. APase-deposits occur in the multivesicular bodies (arrows) around the Golgi apparatus (a) and in the vesicles in the peripheral area (arrows) (b). One of the vesicles is connected with the plasma membrane (arrowhead) (b). GO: Golgi apparatus. a; $\times 20,000$. b; $\times 24,000$.

DISCUSSION

The simultaneous detection of APase activity and LH-immunoreactivity in the present study clearly indicated that in swine adenohypophysis, APase-positive cells contain LH. Since it has been demonstrated that LH and FSH are detected in the same gonadotropic cells in swine

[2, 4, 5], the APase-positive cells are evidently identified as gonadotropic cells well in harmony with the previous findings that the APase-positive cells correspond to LH/FSH cells in swine adenohypophysis [31].

As reported in other tissue, APase is a marker enzyme of the plasma membrane and plays an important role in transport action of various substances across the membrane [1, 32]. Recently, evidences have been put forward that APase is anchored to the plasma membrane by a phosphatidylinositol glycan moiety [12, 15]. Present observations showed that in SGT cells, precipitates showing positive APase activity was tightly localized on the outer surface of the membrane supporting the view that histochemically active plasma membrane-associated APase in SGT cells may exist as a membrane-bound form though requiring a further biochemical study to confirm.

In the present study, the plasma membrane-associated APase activity in SGT cells was observed preferentially on the site associated with the cell-to-cell contact. Stronger activity was related to more developed endoplasmic reticulum and lesser storage of secretory granules in SGT cells. The fine structure of SGT cells showing an intense APase activity on their plasma membrane was very similar to that of hypertrophied gonadotropic cells after castration in swine [9], though not similar to that of rat castration cells, so-called signet-ring cells [33]. Therefore, the plasma membrane-associated APase activity appears to be strengthened with an increase in function of SGT cells and to be involved in the cellular interaction.

Intracellular organelles such as endoplasmic reticulum and Golgi apparatus usually display no APase reaction although a few exceptional cases have been reported in intestinal epithelial cells [13, 14], neutrophils [29] and cultured cancer cells [24–26]. In the present study, the APase activity was demonstrated in multivesicular bodies and in some vesicles, but not in the endoplasmic reticulum and the Golgi apparatus. The intracellular localization of APase in SGT cells generally fitted the findings being reported in other tissue by several investigators [14, 24, 26]. Multivesicular bodies with APase activity were located around the Golgi apparatus, and some of APase-positive vesicles attached to the plasma membrane. Multivesicular body sometimes contains rough endoplasmic reticulum and trans-Golgi vesicles, and functions as a large heterolysosome [27]. The majority of multivesicular body-vesicles display similar enzyme reactions as do vesicles located along the convex surface of the Golgi complex [8]. From these findings, it is speculated that the presence of APase activity in the multivesicular body indicates the APase synthesis in SGT cells, and that APase is transported to the plasma membrane. However, some of the APase-positive vesicles may be the vesicles formed by the plasma membrane recycling.

In our previous study by light microscopy, the Golgi area was occasionally APase-positive in the SGT cells showing an intense activity on the plasma membrane. The APase activity in the Golgi area is stronger by the azo dye method than by the lead citrate method [29, 30]. Recently,

several investigators demonstrated that intracellular APase may be physiologically cryptic inside the membrane, and that saponin treatment is effective in demonstrating all the cryptic APase activity [24, 26]. When swine adenohypophysial cells were disaggregated with collagenase and hyaluronidase solution and were stained with APase reaction after saponin treatment, most of SGT cells exhibit the APase activity in the Golgi area (Yamaguchi, unpublished data), suggesting that the Golgi apparatus in SGT cells is essentially positive for APase and that APase is generally cryptic in the Golgi area. Therefore, the discrepancy between electron microscopical observation and light microscopical observation on the APase activity in the Golgi apparatus in SGT cells is likely due to technical differences such as type of fixatives, duration of exposure to fixation, thickness of sections incubated and rate of penetration by the substrate.

In intercellular spaces in SGT cells, APase was detected as a soluble form. Recently, it has been elucidated that the released and/or soluble form of APase is produced by enzymatic cleavage of the glycan-phosphatidylinositol anchoring moiety in the membrane form [7, 10, 12, 15]. This indicates that the intercellular APase in SGT cells may have been released to the environment following cleavage by phospholipase C. On the other hand, an intracellular transport process of APase has been clarified in cultured tumor cells treated with cycloheximide, a protein synthesis inhibitor, and monensin, an inhibitor of protein transport under the induction of APase with saponin [26]. This also gives us a chance to confirm the intracellular APase movement in SGT cells. However, in the present study, it was impossible to investigate the intracellular transport of APase, since the experiment was carried out using tissue sections.

The present study provided us some new observations on the subcellular localization of APase in SGT cells. However, possible roles of APase in SGT cells could not be elucidated in detail. Therefore, in SGT cells, additional studies are needed to obtain a line of direct evidence of APase biosynthesis and to explore the intracellular transport process of APase by visualization of cryptic intracellular enzyme activity.

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