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Cytochemical Localization of NAD(P)H Oxidase in the Myoepithelial Cells of Salivary and Other Exocrine Glands

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Cytochemical localization of NAD(P)H oxidase, a hydrogen-peroxide generating enzyme, has been investigated using the cerium method in several mammalian exocrine glands: (a) salivary glands of the rat, Mongolian gerbil, house musk shrew and man, (b) lactating mammary glands of the rat and Mongolian gerbil and (c) exorbital lacrimal gland of the rat. The NAD(P)H oxidase activity could be exclusively localized in association with the myoepithelial cells (MEC) of all the exocrine glands examined. The reaction products in the MEC were found mostly on the plasma membranes facing the neighboring cells (acinar cells, ductal cells and MEC) and in the caveolae. These localizations imply that a membrane-bound NAD(P)H oxidase specific for the MEC could exist in mammalian exocrine glands, and that it may be involved in some intercellular regulatory system between MEC and other parenchymal cells of the glands. With regard to the reactivities on MEC, there were apparent diversities among different glands. Intense and constant activities were found in both the lactating mammary glands of the rat and Mongolian gerbil and the salivary glands of house musk shrew, in which the MEC are extremely developed. The usefulness of NAD(P)H oxidase as a marker enzyme of MEC and the significance of its localization in MEC are discussed.

Key words: NAD(P)H oxidase, Myoepithelial cells, Salivary glands, Exocrine glands, Cerium cytochemistry

I. Introduction

Cytochemical localization of NAD(P)H oxidase, a H_2O_2-generating enzyme, has been shown most extensively in leukocytes and macrophages, where it plays an important defense mechanism against infectious agents [23]. Also, NAD(P)H oxidase has been known to localize on the plasma membranes of several epithelial cells, e.g., the thyroid follicular cells [3], the epithelial cells of the endometrium [10], the hepatocytes [15], and the absorptive cells of the duodeno-jejunum [9]. In the thyroid follicular cells, NADPH oxidase is known to be involved in the biosynthesis of thyroid hormone [5]. We found in a previous study about the H_2O_2 generating sites in the parotid gland of the Mongolian gerbil that NAD(P)H oxidase could be exclusively localized on the myoepithelial cells (MEC) in the parenchyma of the gland [13]. Until then, in the MEC of exocrine glands, there had been no report demonstrating the localization of NAD(P)H oxidase. So far, in order to identify MEC using the enzyme-histochemical method, alkaline phosphatase or Mg^{2+}-ATPase have been mainly used. However, the use of either of these enzymes as markers of MEC cannot be always helpful because their reactivities are not universal throughout different investigations {reviewed in [6, 20]}. The purpose of this report is to evaluate whether NAD(P)H oxidase can be useful as an enzyme-cytochemical marker of MEC in several mammalian exocrine glands.

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II. Materials and Methods

The mammalian exocrine glands used for this study are listed in Table 1. Wistar rats (*Rattus norvegicus albinus*), Mongolian gerbils (*Meriones unguiculatus*) and house musk shrews (*Suncus murinus*) were bred in the Laboratory Animal Center for Biomedical Research, Nagasaki University School of Medicine. These animals were anesthetized by an intraperitoneal injection of sodium pentobarbital, and tissues were excised.

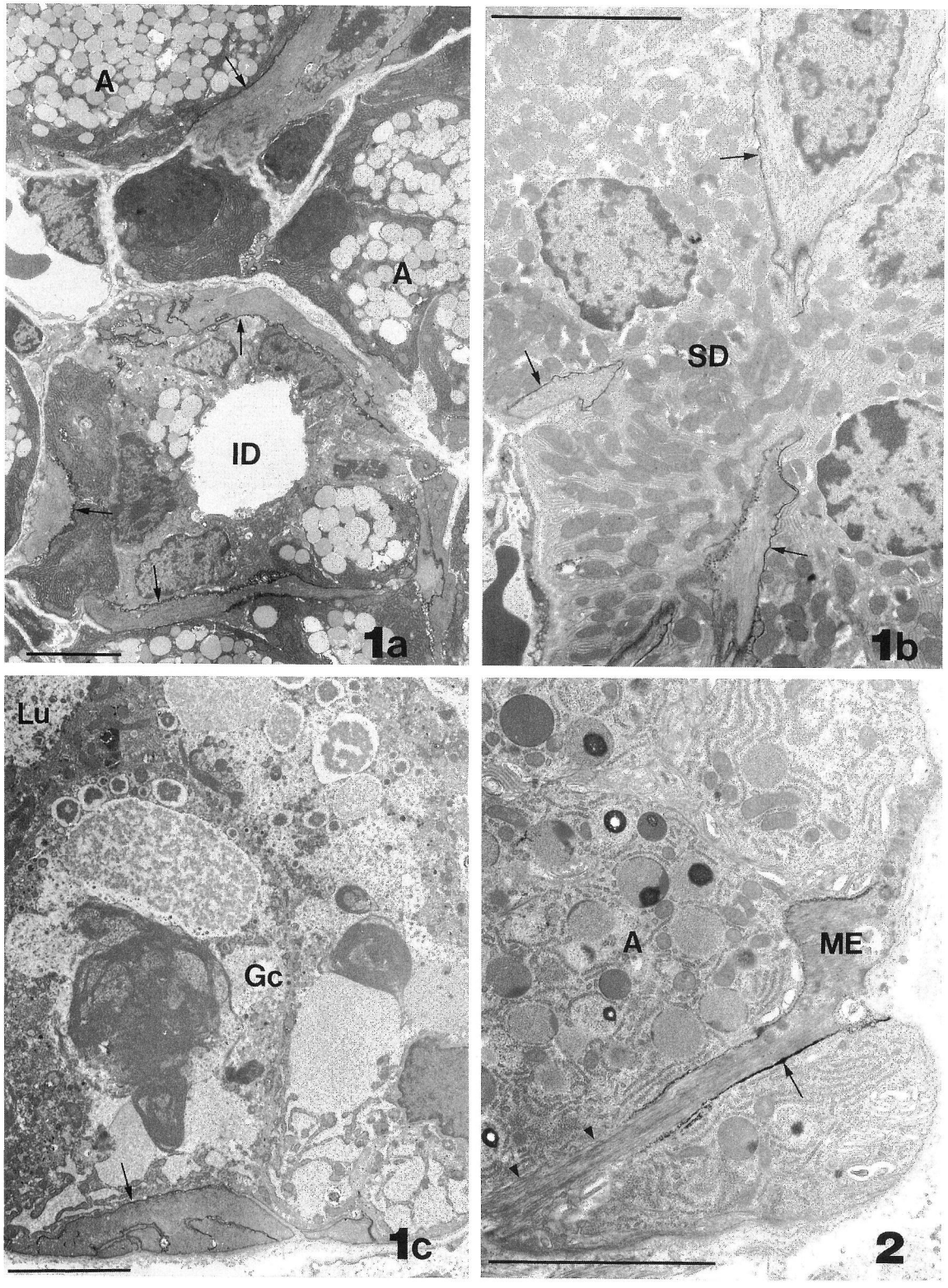

Fig. 1. NAD(P)H oxidase activities in the salivary glands of house musk shrew. **a**: Terminal portions of the sublingual gland. A: acini, ID: an intercalated duct. **b**: A part of a striated duct (SD) of the parotid gland. **c**: A part of a granular duct of the submandibular gland. Gc: granular duct cells, Lu: lumen. Reaction products are found in association with the plasma membranes of the myoepithelial cells (arrows). **a** ×3,100, **b** ×6,300, **c** ×4,000. Bars=5 μm.

Fig. 2. NAD(P)H oxidase activity in the human sublingual gland. Acinar (seromucous) cells (A) and an associated myoepithelial cell process (ME). Activity is found on the plasma membrane of the myoepithelial cell (arrow), but part of the membrane is devoid of reaction product (arrowheads). ×7,500. Bar=5 μm.

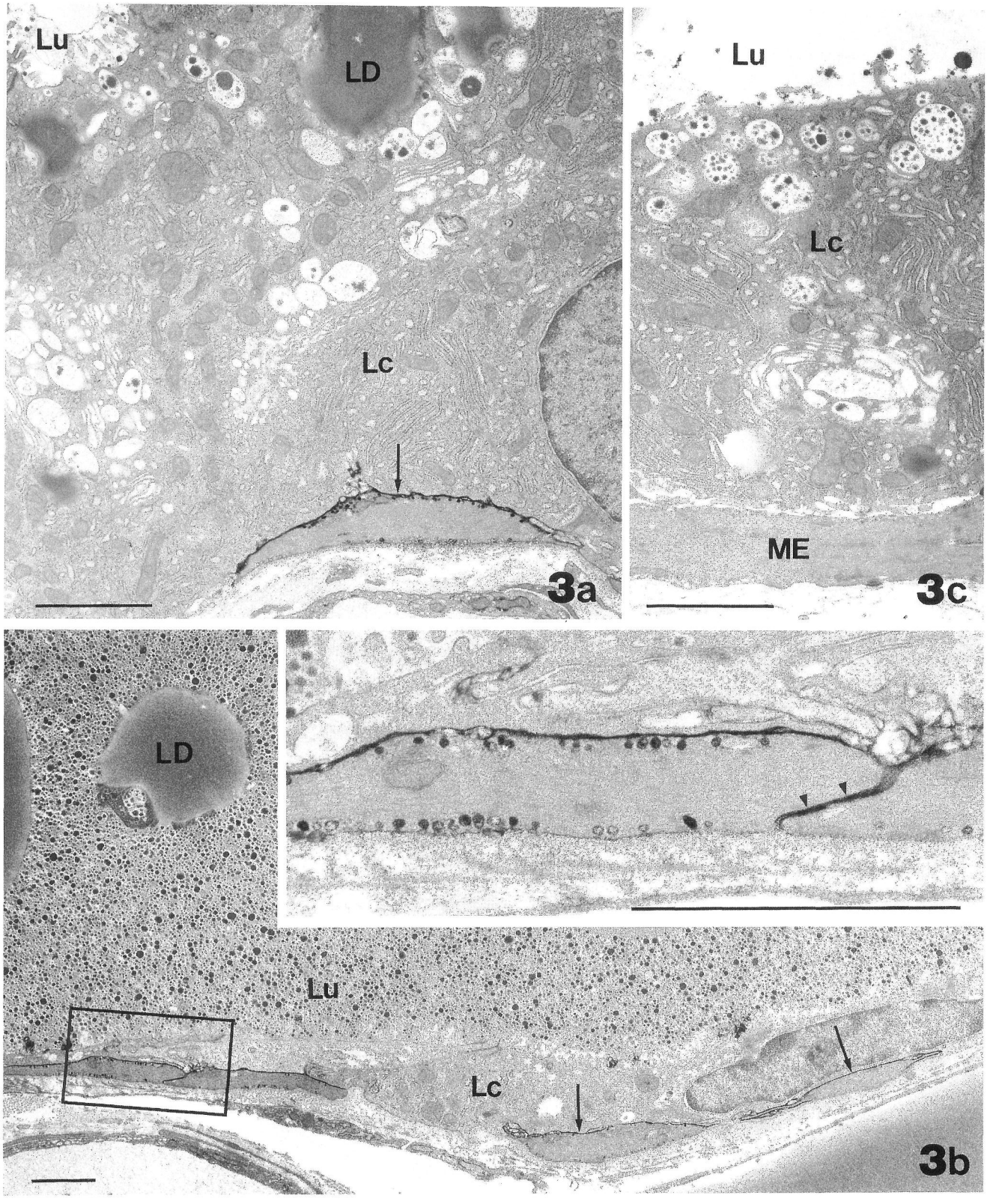

Fig. 3. Electron micrographs of the lactating mammary gland incubated in a NAD(P)H-cerium medium (**a**, **b**) or in a substrate-free medium (**c**). Lu: lumen, Lc: lactocytes, LD: lipid droplet. **a**, **b**: NAD(P)H oxidase activities are found on the plasma membrane and caveolae of the myoepithelial cells (arrows) of acini both before (**a**) and after (**b**) secretion. Inset: High magnification of the outlined area. Reaction products are also seen on the plasma membrane of myoepithelial cells that contact each other (arrowheads). Small amounts of deposits are found diffusely on the basal plasma membrane of the myoepithelial cell and on the basement membrane. **c**: No activity is found on the myoepithelial cell process (ME). **a** ×9,900, **b** ×5,300 (Inset: ×28,000), **c** ×10,700. Bars=2μm.

Specimens of the human sublingual gland were surgically excised from three patients (women; 17, 48 and 58 years old) during general anaesthesia at Nagasaki University Hospital of Dentistry, and were of histologically normal appearance.

The tissues were immediately fixed for 1 hr in a cold 1% glutaraldehyde and 2% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.4. NAD(P)H oxidase cytochemistry was performed using a previously described cerium method [13]. Some control incubations were performed as follows: (1) incubation in a substrate-free medium; (2) incubation in a complete medium containing 10 mg/ml catalase (bovine liver, C-40, Sigma), a scavenger of H_2O_2; and (3) heating for 5 min at 65°C prior to the incubation. The reacted sections were rinsed and postfixed for 1 hr with 1% osmium tetroxide in 0.05 M cacodylate buffer, pH 7.4, followed by dehydration and embedding in Epon-Araldite resin. The ultrathin sections, stained briefly with uranyl acetate, were examined using a Hitachi H-800 electron microscope.

III. Results

NADH- and NADPH- dependent reaction products were found exclusively associated with the MEC of all mammalian exocrine glands examined. In the MEC, intense reaction products were localized to the outer surface of the plasma membranes, especially where facing parenchymal (acinar and ductal) cells (Figs. 1, 2, 3a, b). The plasma membranes of the cellular processes of MEC that overlap and attach to each other also showed intense reaction (Figs. 1, 3b). Only min amounts of deposits were found diffusely on the basal plasma membranes of MEC and on the basement membranes which face the MEC (Fig. 3a, b). The reaction products were also found densely in most of the caveolae along both the apical and basal surface of MEC (Figs. 1, 3a, b). However, some caveolae revealed few reaction products (Fig. 3b). In control specimens incubated in a medium that omitted the substrates (NADH and NADPH), no reaction products associated with the MEC were present (Fig. 3c). Reactions on the MEC were almost extinguished by the addition of catalase, a scavenger of H_2O_2, or by heating prior to incubation. Although electron dense deposits, apart from the reaction on MEC, were sometimes found both on the luminar plasma membranes and on the mitochondria of all glands' parenchymal cells, these were formed independently of the substrates.

With regard to the reactivities, there were apparent differences both among MEC in different glands and among different MEC in the same gland as follows (Table 1). (1) Reaction products on a NAD(P)H oxidase-positive MEC were clearly and continuously present in the exocrine glands of rat, Mongolian gerbil and house musk shrew (Figs. 1, 3a, b). In particular, the intensity of the reaction product was heaviest in the lactating mammary gland of the rat and Mongolian gerbil. In contrast, reaction products on MEC in the human sublingual glands were visible but sometimes sparse or discontinuous. (2) Reactivities on different MEC in the same gland were consistent both in the lactating mammary gland of the rat and Mongolian gerbil and in the salivary glands of house musk shrew. However, MEC without any reaction product were occasionally seen in the glands of the rat and Mongolian gerbil except for the mammary glands, and many MEC in the human sublingual gland were unreactive.

Table 1. *Reactivities of NAD(P)H oxidase cytochemistry on myoepithelial cells (MEC) in several mammalian exocrine glands*

Exocrine glands	Reactivity[1]	Universality[2]
rat		
parotid gland	+	+
submandibular gland	+	+
sublingual gland	+	+
exorbital lacrimal gland	+	±~+
(lactating) mammary gland	++	++
Mongolian gerbil		
parotid gland[3]	+	+
submandibular gland	+	+
sublingual gland	+	+
(lactating) mammary gland	++	++
house musk shrew		
parotid gland	+	++
submandibular gland	+	++
sublingual gland	+	++
human		
sublingual gland	±~+	±

1) Reactivity is expressed by the amount and continuity of deposits in a NAD(P)H oxidase positive MEC: heavy and continuous, ++; visible and continuous, +; visible but irregular, ±
2) Universality of reactions on different MEC in the same gland is expressed as follows: almost all the MEC are reactive, ++; some MEC are nonreactive, +; few MEC are reactive, ±
3) Data from our previous report [13]

IV. Discussion

The reactions associated with MEC were, in principle, the same as those reported in our previous study on the Mongolian gerbil parotid gland [13], and suggest that some H_2O_2-generating system by NAD(P)H oxidase may be present in association with the MEC of the mammalian exocrine glands. So far there has been no information about the functional significance of the H_2O_2 generation system in MEC. The NAD(P)H oxidase activity on the apical plasma membranes of MEC was more intense than that of the basal plasma membranes. It is not known whether this different amount of activity reflects a true variation in concentration of the enzyme or a functional

differentiation on each side of the membrane. The distribution of activity in MEC, which is densely associated with the neighboring cells, implies that NAD(P)H oxidase is involved in some intercellular regulatory system. A possibility that the weak activity of the basal plasma membranes of MEC is due to diffusion at the exposed surface must be considered, because only the caveolae showed intense activity at the basal surfaces. The activity of the basal surfaces may also be involved in some intercellular communication between the MEC and stromal elements such as the endothelial cells of the capillaries.

It has been reported that some active oxygen species (e.g., H_2O_2, O_2^-, etc.)-generating enzyme systems are involved in several significant physiological functions [4, 19]. For example, the plasma membrane-bound NADPH oxidase system is well known to be essential for the microbicidal activity in leukocytes [23] or for the biosynthesis of thyroid hormones in thyroid follicular cells [5]. Similar membrane-bound NAD(P)H oxidase systems have also been recognized in several epithelial cells [9, 10, 15], fibroblasts [11], osteoclasts [22], endothelial cells [16] and smooth muscle cells [7]. The existence of the NAD(P)H oxidase system in smooth muscle cells is interesting to us because MEC have been shown to share many features with smooth muscle cells [6, 20]. The membrane-bound NAD(P)H oxidase system in vascular smooth muscle cells [7, 18], as well as that in vascular endothelial cells [16], has been suggested to be an important contributor to the regulation of smooth muscle cell proliferation or of vascular tone. The cytochemical localization of NAD(P)H oxidase in MEC of exocrine glands is very similar to that of adenylate cyclase in the lactating mammary glands and in the palatine glands, which were reported by Sopel [21] and Han *et al.* [8], respectively. They suggested that the adenylate cyclase in MEC might be involved in the regulation of cell contraction. It is possible that NAD(P)H oxidase may take part in the contractile system of MEC in cooperation with adenylate cyclase.

It is noteworthy that NAD(P)H oxidase activity in the lactating mammary glands was intense and consistent, but on the contrary, that in the human sublingual glands was weak and inconsistent. The activities of lactoperoxidase [1] or alkaline phosphatase [2] in the rat mammary gland are histochemically known to be closely related to active lactogenic stages. NAD(P)H oxidase activity may also be concerned with the functional state of the glands. Possibly, low activity of NAD(P)H oxidase in the human sublingual glands from patients may be due to low level of functional activity, although their histological appearance was normal. In many cell types, the release of active oxygen species (H_2O_2 and O_2^-) by NAD(P)H oxidase systems occurs by stimulation with various agents {e.g., cytokines, Ca^{2+}-ionophores, growth factors, etc. [4, 17]}. Cytochemical study about the localization of peroxidase and NAD(P)H oxidase in thyroid follicular cells revealed that both enzymes are activated by TSH-stimulation [15]. Investigations under such stimulated conditions will be needed to understand the inconsistencies of NAD(P)H oxidase activity on MEC. It is also interesting that consistent NAD(P)H oxidase activity was seen in the salivary glands of house musk shrew (Fig. 1a–c), in which MEC are known to be extremely developed and have a wide coverage from acini to ducts [12].

As described above, NAD(P)H oxidase-cytochemistry on MEC showed various reactivities among the different exocrine glands. Similar inconsistencies of activity are also known in the histochemical studies of several phosphatases (e.g., alkaline phosphatase, Mg^{2+}-ATPase) which have been used as useful marker enzymes of MEC {reviewed in [6, 20]}. However, there is a noticeable difference between NAD(P)H oxidase and phosphatases concerning the localization of reaction products. That is to say, NAD(P)H oxidase activity could be exclusively localized on MEC in the parenchyma of the glands, but on the other hand, activities of phosphatases have been reported to be occasionally associated with the plasma membranes of parenchymal elements. Therefore, it may be suggested that NAD(P)H oxidase may be enzyme specific to MEC in the parenchymal elements of exocrine glands. It may be possible that NAD(P)H oxidase can be used as a cytochemical marker enzyme of MEC. To our regret, in this case, we had to make allowances for the lack of reaction constancy as well as the nonspecific reactions described above for now. Extensive studies, including methodological modifications of the present cytochemistry, will be required in order to evaluate the significances of the localization of NAD(P)H oxidase in MEC.

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VI. References

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