

Phosphodiesterase 10A Immunoreactivity in the Granular Convoluted Tubule (GCT) of Rat Submandibular Gland

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Abstract

Immunoreactivity of phosphodiesterase 10A (PDE10A), cGMP-binding cAMP PDE and cAMP-inhibited cGMP PDE, was examined in the rat submandibular gland (SMG). The intense immunoreactive signals were observed in specialized segments of the duct system of SMG, or granular convoluted tubule (GCT). Each GCT cell was apparently positive but not uniform in signal intensity; that is, in some GCT cells dense immunoreactive granules were distributed throughout the cytoplasm with preferential accumulation in the basal portions, while in the others the immunoreactive granules were scanty in cytoplasm. A few, small, solitary cells of the intercalated ducts (ID) showed immunoreactivity clearly. Cells of both the interlobular excretory ducts and the intralobular striated ducts (SD) exhibited feeble but discernible immunoreactivity. No immunoreactivity was observed in saliva-producing acinar cells in SMG. These results provide information that PDE10A is involved in the special function(s) of GCT cells, and that GCT cells of the rat SMG can be a model for analyzing PDE10A function(s) in the intracellular signal transduction system closely associated with the cell physiology, as well as the molecular aspects of PDE10A.

Key words: Phosphodiesterase 10A, Submandibular gland, Granular convoluted tubule, cAMP, cGMP

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Introduction

The granular convoluted tubule (GCT) is a specialized segment of the duct system of the submandibular gland (SMG) frequently seen among rodents, including laboratory animals. The similar structures have been also reported in a few species of insectivores and one species of lagomorphs¹⁾. The duct systems of rat and mouse SMGs are divided into 6 segments along the direction of saliva secretion; (1) intercalated duct (ID) next to SMG acinar cells, (2) granular convoluted tubule (GCT), (3) striated duct (SD), (4) excretory duct, (5) main excretory duct (MED), and (6) salivary bladder (expanded portion of the MED)^{2, 3)}. Among them, the GCT segment is distinctive in morphology and biochemistry, because GCT cells differentiate from portions of SDs into different type of mature cells at the onset of puberty and are under complex developmental and multihormonal regulation. In some species GCT cells and the SD predecessor cells are androgen-sensitive particularly in males and undergo the destined morphological changes; the GCT segments of the SMG duct system display the sexual or gender dimorphism^{1, 4, 5)}.

The morphological changes of GCT cells during the postnatal development are in harmony with the biochemical properties that these cells express biologically active polypeptides such as nerve growth factor (NGF), epidermal growth factor (EGF), insulin-like growth factor I (IGF-I), hepatocyte growth factor (HGF), transforming growth factor β -1 (TGF β -1), colony-stimulating factor (CSF), renin, erythropoietin, and endothelin^{4, 6)}. These phenotypical properties of GCT cells as well as the other cells of adjacent components of the ducts are regulated by hormones such as androgen, thyroid hormone and corticosteroids^{1, 4, 6)}. Several factors from these cells are secreted into saliva under physiological conditions of autonomic nerve stimulation^{7, 8)}, but their physiological roles in it are not fully understood. Furthermore, some biologically active factors described above are found in the circulation under certain conditions such as aggressive behavior and experimental stimulations⁹⁻¹²⁾. These elevated serum contents are dependent on the SMG and this issue needs to be clarified whether those factors are released in an endocrine fashion¹³⁾.

Although the peculiar property of GCT cells is intriguing, in salivary glands the most important function is the secretion of saliva containing digestive enzymes for foods. Saliva secretion is regulated by autonomic nerves, i.e., sympathetic and parasympathetic. β -Adrenergic stimulation increases intracellular cAMP via GTP-binding protein, activates cAMP-dependent protein, and then leads to secretion of protein-rich saliva^{14, 15)}. α -Adrenergic and muscarinic cholinergic stimulations activate phospholipase C via GTP-binding proteins to produce inositol triphosphate. This intracellular signaling pathway causes the increased intracellular Ca^{2+} and results in secretion of watery and electrolyte-rich saliva¹⁶⁾. Muscarinic cholinergic stimulation increases intracellular cGMP and lowers the cAMP concentration raised by β -adrenergic stimulation¹⁷⁾. These intracellular signaling molecules are elaborately regulated by activating adenylyl cyclases or guanylyl cyclases and by activating catalytic enzymes, or phosphodiesterases (PDE), to hydrolyze cyclic nucleotides. In addition, the GCT of SMG is also innervated by sympathetic nerves and excretes granular contents into saliva by either α - or β -adrenergic receptor stimulation¹⁸⁾, and hormones as well. As for the catalytic enzymes of cyclic nucleotides, a repertory of phosphodiesterases has been established in mammals; 11 families with several subtypes and their own splice variants¹⁹⁾. A recent biochemical study in the whole SMG has demonstrated in rat that the major isozyme of PDE is PDE 4 and also the other isozymes (PDE 1, PDE 2, PDE 3 and PDE 5) are expressed in a small amount²⁰⁾. The multiple isozymes and numerous splice variants may, however, imply possible participation of the other isozymes in the rat SMG functions; proliferation and differentiation for replacement of acinar and ductal cells as well as salivary secretion.

The present study is a part of a series of studies to know the roles of PDE10A in specific cell functions. A tissue-survey of PDE10A expression using the antibody revealed that a restricted number of tissues and specific cell populations displayed immunoreactive signals for the enzyme. GCT cells of the rat SMG is one of the 4 major tissues expressing PDE10A. The present study is the first that PDE10A expressing cells are demonstrated immunohistochemically at cellular level.

Materials and Methods

Animals

Male Sprague-Dawley rats (10 to 13-week-old, ranging from 350 to 450 g in body weight) were used in the present study. Under deep anesthesia (pentobarbital sodium, i. p., 50 mg/kg in body weight), the animals were perfused transcardially with 150 mL 0.9% saline, followed by 450 mL 4 % paraformal-dehyde in 0.1 M phosphate buffer (pH 7.2). After dissected out, the submandibular glands (SMGs) were postfixed in the same fresh fixative at 4 °C for 18-24 hours, and rinsed in phosphate-buffered 20% sucrose at 4 °C for another 18-24 hours. The SMGs were frozen in powdered dry ice, and serial sections were cut at 15 μ m in thickness on a cryostat and thaw-mounted on slides. Slides were stored at -40°C until use. The handling and treatment of the animals conformed to the guidelines of the Animal Study Committee of the Kagawa Prefectural College of Health Sciences.

Antibody of phosphodiesterase 10A

The polyclonal antibody of phosphodiesterase 10A was generated in a rabbit against a synthetic peptide (14 amino acids), which corresponds to the carboxyl terminal sequence of rat PDE10A²¹⁾. The synthetic peptide was coupled to Keyhole Limpet Hemocyanin and administered as antigen complex into a rabbit intracutaneously. Usable titers of antibody were established approximately one month after antigen injection. The antiserum was tested if it could recognize the natural PDE10A in rat tissue repertoire²²⁾. The immunoreactive staining was observed in a restricted number of tissues and localized in specific cells and structures of the tissues; that is, brain, testis, submandibular gland, and kidney. It is promising for the antibody because the PDE10A gene was cloned in the testis and a large amount of PDE10A mRNA expression has been reported in the brain and testis. No immunoreactivity in all tissues examined was observed when tissues were incubated with naïve rabbit serum collected before immunization. Furthermore, Western blot analysis of various brain tissues revealed that the antibody detected a single band corresponding to the molecular size reported previously (approximately Mr 88,000).

Immunohistochemistry

Sections of rat SMGs were processed routinely in combination with ABC kits (VectorStain, CA; Santa Cruz Biotechnology, Inc., CA). The procedure was carried out at room temperature (22 to 24°C) unless otherwise mentioned. In brief, to suppress endogenous peroxidase activity sections were immersed in 0.1% hydrogen peroxide in methanol for 30 minutes, followed by immersion in 5 % skim milk in phosphate-buffered saline containing 0.3% Triton X-100. For specific antigen-antibody reaction, sections were incubated at 10°C with anti-PDE10A antibody (ca. 1 μ g/mL IgG fraction purified through the protein-A column) for 18-24 hours and then with the secondary antibody (1:500 dilution) supplied in the ABC kits at 10°C for another 18-24 hours. Following incubation with avidin-biotin complex (1:1:100 dilution) for 30 minutes, sections were processed for diaminobenzidine (DAB) reaction to visualize peroxidase-mediated signals. Some sections were stained with haematoxyline dye for histological identification.

Results

Histology

In rat the submandibular and sublingual glands (SMG and SLG) constitute a single ovoid continuum of salivary gland in loose connective tissues of the anterior cervical region. Unlike human, the SMG and SLG are not separated macroscopically and furthermore loosely organized glandular tissues extend laterally from the main portion of the SMG and SLG continuum. The SLG constitutes the rostral cap (ca. 10-15%) of the continuum and the remaining caudal portion is the SMG. Microscopically, however, acinar cells in lobules of the SMG and SLG were clearly demarcated by a boundary connective tissue space from each other (Fig. 1). The SMG mainly consists of serous acinar cells, whereas the SLG mucous acinar cells (Fig. 1). The SMG also develops specialized and characteristic cells, or granular convoluted tubule (GCT) cells, in the duct system of SMG. (Fig. 1 and 2 A). The special GCT segments are situated between the intercalated duct (ID) and the striated duct (SD) (Fig. 2 A).

The GCT cells contained a lot of secretory granules in the luminal two-thirds of the cells and their nuclei

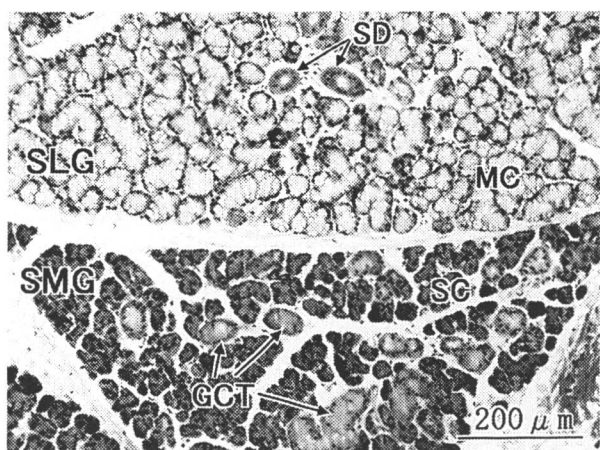


Fig. 1 Photomicrograph showing the boundary between the submandibular and sublingual glands (SMG and SLG). SMG and SLG in rat form a single continuum in the loose connective tissue of the anterior cervical region. SMG comprises primarily serous acinar cells whereas SLG mucous acinar cells. SMG also contains the granular convoluted tubules (GCT), or specialized striated duct cells, in addition to regular segments of the duct system.

were located in the cytoplasm-rich region of the basal third (Fig. 2 B). The cytoplasm of GCT cells was eosinophilic, while the serous acinar cells of SMG stained dark with a basophilic dye Haematoxyline (Fig. 2). Therefore, the two types of SMG cells were well-contrasted with each other. The serous acinar cells also aggregate a large amount of secretory granules in the cytoplasm and their nuclei were located close to the basal ends of the cells.

The SD and ID of SMG showed typical cytological features of the duct system seen in many excretory glands (Fig. 2 C, D and E). SD cells displayed basal striations and their nuclei were located in the middle of the cells (Fig. 2 C). Although GCT and SD cells are homologous by nature, the two types of duct cells are clearly distinguishable in morphology in rat, as well as other species of rodents, in particular in male after puberty (ca. postnatal 4 weeks in rat) (Fig. 2 A, B and C). The IDs are the initial segments of the duct system, which intervene between serous acinar cells and GCT cells, and carry out saliva secreted from acinar cells. ID cells are small in size and have a round or elliptic nucleus surrounded by

eosinophilic poor cytoplasm (Fig. 2 D and E). Another type of acinar cells was rarely observed in cluster in some lobules of SMG. They displayed the cytological features of mucous acinar cells seen typically in SLG (Fig. 2 F). Cytoplasm of these cells stained pale and their nuclei were pressed to the basal sides or the corners of the cells. All cells proper in rat SMG described above have their distinctive features and are discernible on the basis of shape, size and location.

Immunohistochemistry

Immunoreactivity of PDE10A was observed mostly on GCT cells of the duct system of SMG (Fig. 3 A). The other segments of the excretory ducts also showed immunoreactivities; that is, the interlobular excretory ducts, intralobular SDs (Fig. 3 A), and IDs (Fig. 3 B; small arrows). Most cells in the excretory and SD ducts contained feeble and invariable signals in the entire cell bodies, in contrast to the immunoreactivities of GCT cells (Fig. 3 A, C and D). In IDs, a few, small, solitary cells were labeled clearly and distinguished among unlabeled adjacent ID cells and SMG serous acinar cells (Fig. 3 B; small arrows). GCT cells were densely labeled and clearly discernible among unlabeled serous acinar cells (Fig. 3 C). Some GCT cells contained a large number of immunoreactive granules in the entire cell bodies with preferential accumulation in the basal portions, while the others contained sparsely spread immunoreactive granules in the cytoplasm (Fig. 3 D). No immunoreactivity was seen on serous acinar cells of SMG.

Discussion

GCT cells

The present study is the first that PDE10A immunoreactivity was localized in tissues at cellular level. In the rat SMG PDE10A immunoreactive cells occurred in four segments of the duct system; ID, GCT, SD and the excretory duct. Immunoreactivity of GCT cells was outstanding among them and the cytoplasm-rich GCT cells were immunostained to variable extent. Many PDE10A-positive GCT cells contain immunoreactive granules densely throughout the entire cytoplasm, some accumulate the positive signals in the basal third

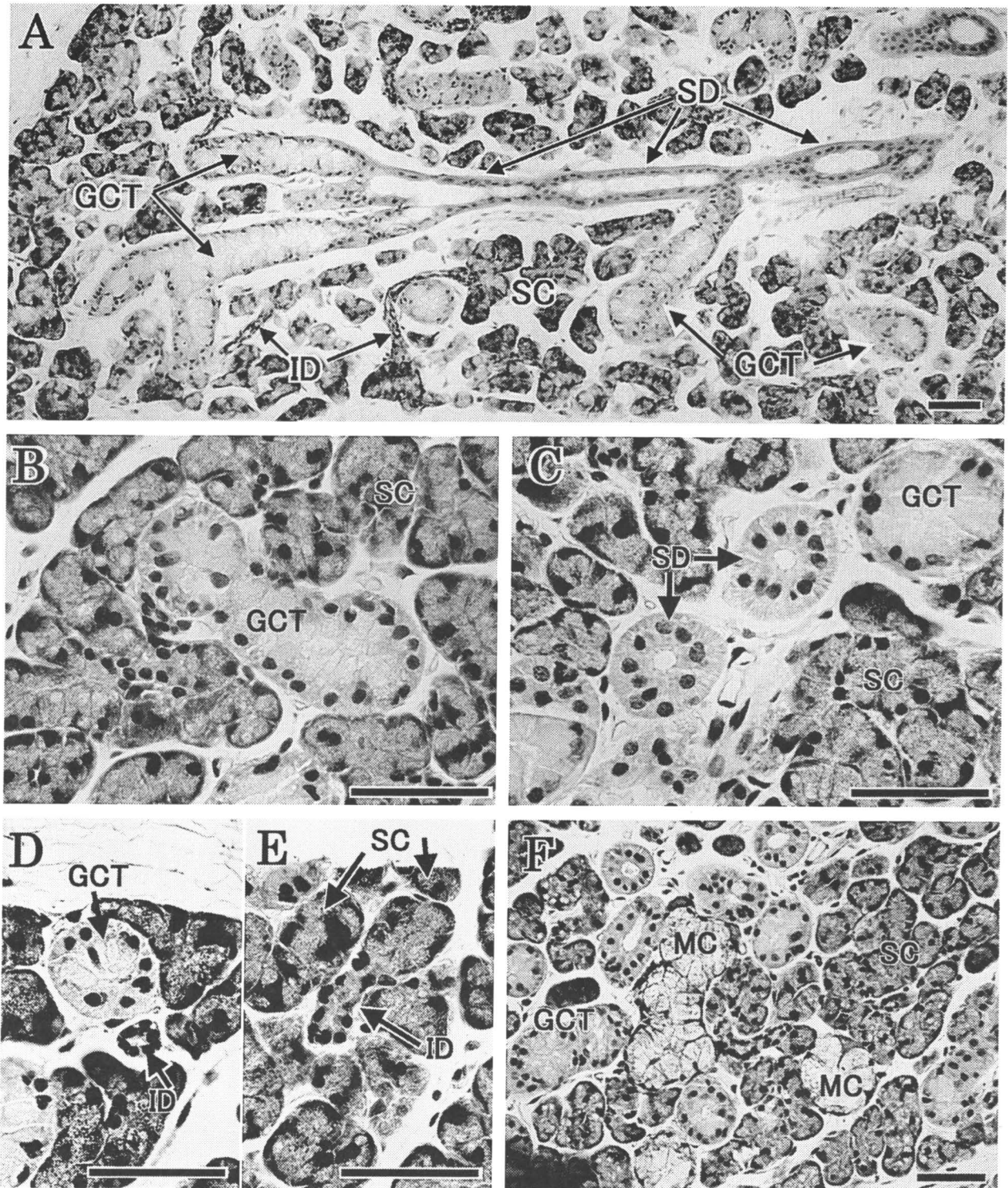


Fig. 2 Photomicrographs of the duct system of the submandibular gland (SMG). A: Arborization of the duct system in SMG lobule. Special segments or the granular convoluted tubule (GCT) are situated between the striated duct (SD) and the intercalated duct (ID). B: GCT cells have eosinophilic cytoplasm where secretory granules are located in the apical two-thirds and a densely stained nucleus in the basal third. GCT cells are well-contrasted with basophilic dark cytoplasm of serous acinar cells (SC). C: SD cells are typically characterized by basal striation and contain a round nucleus in the middle of cell. GCT cells, SD cells and SC cells display the indistinctive cytological features. D and E: ID cells have eosinophilic poor cytoplasm and constitute thin ducts (i.e., ID) which intervene between SMG acini and GCTs. Cross in D and longitudinal in E. F: Clusters of mucous acinar cells rarely appear in SMG. The cytological features are the same to the mucous acinar cells of the sublingual gland. GCT; granular convoluted tubule, ID; intercalated duct, MC; mucus acinar cell, SC; serous acinar cell, SD; striated duct, SMG; submandibular gland. A bar in each panel equals 50 μ m.

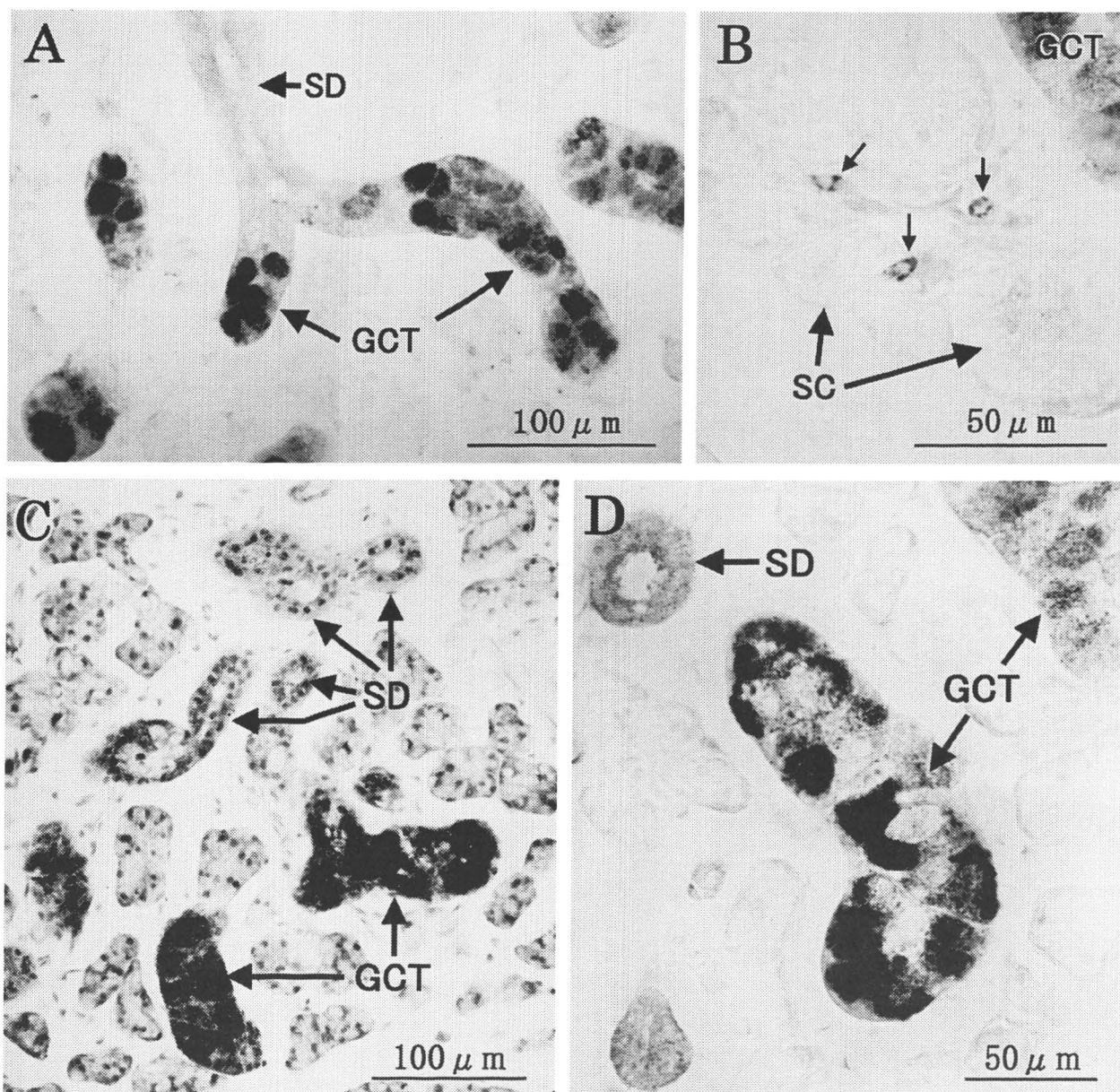


Fig. 3 Photomicrographs of phosphodiesterase 10A immunoreactive cells in the submandibular gland (SMG).

A: Intensive immunoreactivities were localized in the granular convoluted tubule (GCT) cells of the SMG duct system. The striated duct (SD) also showed weak immunoreactivities. B: A few small cells in the intercalated duct (ID) (small arrows) were occasionally immunoreactive. C: Section stained with Haematoxylin for cytological identification showed immunoreactivities were localized primarily in GCT cell and less intensively in SD cells. D: Some GCT cells gather a large number of immunoreactive granules in the entire cell bodies, while the others contained scattered positive granules in their cytoplasm. Some other GCT cells have pale (no granule) cytoplasm in the apical region and densely packed granules in the basal third. Weak immunoreactivities of SD cells were also observed. GCT; granular convoluted tubule, SC; serous acinar cell, SD; striated duct.

with pale apical cytoplasm, and the others scatter only a small number of positive signals throughout the cytoplasm. Such cell-dependent immunostaining are also seen in cases of EGF²³⁾, Protease A²⁴⁾ and a true tissue kallikrein (mK 1)^{25, 26)}.

Pinkstaff²⁾ distinguished the so-called pillar cells in

GCTs as well as the dark cells in SDs and the excretory ducts. Intense immunoreactivities for S100 protein and calmodulin were localized in the pillar cells of GCTs and SDs²⁷⁾. However, the principal and pillar cells in GCTs were indistinguishable with anti-PDE10A antibody in the present study. A zone of transition

cells (a most distal portion of GCTs), exhibiting characteristics of both GCT and SD cells, was found between the ID and GCT segments¹⁸⁾. With some difficulties to exhibit the continuation from GCTs to IDs histologically, the transition cells were immunostained with the PDE10A antibody.

Although there may be some interspecies differences in cellular components of GCTs between animals²⁸⁾, GCTs in mouse and rat, as a whole, differentiate from the special portions of SDs peripubertally. In adult mouse GCT cells also differentiate from ID cells²⁹⁻³¹⁾. The differentiation from SD cells to GCT cells was accelerated in males after the onset of puberty but not in females, resulting in a clear sexual dimorphism in adults³²⁾. GCTs of SMG are under multihormonal regulation, and are sexually dimorphic, being larger in males than in females. Correspondingly, levels of its various secretory products are more abundant in males than in females. On the contrary, GCT cells containing the true tissue kallikrein (mK1) are much more abundant in the female SMGs than the male's, and display a sexually dimorphic mosaic distribution of this isozyme in the mature SMG^{25, 33)}. Castration of male mice restores immunoreactivity of the enzyme in GCTs, whereas administration of testosterone suppresses the true tissue kallikrein expression and decreases immunoreactive GCT cells in SMGs of both genders. With maturation to adult male mouse, however, it has been reported that GCT cells decrease and then disappear from male SMG, due to their conversion into the non-granular type²⁸⁾.

GCT cells of SMG are characterized by a wide variety of biologically active proteins (CSF, EGF, IGF-I, NGF, TGF- α , TGF- β 1, HGF, basic fibroblast growth factor, calmodulin, C-type natriuretic peptide, endothelin, erythropoietin, renin, S100 protein and etc.). Most factors display differential expressions along the course of postnatal development; many of them fluctuate showing elevated levels of the expressions in harmony with pubertal events, some factors are declined to a adult level before the puberty, and the others appear to show time-dependent fluctuations under the circadian regulation or daily behaviors. Furthermore, GCT cells are under multihormonal regulation by androgens, thyroid hormones, and adrenocortical hormones²¹⁾. Accordingly, the time-

dependent oscillations of some factors, such as NGF and EGF, in saliva and GCT cells seem to be under the circadian regulation; diurnal and nocturnal phases^{34, 35)}.

The regulatory or inhibitory factors and information are scanty about functions of SMG, despite a large number of stimulatory factors to elevate the cellular activities. It is well known that various extracellular signals work for activating adenylyl and guanylyl cyclases to generate cAMP and cGMP, respectively. On the other hand, the degradation enzymes of these cyclic nucleotides are phosphodiesterases (PDE) which comprise of 11 families with subtype variations expressing their own splice variants. The present study has revealed immunocytochemically that PDE10A isozymes participate in regulation of cyclic nucleotide-mediated intracellular signaling cascades in rat GCT cells. PDE10A isozymes have been cloned originally from testis because of abundance of PDE10a transcripts, and also known being rich in the striatum of brain²¹⁾. Only limited information is available about PDE10A in body tissues. Recently a biochemical study has reported that other PDE isozymes are detected in the rat SMG. The analysis of the whole extract of SMG reveals that the major isozyme of PDEs is PDE4 and the minor ones are PDE1, PDE2, PDE3 and PDE5. Since in SMG many biologically active molecules are produced, these degradation isozymes may be associated with some specific functions of different types of cells in SMG.

ID cells

In adult animals the parenchyma of SMG is self-renewing with newly formed acinar cells. Based on the different expression of perinatal secretory proteins (SMG-B1, -C and, -D), ID cells are phenotypically diverse by nature and multipotent to differentiate into different parenchymal cells of SMG. GCT cells are also believed to differentiate from ID cells. In tritiated thymidine autoradiography, proliferating cells were found mostly in ID cells and primarily in cells lacking the perinatal proteins³⁶⁾. Man et al.³⁶⁾ also found proliferous signals over parenchymal acini as well as the junctional zones where ID cells differentiate into both acinar and GCT cells. Some ID cells, therefore, subserve for replacement of SMG parenchymal acinar cells and adjacent GCT cells. In the present

study, a few small solitary ID cells showed PDE10A immunoreactivities, in addition to GCT and SD cells. It may be assumed that these PDE10A-positive ID cells display the intrinsic nature to differentiate into GCT cells, i.e., progenitors committed to differentiate into mature GCT cells. If it is the case, PDE10A would function for differentiation in consonance with other intracellular signaling molecules in progenitor ID cells.

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