Restricted expression of somatostatin receptor 3 to primary cilia in the pancreatic islets and adenohypophysis of mice

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ABSTRACT
The primary cilium is now considered to function as a fundamental, not rudimentary, structure for mechanical and chemical sensing by individual cells. Primary cilia in neurons express type III adenylyl cyclase (ACIII) and GPCRs for somatostatin (somatostatin receptor 3, SSTR3), serotonin, and melanin-concentrating hormone. The present immunohistochemical and electron microscopic study revealed an abundant occurrence of SSTR3-expressing solitary cilia in insulin- and growth hormone-secreting cells of the mouse. The SSTR3 immunoreactivity was restricted to the plasma membrane of cilia in both cell types, differing from previously reported immunohistochemical localization of SSTRs to cell bodies. The primary cilia in the islet cells were longer than those in the pituitary cells and extended for a long distance in the intercellular canalicules endowed with microvilli. No other endocrine organs were provided with the SSTR3-expressing primary cilia, while the primary cilia in these organs were frequently immunolabeled with ACIII antibody. Since the somatostatin inhibition of both insulin and GH release is regulated mainly by SSTR1 and SSTR5, the primary cilia expressing SSTR3 may be involved in a signaling which differs from that via other SSTR subtypes expressing in cell bodies.

Morphologists have long observed a single cilium, termed the primary cilium, in a variety of cells under light and electron microscopes. The primary cilium is an immotile cilium that is extended from the centriole—usually surrounded by the Golgi apparatus—in almost all cell types (38). The primary cilia in sensory cells serve specialized sensing functions, as represented by photoreceptor cells, auditory hair cells, and olfactory receptor cells. However, evidence is accumulating that raises the idea that the primary cilium in non-sensory cells functions as a sensory device at a cellular level. Long solitary cilia on epithelial cells in the collecting tubules of the kidney act as mechanoreceptors to detect and respond to fluid flow in the lumen (21, 26, 27). Bending of the ciliary axoneme by fluid movement has been shown to induce a Ca²⁺-response in the cells of the collecting tubules (26). Neuronal primary cilia in the brain express G protein-coupled receptors (GPCRs), including somatostatin receptor 3 (SSTR3) (11), 5-HT₆ serotonin receptor (10), and melanin-concentrating hormone receptor 1 (MCHR1) (3), suggesting an important role for the primary cilium in the extra-synaptic signaling of neurons.

The five SSTRs identified and characterized to date predominate in having inhibitory actions (6, 23). Neurons in the central nervous system express all SSTR subtypes on the plasma membrane of somata and proximal dendrites and on beaded fibers—including axons (17), while the immunoreactive SSTR3 gathers in the primary cilia of neurons in several regions of the brain (11, 31). In addition to SSTR3, the primary cilia of neurons can be easily labeled by immunostaining for type III adenylyl cy-
clase (ACIII) in vitro and in vivo (3, 4). Immunostaining for ACIII and tubulin, though not SSTR3, has been applied to detect the primary cilia in visceral organs such as the pancreas and kidney.

Somatostatin is widely distributed outside of the brain and exerts various inhibitory actions for endocrine and exocrine secretions (28). Among the endocrine cells and organs, somatostatin is produced and released from the gastro-entero-pancreatic (GEP) endocrine system, thyroid gland (parafollicular cells), adrenal medulla, and dispersed endocrine cells of the respiratory and urogenital tracts. In most regions, somatostatin functions as a local hormone which is conveyed by local circulation or diffusion through an intercellular space to regulate neighboring cells. Many electron microscopic observations have demonstrated the solitary cilium in endocrine cells such as pancreatic islet cells (39), pituitary endocrine cells (2), and adrenal endocrine cells (37, 38). However, it remains unknown whether the primary cilia in these endocrine cells express SSTR3 and other signal molecules for sensing.

The present immunohistochemical study examined the existence of primary cilia expressing SSTR3 and ACIII in a variety of endocrine cells. Insulin-secreting cells in the pancreas and growth hormone (GH)-secreting cells in the adenohypophysis of mice were found to develop the primary cilia expressing SSTR3, suggesting the involvement of primary cilia in a special sensing of somatostatin in the mouse.

MATERIALS AND METHODS

Immunohistochemistry. Adult male BALB/c mice, weighing about 25 g each, were used. For immunohistochemistry, five mice were perfused with a physiological saline through the heart, followed by 4% paraformaldehyde plus 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. The hypophysis, pancreas, other endocrine organs, and sexual glands were dissected out and immersed in the same fixative for an additional 8 h. The stomach, duodenum, lung, and urethra containing dispersed paraneuronal cells were processed according to the same protocol. In addition, the pancreas was collected from male rats (Wistar strain, 250 g in body weight), hamsters (Syrian strain, 300 g), and guinea pigs (Hartley strain, 350 g) and processed as mentioned above. A mouse cell line secreting insulin, MIN6, was fixed for 3 h with 4% paraformaldehyde. All experiments using animals were performed under protocols following the Guidelines for Animal Experimentation, Hokkaido University Graduate School of Medicine.

The fixed tissues were dipped in 30% sucrose solution overnight at 4°C, embedded in OCT compound, and quickly frozen in liquid nitrogen. Frozen sections of 10 μm in thickness were mounted on poly-l-lysine-coated glass slides. After immersion in 0.01 M phosphate buffered saline containing 0.3% Triton-X 100, the sections were preincubated with a normal donkey serum. For double immunofluorescence, sections were stained with one of following antibodies: rabbit anti-SSTR3 (1 : 5,000 in dilution; Gramsch Laboratories, Schwabhausen, Germany), rabbit anti-ACIII (0.2 μg/mL; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or mouse anti-acetylated tubulin (Clone 6-11B-1, 1 μg/mL; Sigma-Aldrich, St. Louis, MO). The sections were then incubated with one of the following antibodies: guinea pig anti-insulin (1 : 100; Invitrogen, Camarillo, CA), guinea pig anti-glucagon (1 : 600, Progen, Heidelberg, Germany), goat anti-somatostatin (1 : 750, Santa Cruz Biotechnology), goat anti-GH (1 : 250; R&D Systems, Inc., Minneapolis, MN), or rabbit anti-GH antisera (1 : 100; Zymed Laboratories, South San Francisco, CA). The sites of antigen-antibody reactions were detected by incubation with a combination of Cys3-labeled anti-rabbit or mouse IgG and FITC-labeled anti-guinea pig or goat IgG (Jackson ImmunoResearch, West Grove, PA). Stained sections were mounted with glycerin-PBS and observed under a confocal laser scanning microscope (Fluoview; Olympus, Tokyo, Japan). Some of immunostained sections were counterstained with SyTO 13 (Invitrogen) before observation. SSTR3 and ACIII antibodies used specifically labeled the primary cilia on neurons in several regions of the mouse and rat brain, in agreement with previous studies (4, 11). The specificity of immunoreactions with the anti-SSTR3 antibody was confirmed by disappearance of the immunoreactivity when the antibody was preincubated with a synthetic peptide antigen (Yanaihara Institute, Shizuoka, Japan).

Silver-intensified immunogold method for electron microscopy. Frozen sections of 15 μm in thickness were mounted on poly-l-lysine-coated glass slides, incubated with the rabbit anti-SSTR3 (1 : 2,000 in dilution; Gramsch Laboratories), and subsequently reacted with goat anti-rabbit IgG covalently linked with 1-nm gold particles (1 : 200; Nanoprobes, Yaphank, NY). Following silver enhancement using a kit (HQ silver; Nanoprobes), the sections were osmicated, dehydrated, and directly embedded in Epon (Nissin EM, Tokyo, Japan). Ultrathin sections were prepared and stained with both uranyl acetate
and lead citrate for observation under an electron microscope (H-7100; Hitachi, Tokyo, Japan).

Scanning electron microscopy (SEM). Under pentobarbital anesthesia (40 mg/kg body weight), another five mice were perfused transcardially with Locke’s solution and subsequently with a mixture containing 2.5% glutaraldehyde and 1.0% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The hypophysis and pancreas were removed and immersed in the same fixative overnight. The anterior lobe of the hypophysis and the islets of the pancreas were cut out of the fixed tissues under a dissecting microscope, and rinsed in 0.1 M phosphate buffer (pH 7.4). The specimens were macerated with 6N NaOH at 60°C for 18 min (34). After maceration, the tissue pieces were rinsed in 0.1 M phosphate buffer and postfixed with 1% tannic acid in the phosphate buffer for 1 h, followed by 1% OsO₄ buffered with phosphate (0.1 M, pH 7.2) for 1 h. The osmicated specimens were dehydrated through a graded series of ethanol, transferred to isoamyl acetate, and critical-point-dried with liquid CO₂. The dried specimens were coated with osmium in a plasma osmium coater (Nippon Laser and Electronics Laboratory, Nagoya, Japan), and examined in a Hitachi H-4500 scanning electron microscope (Hitachi, Tokyo, Japan) at an acceleration voltage of 10 kV.

RESULTS

Immunohistochemistry of SSTR3 in mice

We first immunostained using the anti-SSTR3 antibody major endocrine organs (the hypophysis, pineal body, thyroid/parathyroid glands, adrenal gland, and pancreas), gonads (ovary and testis), and some visceral organs containing dispersed endocrine cells. The primary cilia immunoreactive for SSTR3 were found only in the pancreatic islets and the anterior lobe of adenohypophysis (Fig. 1a-c). This restricted staining dose not imply a lack of primary cilia, since the antibody against ACIII—another marker of the primary cilia—detected many primary cilia in the intermediate lobe of adenohypophysis, thyroid parafollicular cells, adrenal medulla (Fig. 1d), and corpus luteum in the ovary. Interestingly, the primary cilia in the adenohypophysis were also immunolabeled by the ACIII antibody with the same staining pattern as SSTR3 while those in pancreatic islets were immunonegative for ACIII.

In the pancreas, cells with SSTR3-immunoreactive cilia were restricted to the islets of Langerhans, in which all insulin cells possessed SSTR3-immunoreactive cilia (Fig. 1a). A population of glucagon cells possessed the primary cilia with SSTR3 immunoreactivity, whereas other populations of glucagon cells lacked SSTR3-expressing cilia (Fig. 1c). No somatostatin cells appeared to possess the immunoreactive primary cilia. In the adenohypophysis, cells with the SSTR3-expressing primary cilia corresponded to GH-secreting cells (Fig. 1b), though it was difficult to deny the existence of SSTR3-expressing cilia in other hormone-secreting cells.

In the silver-intensified immunogold method, SSTR3-immunolabeled primary cilia were easily found under the electron microscope (Fig. 1e, f). Many pancreatic B cells possessed a very long primary cilium extending from the deeper region of the cytoplasm into intercellular spaces among islet cells. The primary cilia possessed two centrioles, the proximal centriole and the distal centriole, the latter being connected with the axial fibrils of primary cilium (an arrow in Fig. 1e). In the adenohypophysis, immunolabeled primary cilia were found in endocrine cells filled with large secretory granules characteristic of GH cells (Fig. 1f). Unlike the B cells, the primary cilia of GH cells tended to project from the centrioles close to the cell surface, thus being shorter than those of B cells. The plasma membrane of cell bodies in both B cells and GH cells completely avoided any immunolabeling by the anti-SSTR3 antibody.

Scanning electron microscopy

Scanning electron microscopy (SEM) confirmed primary cilia extending from pancreatic islet cells (Fig. 2a–d) and pituitary endocrine cells of mice (Fig. 2e, f). Our specimens for SEM revealed the whole image of cilia after removing the extracellular matrix and basement membrane by an alkaline maceration. B cells occupying the central region of pancreatic islets displayed large angular shapes with many dark spots disseminated on the cell surface. These spots indicate the presence of insulin granules that only poorly emit secondary electrons (Fig. 2c, d). The B cells issued single long primary cilia measuring 7–14 μm in arbitrary directions (Fig. 2a, b). The cilia of the B cells occurred on the cell surfaces apposed to islet cells and not on those surrounding vessels. The cilia were projected from invaginations of the plasma membrane, subsequently pursued tortuous courses in narrow intercellular gaps, and ultimately entered a canicular space bordered by microvilli of the endocrine cells (Fig. 2b). Such a microvillous channel coursed along every cell edge where three or four endocrine cells met, to consti-
cluding large round cells which corresponded closely to GH-secreting cells in shape—were seen to extend a short primary cilium ranging in length from 2.8 to 7.5 μm (Fig. 2e, f). The round endocrine cells displayed many bright spots on the cell surfaces, suggesting the presence of secretory granules that emit excess amounts of secondary electrons (Fig. 2e).

In the hypophysis, all the parenchymal cells—"tute a reticular system of intercellular canalicule that communicated with perivascular spaces in the islets. Some cilia in the intercellular passages were directed toward the blood vessels, while others were extended in the opposite direction (Fig. 2c). Occasionally, double primary cilia were found to protrude from a single invagination of the plasma membrane (Fig. 2d).

In the hypophysis, all the parenchymal cells—including large round cells which corresponded closely to GH-secreting cells in shape—were seen to extend a short primary cilium ranging in length from 2.8 to 7.5 μm (Fig. 2e, f). The round endocrine cells displayed many bright spots on the cell surfaces, suggesting the presence of secretory granules that emit excess amounts of secondary electrons (Fig. 2e). Primary cilia always originated from the contact surfaces between endocrine cells. The cilia occurred

"Fig. 1 Immunohistochemistry of primary cilia in pancreatic islets (a, c, e), adenohypophysis (b, f), and the adrenal gland of mice (d). The pancreas and hypophysis were double-stained for somatostatin receptor 3 (SSTR3, red) together with insulin, glucagon or GH. An antibody against SSTR3 labels primary cilia in insulin cells (a) and pituitary GH cells (b). Some, though not all, glucagon cells appear to possess SSTR3-immunoreactive cilia (c). Adrenal chromaffin cells possess short primary cilia immunoreactive for type III adenylyl cyclase (but not for SSTR3) (d). Electron microscopically, gold particles showing the existence of SSTR3 gather selectively in the plasma membrane of primary cilia of a B cell (e) and a GH cell (f). An arrow indicates centrioles located at the base of a primary cilium in the B cell (e). N: nucleus Bars 20 μm (a–d), 1 μm (e, f)"
Primary cilia in pancreatic islets

Primary cilia in pancreatic islets of other animal species and MIM6 cells

To examine the existence of SSTR3-expressing cilia in other animal species, the pancreas of the rat, hamster, and guinea pig was stained using the same antibody against the C-terminal amino acids of SSTR3 which are common to the mouse and rat molecules. At first, we stained the brain of these animals for checking the crossreactivity of the anti-

solitarily, or in small groups. In the latter case, two to four endocrine cells projected their own cilium into a narrow space that was surrounded by their small facets sparsely covered with microvilli and microplicae. Such intercellular spaces were separated from one another instead of forming an interconnected system of canalicule.

Fig. 2 Scanning electron micrographs of primary cilia in pancreatic islet cells (a–d) and pituitary endocrine cells of mice (e, f). The extracellular matrix has been thoroughly removed by alkaline maceration to expose the surfaces of cells. Arrows indicate exposed primary cilia. One long primary cilium projects from each islet cell, pursues a tortuous course, and frequently enters an intercellular canalicule endowed with microvilli (a–c). Occasional islet cells possess double primary cilia projecting from a deep invagination of the cell membrane (d). Primary cilia of pituitary endocrine cells are shorter and less flexuous than those of islet cells (e, f). Bars 5 μm (a, e), 1 μm (b–d, f)
Hypothalamic somatostatin is released at the median eminence, conveyed by the hypophysial portal system, and regulates GH secretion in the adenohypophysis. In the pancreas, somatostatin is secreted from D cells and exerts inhibitory actions against islet hormone secretion, possibly in a paracrine manner. It is worth noting that the localization of SSTR3 in the primary cilia of endocrine cells is restricted to the two main target cells of somatostatin in the body. Among other endocrine cells, somatostain is secreted by thyroid parafollicular cells (13), adrenal chromaffin cells (15), gut endocrine cells (12), and dispersed endocrine cells in the respiratory (8) and urogenital tracts (29). However, no adjacent endocrine and exocrine cells were provided with the SSTR3-expressing primary cilia in endocrine systems other than the adenohypophysis and pancreas, though the primary cilia immunoreactive for ACIII

**Fig. 3** Primary cilia in the pancreatic islets of other animal species and a murine insulin cell line. Primary cilia and insulin cells are labeled red and green, respectively (a–c). SSTR3 antibody stains many but short primary cilia on insulin cells of the hamster (a). In Fig. 3b and 3c, sections from the guinea pig and rat pancreas are double-stained for tubulin (red) and insulin (green). The tubulin-immunoreactive cilia are largely localized on insulin cells in the guinea pig (b), while in the rat (c) they are restricted to the periphery of an islet, namely the glucagon cell region (asterisks). Arrowheads indicate nerve fibers immunoreactive for tubulin (c). A mouse insulin cell line (MIN6) possesses the primary cilia immunoreactive for tubulin (d). In Fig. 3d, nuclei are stained green with a nucleic acids-sensitive dye. Bars 20 μm
were found in a variety of cells, such as the adrenal chromaffin cells and thyroid parafollicular cells.

There are five subtypes of somatostatin receptors (SSTRs) that have been identified so far, each having different sequence homologies, distribution patterns, and affinities to antagonists and agonists. Expressions of all SSTR subtypes can be recognized in both the pancreatic islets (33) and adenohypophysis (40). Immunohistochemical studies for SSTRs—
including SSTR3—localized the immunoreactivities in the whole cell bodies of endocrine cells in pancreatic islets (16, 18, 25) and adenohypophysis (17, 19), except for one study showing the SSTR2A immunoreactivity in the plasma membrane of islet cells in the rat and human pancreas (14). In contrast, the present study showed the selective localization of SSTR3 to the primary cilia of both insulin cells and GH cells, with a heavy immunolabeling for SSTR3 along the plasma membrane of the primary cilia, while the plasma membrane of cell bodies lacked any immunoreactivity for SSTR3. Recent studies have identified several proteins, such as Bardet-Biedl syndrome (BBS) proteins, which mediate vesicular transport to the primary cilia (20). BBS proteins in neurons of the mouse brain are required for functional development of the primary cilia; the genetic deletion of BBS proteins failed to gather SSTR3 and MCHR1 into the primary cilia of neurons, while another membrane-bound ciliary protein, ACIII, was normally localized to the primary cilia in the knockout mice (3). BBS is characterized by obesity as well as renal anomalies (35), and MCHR1 is involved in the regulation of feeding and energy balance (24). Therefore, an interesting inquiry to be addressed in future studies is whether BBS patients and BBS protein-knockout mice normally express SSTR3 on the primary cilia of insulin cells.

Cells of pancreatic ducts and islets possess the primary cilia, but acinar cells of the exocrine pancreas apparently lack them (1, 5, 22, 39). An electron microscopic study by Yamamoto and Kataoka (39) has reported in detail the ultrastructure of primary cilia in islet cells of some animal species. The primary cilia were projected from the Golgi region to intercellular spaces and their basal part was usually surrounded by deep invaginations of the plasma membrane. In an immunostaining of mouse pancreatic islets with an antibody against acetylated tubulin, the primary cilia were mostly localized in the lateral surface of B cells away from capillaries (9), in agreement with the present SEM study. It is generally believed that somatostatin released from pancreatic D cells is conveyed by local blood circulation or via intercellular spaces to act upon other islet cells in a paracrine manner. Some morphologists have recognized the development of a special intercellular canalicular system apart from the perivascular spaces within pancreatic islets (7, 39). As shown by the present SEM observation, the primary cilia were usually located in these intercellular canalicules, i.e., microvillous channels among islet cells, but did not face the perivascular spaces. Thus, the primary cilia on insulin cells are well situated to detect directly somatostatin released from D cells and undiluted by blood. In contrast, pituitary GH cells must be regulated by somatostatin which is conveyed by the pituitary portal system. This fact may affect the length of primary cilia: short primary cilia on GH cells is enough for sensing.

The use of SSTR subtype-selective drugs and gene-knockout studies suggest that the inhibition of insulin secretion by somatostatin is mediated predominantly by SSTR1 and SSTR5 in rodents (32, 33, 36). An in vitro study of human pancreatic islets also reported that somatostatin inhibited both insulin and glucagon release through SSTR1, SSTR2, and SSTR5 (30). Thus, it is likely that SSTR3 on the primary cilia may regulate insulin secretion and other B cell functions via a unique mechanism which differs from the reception of somatostatin on the cell body of B cells. This idea accords with a finding that a genetic deletion of the primary cilia with inactivated Kif3a in the whole pancreas induced severe dilation of the duct system in the exocrine pancreas but did not affect basic islet formation (arrangement of A and B cells) and functions (glucose tolerance tests) (5). Since a cell line of murine insulin cells (MIN6) consistently possessed the primary cilia expressing SSTR3, the precise function of the primary cilia in B cells may be verified in future studies by cell-biological methods including a controlled formation of cilia.

There was a species difference in the existence of primary cilia that must be linked to species difference in the function of somatostatin/SSTRs. The lack of SSTR3-immunoreactive primary cilia in the pancreatic islets of guinea pigs may be due to non-crossreactivity of the antibody raised against the mouse/rat SSTR3. However, this dose not hold true of the rat, because the antibody could detect SSTR3 expressing primary cilia in various regions of the rat brain (11 and the present study). Instead, insulin cells in the rat pancreas did not possess the primary cilium itself, which should be detectable by immunostaining for tubulin. It is considered at present that the rat is a unique species in regard to the pres-
ence of primary cilia on B cells.

In conclusion, the primary cilium is a fundamental apparatus of endocrine cells and SSTR3-expressing cilia on insulin and GH cells serve for sensing the inhibitory signal, somatostatin, in the mouse.

REFERENCES