Subcellular redistribution of AQP5 by vasoactive intestinal polypeptide in the Brunner’s gland of the rat duodenum

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Parvin, Most. Nahid, Shingo Kurabuchi, Kwartarini Murdiastuti, Chenjuan Yao, Chisato Kosugi-Tanaka, Tetsuya Akamatsu, Norio Kanamori, and Kazuo Hosoi. Subcellular redistribution of AQP5 by vasoactive intestinal polypeptide (VIP) in vitro significantly increased the amount of AQP5 in the apical membrane fraction in a dose- and time-dependent manner with the amount reaching a plateau at 100 nM VIP and becoming near maximal after a 30-s incubation. Protein kinase inhibitors, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7, 50 μM), and N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89; PKA-specific, 1 μM) blocked this increase, but PKC-specific inhibitor calphostin C did not, implying the involvement of PKA but not PKC in this cellular event. Intravenous injection with VIP (40 μg/kg body wt) provoked dilation of the lumen of the Brunner’s gland at 2 and 7 min and increased the staining intensity of AQP5 in the apical and lateral membranes. AQP1 (both nonglycosylated and glycosylated forms) was also found to localize in the apical and basolateral membranes of cells of Brunner’s gland. VIP, however, did not provoke any significant change in the AQP1 level in the apical membrane, as judged from the results of the above in vitro and in vivo experiments. These results suggest that VIP induced the exocytosis of granule contents and simultaneously caused translocation of AQP5 but not of AQP1 to the apical membrane in Brunner’s gland cells.

COMPLEMENTARY DNAs of members of the large family of aquaporins (AQPs) have been cloned recently from a variety of mammalian tissues (29). Proteins of this AQP family selectively transport water and other components, such as urea and glycerol (29). Recent investigations have uncovered the molecular basis of the water transport across the cell membrane; e.g., the AQP5 molecule, one of the exocrine-type AQPs, was demonstrated to be increased in concentration at the apical membrane of the salivary gland in response to the stimulation of muscarinic receptors or to an increase in the intracellular level of calcium ions (10, 11, 28). AQP1 in rat bile duct cells and AQP2 in rat renal collecting duct cells were reported to traffic from an intracellular location to the apical membrane in response to secretin and vasopressin, respectively (16, 21, 30).

A recent study of ours (22) demonstrated AQP5 to be present in the duodenum, where it was localized in the apical and lateral membranes of the secretory cells of Brunner’s gland. In the duodenum, epithelial cells and cells of Brunner’s gland secrete bicarbonate and mucin, respectively, into the duodenal lumen, which secretion is thought to play an important role in mucus protection against gastric acid. Mucin and water are known to combine together to create a viscoelastic gel that becomes infiltrated with bicarbonate, thus forming a physicochemical barrier to hydrogen ions and proteolytic enzymes and protecting the mucosal lumen from ulceration (8, 27). In addition to such mucosal protection, the Brunner’s gland also appears to play important roles in proliferation and healing, mucus defense, and digestive control, because several growth factors, including epidermal growth factor (23), immunoglobulin (6), lypoxygenase (6), and trypsin inhibitor (2) are known to be present in this gland. The water secretion is important there because such secretion would contribute to the flow of the above-mentioned factors as well as to that of bicarbonate and mucin. Brunner’s gland is innervated by cholinergic vagal fibers (19) and by a network of vasoactive intestinal polypeptide (VIP)-immunoreactive nerve fibers around its acini (12). Actually, Kirkegaard et al. (12) reported that infusion of VIP in vivo significantly increased the flow rate as well as bicarbonate and protein output from the Brunner’s gland in rats. However, little is known about the effect of VIP and other intestinal hormones on the regulation of water secretion via AQPs in the gastrointestinal tract.

In the present study, we examined which AQP including AQP5 might be expressed in the Brunner’s gland and how these AQPs are regulated by gastrointestinal hormones. Our special goal in the present study was to determine whether or not the trafficking and/or translocation of AQP5 in the Brunner’s gland could be provoked by hormonal stimulation.

MATERIALS AND METHODS

Reagents. An enhanced chemiluminescence detection kit was from Amersham Pharmacia Biotech (Little Chalfont, UK), whereas Protran nitrocellulose transfer membranes came from Schleicher & Schuell (Dassel, Germany). Complete EDTA-free protease inhibitor cocktail tablets were from Boehringer-Mannheim (Mannheim, Germany). RX X-ray film was procured from Fuji Film (Kanagawa, Japan), and silane-coated slide glasses were from Matsunami (Tokyo, Japan).

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Vectastain Elite ABC kit including avidin-biotin peroxidase complex was obtained from Vector Laboratories (Burlingame, CA); and Entellan was from Merck (Rahway, NJ). LR white was procured from London Resin (Basingstoke, UK). Streptavidin conjugated with 10 nm of gold particles was from Aurion (Wageningen, The Netherlands); and peptide N glyclosides F (PNGaseF) was from New England Biolabs (Beverly, MA). PMSF and aprotinin were purchased from Wako Pure Chemicals (Osaka, Japan); and pepstatin A, leupeptin, and VIP were from Peptide Institute (Osaka, Japan). 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) and calphostin C came from Sigma-Aldrich (St. Louis, MO). N-[2-(p-bromocinnamylamino)ethyl]-5-isouquinolinesulfonamide (H-89) was from Seikagaku (Tokyo, Japan). PepTag nonradioactive PKC assay kit supplemented with purified rat brain PKC was obtained from Promega (Madison WI). The AQP5 and AQP1 COOH-terminal peptides were synthesized by Mimotopes (Victoria, Australia). The protein assay kit was obtained from Bio-Rad (Hercules, CA).

Antibodies against AQP5 and AQP1. Antibodies against AQP5 antiserum was previously prepared in our laboratory, and its specificity for AQP5 protein was confirmed (22, 28). The anti-AQP1 antiserum was also prepared in our laboratory; it was raised in rabbits by injecting them weekly with keyhole limpet hemocyanin and a synthetic peptide, NH$_2$-CEEYLDLDADDINSRVEMKPK-COOH, that corresponds to the COOH-terminal sequence of rat AQP1. For both anti-AQP1 and anti-AQP5 antibodies specificity was tested, and we confirmed that they did not cross-react with each other (see RESULTS).

Animals. Eight-week-old male Sprague-Dawley rats, purchased from Japan SLC (Shizuoka, Japan), were used throughout the present study. Animals were kept in our animal facility and given water and laboratory chow ad libitum. Illumination of the animal house was periodically controlled so that it was lighted during the period of 0600–1800. The protocol applied for the present animal experiment was approved by the Institutional Review Board (IRB) of the Animal Committee of the University of Tokushima.

Preparation of the total membrane fraction. The total membrane fraction was prepared from the duodenum of male rats as described previously (22). Briefly, the tissue homogenate was prepared in ice-cold homogenization buffer (referred to as buffer A, comprising 5 mM HEPES buffer, pH 7.5, 50 mM mannitol, 0.25 mM MgCl$_2$, 1 mM PMSF, 1 µg/ml aprotinin, 2 µg/ml pepstatin A, 2 µg/ml leupeptin, and 1 tablet/25 ml of Complete EDTA-free protease inhibitor cocktail) by use of a glass mortar fitted with a Teflon pestle (Wheaton Science Products, Millville, NJ). The homogenate was centrifuged at 800 g for 5 min, and the resulting supernatant was ultracentrifuged at 105,000 g for 1 h. The pellet obtained served as the total membrane fraction.

Preparation of rat duodenal slices, incubation with VIP, and preparation of apical membrane fractions. The proximal part of the duodenal tissue (~1.5 cm in length starting immediately after stomach) was used for experiments, because this area contains many Brunner’s glands and the AQP5-positive reaction was restricted to these glands. The distal part of the duodenum was also examined, but no reaction for anti-AQP5 was seen at all, except in the Brunner’s glands. The distal part of the duodenum was also examined, but no reaction for anti-AQP5 was seen at all, except in the Brunner’s glands, in which the number was very sparse.

The duodenum from 24 rats was cut into slices of 1.5-mm thickness by using a McIlwain tissue chopper (Mickle Laboratory Engineering, Guildford, England), and the slices were pooled and then divided into 12 parts; and each part (~300 mg) was thereafter transferred into a flask containing 10 ml of Krebs-Ringer-Tris solution (in mM: 120 NaCl, 4.8 KCl, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 2.0 CaCl$_2$, 16 Tris·HCl, pH 7.4, and 5 glucose) that had been bubbled with O$_2$ gas at 0°C for >20 min (10). The slice suspension was incubated at 37°C in the presence or absence of various concentrations of VIP for the desired times. Alternatively, it was incubated for 2 min with or without VIP (100 nM) in the presence or absence of protein kinase inhibitors, H-7 (50 µM), H-89 (1 µM), or calphostin C (1 µM).

After incubation, the tissue slices were washed with ice-cold incubation buffer and homogenized at 0°C in 20 vol of buffer A. For preparation of the apical membrane fraction, the homogenate was centrifuged at 9,750 g for 10 min to obtain the supernatant, which was next centrifuged at 35,000 g or 30 min. The resulting pellet was resuspended in buffer A and treated with 10 mM MgCl$_2$ (final concentration) to precipitate the basolateral membranes, which were removed by centrifugation at 3,000 g for 15 min, according to the procedure described earlier (15, 22). The supernatant was next centrifuged at 35,000 g for 30 min to obtain the apical membrane fraction (10, 15, 22). The purity of the apical membranes thus obtained had been confirmed previously by measuring marker enzymes (15). The protein concentration of samples was measured with the Bio-Rad protein assay kit, and 10 or 5 µg of each sample was used for Western blotting for AQP analysis, as described previously (22). Western blot analysis. Protein samples (10 or 5 µg each) and standard molecular weight protein markers were electrophoretically resolved in 12% polyacrylamide slab gels containing sodium dodecyl sulfate and then electroblotted onto nitrocellulose filters. The blotted filters were blocked with 1% skim milk, and probed with either anti-AQP5 antiserum (1:500 dilution) or anti-AQP1 antiserum (1:1000 dilution) at 4°C overnight. The filter was incubated with peroxidase-labeled anti-rabbit IgG at room temperature, followed by a luminal-based, enhanced chemiluminescence procedure. The filter thus treated was exposed to X-ray film. For a control experiment to verify the specificity of the band, anti-AQP5 and anti-AQP1 antisera were preabsorbed with AQP5 and AQP1 COOH-terminal peptides, respectively (final concentration: 40 µg peptide/ml of the diluted antiserum), and reacted with the blotted filter in place of the nonpreabsorbed antiserum.

Injection of VIP and immunohistochemistry. For light microscopy, rats anesthetized under ether were injected with VIP (40 µg/kg body wt) intravenously, and the duodenal tissue was removed after 2 or 7 min. For the 7-min experiment, VIP was injected two times to maintain a high blood level of VIP, with the same amount as above for each injection; the second injection was given at 3 min after the first injection. Fresh tissue specimens were immersed in periodate-lysine-parafomaldehyde (PLP) fixative (4% paraformaldehyde, 0.075 M lysine solution, pH 7.4, 0.037 M phosphate buffer, pH 7.4, and 2.14 mg/ml sodium periodide; Ref. 18). They were then irradiated with a microwave at 0°C three times, each time for 20 s. Samples were kept in the same fixative at 4°C overnight. After having been washed and dehydrated by passage through a graded series of ethanol solutions, they were embedded in Paraplast.

Sections of 5 µm thickness were cut and mounted on silane-coated slides. The sections were deparaffinized with xylene, washed with distilled water, and incubated for 30 min in a solution of 0.3% H$_2$O$_2$ in methanol to inactivate the endogenous peroxidase activity. Sections were washed in distilled water, then in PBS (0.01 M sodium phosphate buffer and 0.14 M NaCl, pH 7.5), and incubated at room temperature overnight with anti-AQP5 or anti-AQP1 antisera diluted 1:3,000 for immunostaining. They were next immunostained with the avidin-biotin-peroxidase complexes method according to the manufacturer’s protocol. The sections were next gently stained with Mayer’s hematoxylin, dehydrated by passage through a graded ethanol series, and mounted in Entellan. Samples were examined under a normal microscope (Nikon, Japan) equipped with a Nikon digital camera (model DXM1200), and the digital images were taken by use of Color Stacke software (Polaroid).

For electron microscopy, normal rats were anesthetized, and the PLP fixative was circulated throughout the body from the ventricle of the heart for 30 min. The duodenum was dissected out and immersed in the same fixative for further fixation. These samples were dehydrated with a graded ethanol series and then embedded in LR white. Ultrathin sections were cut and mounted on gold grids, etched with 3% H$_2$O$_2$ for 5 min, rinsed with distilled water, and subjected to
immunostaining for AQP5. In this procedure, the samples were first incubated with 20% normal goat serum (Vectorstain Elite ABC kit) for 2 h and then directly transferred into a drop of anti-AQP5 antiserum (1:200). In some cases, samples were reacted with peptide-preabsorbed anti-AQP5 antiserum. All samples were incubated at 4°C overnight. Sections were rinsed 3 times in PBS, and incubated with biotinylated goat anti-rabbit IgG (Vectorstain Elite ABC kit) for 1 h, followed by washing. Finally, they were incubated for 2 h with 30-times-diluted streptavidin conjugated to 10-nm gold particles. The immunolabeled sections were stained with uranyl acetate and viewed with a JEOL transmission electron microscope (model JEM-2000EXII) at 80-kV accelerating voltage.

Statistics. For statistical analysis, Williams’ test was applied for the time course and dose-response studies, because a monotone increase was observed for the former experiments and dose dependency was in the latter. Dunnett’s test was used for the protein kinase inhibitor study.

RESULTS

Detection of AQP5 and AQP1 proteins in the duodenum. Total membrane fractions from the submandibular gland, duodenum, kidney, and peptide N glycosidase F-treated duodenum, and kidney were analyzed by using the anti-AQP5 and anti-AQP1 antisera. The anti-AQP5 antiserum detected a 27-kDa AQP5 protein in the submandibular gland and duodenum (Fig. 1A, left), which did not appear when the antiserum was preabsorbed with the peptide (Fig. 1A, right), thus indicating the specificity of anti-AQP5 antiserum. Also, this result confirmed our previous finding that the rat duodenum expresses AQP5 (22). The anti-AQP1 antiserum detected both 28-kDa and 35- to 50-kDa AQP1 bands in the kidney and duodenum (Fig. 1B, left), which sizes were the same as those for AQP1 reported previously (14). These bands disappeared when anti-AQP1 antiserum was preabsorbed with AQP1 COOH-terminal peptide (Fig. 1B, right), indicating the specificity of the reaction with anti-AQP1 antiserum. The anti-AQP1 antiserum also detected an extra 97-kDa band in the duodenum, which also disappeared by preabsorption; however, the nature of this protein is not clear at present. When the duodenal sample was incubated with PNGaseF glycosidase enzyme, these 35- to 50-kDa bands reactive with AQP1 antiserum completely disappeared, and the intensity of the 28-kDa band, corresponding to the nonglycosylated form of AQP1, increased (Fig. 1B, left, lane 3). This result suggests that these broad bands were glycosylated forms of AQP1 and that both nonglycosylated and glycosylated forms of AQP1 were present in the duodenum.

Cross-reactivity of the two antibodies used was proved by Western blotting and immunohistochemistry experiments; i.e., Western blotting using duodenum samples showed that anti-AQP5 antibody did not cross-react with AQP1 protein, because no positive reaction was found at the position of ~35–50 kDa (Fig. 1A, to which glycosylated forms of AQP1 move. Similarly, anti-AQP1 antibody recognized exactly one band at a position around 28 kDa, and no extra band was observed around this position (Fig. 1B). On the other hand, when frozen sections of the rat stomach were immunostained by using the anti-AQP1 and anti-AQP5 antibodies, the former antibody specifically stained the blood vessels and the latter, the pyloric gland; i.e., the blood vessels did not immunoreact with anti-AQP5, nor did the pyloric gland with anti-AQP1 antibody (unpublished observations). All of these biochemical and immunohistochemical data apparently indicate no cross-reaction of anti-AQP5 with AQP1 antigen or of anti-AQP1 with AQP5 antigen.

VIP-induced increase in AQP5 levels in the duodenal apical membrane fraction. In an in vitro experiment, slices of duodenum were incubated for 2 min in the presence and absence of various concentrations of VIP, and the amounts of AQP5 and AQP1 in the apical membrane fraction were determined by Western blot analysis. There appeared a less intense but specific band immediately above the 27-kDa AQP5 band in the apical membrane fraction (Fig. 2A) but not in the duodenum total membrane fraction (Fig. 1A). Although the nature of this protein was not clear, we assumed that this protein is a derivative of AQP5 because of its close molecular weight to AQP5 and apparent specificity for reaction with AQP5 antibody. We therefore merged this band with the 27-kDa AQP5 band for assessment of Western blot data. The band intensity of these data was quantified by NIH Image software. The VIP-induced increase in the amount of AQP5 in the apical membrane fraction was concentration dependent; it appeared to increase at 1 nM, became maximal at 100 nM, and remained high at 1 μM (Fig. 2, A and C). There was no change elicited by VIP in the amount of the nonglycosylated or glycosylated form of AQP1 in the apical membrane fraction (Fig. 2, B and C).

Because 100 nM VIP was the least concentration that induced maximal level of AQP5 in the apical membrane fraction in this experiment, we selected this concentration of VIP for the time-course study. On incubation with VIP, the amount of AQP5 in the apical membrane fraction increased as early as 30 s, reached its maximal level at 1 min, and remained high until 7 min (Fig. 3, A and C). VIP scarcely affected the amount of AQP1 in the apical membrane fraction at 7 min or at 30 s, 1, 2, and 4 min (Fig. 3, B and C). These data thus suggest that the content of AQP5, but not that of AQP1, in the duodenal apical membrane was altered in response to VIP, one of the gastrointestinal hormones.

In the next experiment, we examined the nature of the signaling pathway in the VIP-provoked AQP5 redistribution in...
the duodenum. First, we incubated duodenal tissue slices with VIP in combination with H-7, a protein kinase inhibitor. Incubation with VIP at a concentration of 100 nM for 2 min significantly and consistently increased the AQP5 level in the apical membrane fraction (Fig. 4B). This increase was completely inhibited by concomitant incubation with 50 μM H-7 (Fig. 4B). The effect of the protein kinase inhibitor itself on the level of AQP5 in the apical membrane fraction was minimal or small, with no statistical difference compared with the control value. The AQP1 level was affected by neither VIP nor H-7 nor by the combination of these two substances (Fig. 4B).

These data suggest the involvement of the protein kinase system in the redistribution of AQP5. Effects of protein kinase inhibitors were examined further to specify the type of the kinase involved. H-89, a selective inhibitor for PKA (4), inhibited the VIP-induced AQP5 increase in the apical membrane fraction at 1 μM concentration (Fig. 5), suggesting the involvement of PKA for AQP5 redistribution. Calphostin C (1 μM, Ref. 3), a PKC-selective inhibitor, did not inhibit such increase (Fig. 5), although this inhibitor, at the same concentration, was verified to inhibit the phosphorylation of fluorescence-tagged peptide (PLSRTL5VAAK) acting on purified rat brain PKC by > 98% (data not shown). This result implies that PKC is not involved in this cellular event.

**Immunohistochemical localization of AQP5 and AQP1, and redistribution of AQP5 by VIP.** From in vitro analysis using Western blot analysis, we found that incubation of duodenal tissue slices with VIP provoked an increase in the AQP5 level in the apical membrane fractions. For the purpose of confirming these biochemical data, we examined the effects of VIP on the AQP5 level in the Brunner’s gland in vivo. Before the VIP experiment, we first examined the localization of AQP5 in the secretory cells of the gland by using duodenal sections from normal rats. The tissue was fixed with PLP solution, and the AQP5 localization was examined by both light and electron microscopic immunohistochemistry. The light microscopic immunohistochemistry showed AQP5 to be localized exclusively in the Brunner’s gland (Fig. 6A). High-power magnification of the gland revealed a positive reaction for AQP5 inside the cells as well as in the apical and lateral membranes of these secretory cells (Fig. 6B). Such locations of AQP5 were confirmed by electron microscopic immunohistochemistry by focusing at the luminal area of the Brunner’s gland in which the secretory granules were localized (Fig. 6C). Although the materials fixed with PLP solution and embedded in LR white showed poor preservation of organelle membranes as well as cell membranes in general, an enlarged picture of one such area (Fig. 6D) clearly showed the presence of a number of gold particles at the peripheral edge around secretory granules as well as on the apical and lateral membranes, indicating the localization of AQP5 in these structures.

At 2 min after intravenous injection with VIP, the AQP5 level was increased at the apical and lateral membranes and decreased inside of the cells, and a small degree of luminal...
dilation also had occurred (Fig. 6E). At 7 min after VIP injection, the AQP5 staining tended to disappear from inside of the cells, whereas the staining at the apical membrane was strongly increased compared with that in the control gland or in the gland at 2 min after the VIP injection (Fig. 6F). Large luminal dilation and irregularly shaped apical membranes were observed (Fig. 6F), indicating the progress of exocytosis in this gland. These results suggest that AQP5 translocation had taken place from the granule membrane to the apical membrane in response to VIP.

Immunohistochemical localization using the antiserum for AQP1 COOH-terminal peptide also demonstrated apparent staining in the duodenum. A strong positive reaction was seen in the Brunner’s gland and also in the blood vessels of the duodenum (Fig. 7A). Under high-power magnification, AQP1 was found to be localized in the apical and basolateral cell membranes of the glandular cells, and there was only faint staining inside these cells (Fig. 7B). The positive staining seen in the Brunner’s gland completely disappeared when the sections were stained with the same concentration of antiserum preabsorbed with the peptide (data not shown), clearly indicating that the positive staining seen in Fig. 7A was due to a specific reaction. At 2 or 7 min after the injection of VIP, the AQP1 staining at the apical membrane did not increase appreciably, although the changes in the inside of the cells were not clear (Fig. 7, C and D). This result is different from that for AQP5 staining. These data therefore suggest that AQP1 was also localized in the Brunner’s gland but that VIP had little effect in causing its redistribution.

DISCUSSION

In the present study, the rat duodenum was shown to express both AQP5 and AQP1 proteins; the former molecular weight was confirmed to be 27 kDa and the latter 28 kDa being accompanied with 35- to 50-kDa glycosylated forms (Fig. 1, A and B) as reported previously (14, 22, 25, 26). Kidney AQP2, salivary gland AQP5, and bile duct AQP1 are suggested to traffic toward the apical membrane in response to their respective ligands (10, 11, 16, 21, 30). Brunner’s gland responds to various intestinal hormones, but we do not know how AQP5 and AQP1 in it are regulated by hormones. Because VIP is involved in water secretion in the gastrointestinal tissue, we decided to determine how this hormone regulates the translocation of AQPs in the duodenal Brunner’s gland.

From the dose-response curve prepared after a 2 min-incubation (Fig. 2), VIP, at a concentration as low as 1 nM, was found to be effective in increasing the AQP5 level in the apical membrane. This concentration is quite low compared with that reported previously (300 nM), which caused dilation of the lumen of the isolated Brunner’s gland in vitro (20). The fact implies that the present experimental condition was not greatly apart from the physiological condition in terms of VIP response. Also, the response of the Brunner’s gland to elevate the
AQP5 level in the apical membrane fraction was very fast; incubation for as short a time as 30 s caused an increase in the AQP5 level to 85% of the maximum level. All these data imply that changes in the AQP5 level in the apical membrane fraction in these experiments are physiologically significant and that VIP induces the translocation of AQP5 in the Brunner’s gland.

In this study, we fixed the tissue using PLP fixative and embedded it in Paraplast. The immunostaining of this sample was essentially the same as that reported previously (Fig. 6 and Ref. 22), except that the present procedure allowed us to detect positive staining at the surface of secretory granules (Fig. 6B), suggesting the localization of AQP5 in the granule membrane. This was also suggested by electron microscopic immunohistochemistry (Fig. 6, C and D). According to our knowledge, this is the first finding of immunohistochemically detectable AQP5 in the membranes of secretory granules present in the cytoplasm.

AQP5 level in the apical membrane fraction was very fast; incubation for as short a time as 30 s caused an increase in the AQP5 level to ~85% of the maximum level. All these data imply that changes in the AQP5 level in the apical membrane fraction in these experiments are physiologically significant and that VIP induces the translocation of AQP5 in the Brunner’s gland.

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Trafficking of AQP5 is thought to be involved in the regulation of water transport via this channel protein; e.g., in the parotid gland, it was reported that AQP5 trafficked from the intracellular vesicles to the apical membrane in vitro in response to stimulation of muscarinic receptors (10). In the same gland, immunostained AQP5 was scattered as clusters in the submembranous area of the acinar cells when the rat was injected with isoproterenol (17). In cells transfected with AQP5 complementary DNA, the AQP5 protein trafficked to the plasma membrane on an increase in intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) (28). All of these reports imply redistribution of AQP5 from inside the cells to the apical membrane on stimulation with secretagogues.

In the duodenum, water is secreted in such a way that its transport is affected by gastrointestinal hormones (such as gastrin, VIP, and others), neurotransmitters, and histamine (20). In the present study, we examined the effect of VIP on the AQP5 level in the duodenal apical membrane fraction and found that VIP immediately (30 s) increased the AQP5 content.
in this fraction (Fig. 3A). This result was supported by our in vivo experiment (Fig. 6, A–E), i.e., VIP injection induced dilation of the apical membrane of Brunner’s gland and strong AQP5 staining appeared at the apical plasma membrane. Although at 7 min after the VIP injection the AQP5 level in the apical membrane remained high, that in the cytoplasm was strongly decreased (Fig. 6F). The appearance of significant luminal dilation by VIP was reported previously (20), and the observed irregularly shaped apical membranes were considered to indicate the exocytosis of secretory granules. Therefore, the strong immunostaining seen at the apical membrane in the Brunner’s gland of rats injected with VIP (Fig. 6, E and F) suggests the translocation of AQP5 from the granule membrane to the apical membrane on exocytosis provoked by this hormone. However, it is not clear how the intensity of AQP5 staining at the apical membrane was increased by VIP stimulation. One possible explanation would be that AQP5 was masked when this channel protein was localized at the granule membrane, and was then unmasked after the granule membrane became the apical membrane. An alternative possibility would be that AQP5, after exocytosis, was condensed at the apical membrane by a mechanism not understood at present. Similar results regarding AQP1 translocation in exocrine pancreatic acinar cells were reported by Cho et al. (5); i.e., on the basis of immunostaining these authors concluded that AQP1, having been present in the membrane of zymogen granules, became localized in the apical area of acini on stimulation with carbamylcholine.

Moore et al. (20) separated Brunner’s glands from epithelial cells and stimulated the isolated glands with VIP, and they found that the gland secretion was affected by this hormone, indicating the presence of VIP receptors in Brunner’s gland. In support of their finding, we observed strong expression of mRNAs for VIP receptor-1 and VIP receptor-2 as detected by RT-PCR using rat duodenal RNA (data not shown). VIP was reported to stimulate protein secretion via PKA and an increase in [Ca^{2+}]_{i} in the lacrimal gland and in other tissues (9). Interestingly, there is a PKA target motif in the AQP5 amino acid sequence.

Fig. 6. Immunohistochemical localization of AQP5 in the duodenum of a rat before and after injection of VIP. The duodenum of control rats (A–D) and of rats at 2 (E) and 7 min (F) after VIP injection were subjected to light and electron microscopic immunohistochemistry using anti-AQP5 antiserum. Photographs A, B, E, and F show the results of light microscopic immunostaining. Their experimental conditions were exactly the same for each sample. Photographs labeled C and D are electron microscopic images of immunostained Brunner’s gland. A: low-power magnification of a duodenal section in which a Brunner’s gland is specifically stained, as indicated by B. B: high-power magnification of A showing moderate staining at the apical and lateral membranes, as indicated by the arrows. The cell interior is also positively stained. C: an electron microscopic photograph of an apical area of cells of a Brunner’s gland, showing deposition of a number of gold particles indicating the presence of AQP5. D: an enlargement of the rectangular area of C; the granule membrane is positively stained, as indicated by arrows; and labeling inside the cells is reduced. E: high-power magnification of a duodenal section from a rat killed at 2 min after having been injected with VIP. Some of the apical lumens appear dilated. Extensive labeling is observed at the apical and lateral membranes, as indicated by arrows; and labeling inside the cells is reduced. F: high-power magnification of a duodenal section from a rat killed 7 min after the first injection of VIP. The apical lumens are extensively dilated and irregular in shape, as indicated by the asterisks. Extensive labeling of the apical membrane is still evident, as indicated by the arrows; and the labeling inside of the cells has almost disappeared. E, epithelial cell; B, Brunner’s gland; M, muscle; L, lumen; AM, apical membrane; LM, lateral membrane; G, granule.
acid sequence but not in the AQP1 sequence (13). The role of this motif in relation to AQP5 function is not clear at present. However, we speculate from these reports that VIP may have induced phosphorylation of AQP5 in the Brunner’s gland. This idea is very plausible, because VIP-induced elevation of AQP5 in the apical membrane was completely blocked not only by a protein kinase inhibitor H-7 but also by H-89, a more selective PKA inhibitor (4) (Figs. 4 and 5). Taken together, the data imply the possibility that VIP may have induced both exocytosis of the secretory granules and phosphorylation at the PKA target motif of AQP5 in the Brunner’s gland. VIP does not appear to induce such exocytosis via PKC activation because a PKC inhibitor, calphostine C, has not inhibited VIP-induced elevation of AQP5 in the apical membrane. It has been proposed that the swelling of the secretory vesicle is important in the process of exocytosis (1, 7). With the use of zymogen granules isolated from exocrine pancreas, Cho et al. (5) suggested that such swelling was mediated by G_{s13} in response to GTP and that AQP1 was present in the membrane of zymogen granules, participating in rapid GTP-induced vesicular water gating and swelling. The possibility that a similar mechanism is involved in exocytosis of AQP5-expressing secretory granule is now being investigated by using isolated rat Brunner’s glands in vitro.

In the present study, we were able to detect the expression of AQP1 protein in the duodenum. Both glycosylated and nonglycosylated forms of AQP1 were detected there by Western blotting. By immunohistochemistry, AQP1 was visualized in the Brunner’s gland as well as in the blood vessels of the duodenum. The localization of AQP1 in the latter tissue is common (24), but its presence in Brunner’s gland was found for the first time in this present study. AQP1 localization in Brunner’s gland was very similar to that of AQP5; but its regulation by intestinal hormones appeared to be different, because the localization of AQP1 was not altered by VIP (Figs. 2–4 and 7). It is not known how Brunner’s gland AQP1 is regulated by hormones or neurotransmitters or how these two AQPs in this gland cooperate with each other. One possible explanation for the origin of this different regulation would be that there may be secretory granules expressing either AQP5 or AQP1. The AQP1-bearing secretory granules may exocytose via a constitutive pathway, whereas those expressing AQP5 may do so via a nonconstitutive pathway (regulated pathway) as implied from the present study. An improved immunohistochemistry that enables the exact intracellular localization of these two AQPs would be indispensable for establishing the validity of such an idea.

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REFERENCES


3. Bruns RF, Miller FD, Merriman RL, Howbert JJ, Heath WF, Koba-


