Desensitization of Noradrenaline-Induced Secretory Response Coincides with Redistribution of Subcellular IP₃ Receptor and Compound Exocytosis in Granular Duct Cell of Rat Submandibular Gland

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ABSTRACT

Double immunohistochemical staining detected inositol 1, 4, 5-trisphosphate receptor type 2 immunoreactivity (IP₃R2 IR) and chromogranin A-like immunoreactivity (CgA-like IR) on the same sections prepared from the isolated and perfused submandibular gland of rat. In the control sections prepared from resting state, an intense IP₃R2 IR was localized at the apical pole of granulated duct cell containing CgA-like IR. Electron-microscopically, the granular cells in the resting state stored numerous membrane-bound granules in the apical cytoplasm. The combined stimulation with 1 μ M noradrenaline and 0.1 μ M acetylcholine caused immediate maximum increase in secretory response (CgA-like IR secretion, flow, and protein secretion). When the sections were prepared from the gland at the peak of secretory response, the apically converged IP₃R2 IR became diffuse and indistinguishable. Ultrastructure of the maximally stimulated cells exhibited extensive compound exocytosis in the apical half of the granular duct cells. The secretory responses to the combined stimulation were significantly inhibited by 100 μ M 2-aminoethyl diphenylborinate, an inhibitory modulator of IP₃-mediated Ca²⁺ release from intracellular stores, and the intense IP₃R2 IR was well preserved in the apical pole of granular duct cells containing CgA-like IR. These results may be compatible with the view that the divergence of apically convergent IP₃R2 with the progress of compound exocytosis may result in decay of [Ca²⁺]_i due to divergence of IP₃R2-sensitive Ca²⁺ pool, and this may provide a novel paradigm for desensitization of G protein-coupled signaling system.

The granular duct cells of the rodent submandibular gland have unique functions: the cells synthesize transforming growth factor α (TGF- α), hepatocyte growth factor, erythroid differentiation factor, endothelin, growth factors including nerve growth factors

(4), and kallikreins (3). We have shown that chromogranin A-like immunoreactivity (CgA-like IR) is stored as well in the granular duct cells, and that highly concentrated CgA-like IR (approximately 1 mM) is secreted by compound exocytosis into saliva in response to 1 μ M noradrenaline (NAd) (6). The secretory response to 3 μ M phenylephrine, an α -adrenergic agonist was hardly inhibited in a Ca²⁺-deficient environment, and was almost completely inhibited by 100 μ M 2-aminoethyl diphenylborinate (2APB) (5), an inhibitory modulator of IP₃-mediated Ca²⁺ release from intracellular store.(2, 5, 7, 8).

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These results are compatible with a view that mobilization of Ca^{2+} from the inositol 1, 4, 5 trisphosphate receptor (IP_3R)-sensitive pool may preferentially be involved in the secretory responses to α -adrenergic agonist. Ca^{2+} mobilization from internal Ca^{2+} pools and increased Ca^{2+} influx across the plasma membrane were confirmed in the granular duct cells of isolated duct preparations (16). The use of streptolysin O-permeabilized ducts showed that adrena-line mobilized entire Ca^{2+} from IP_3 -sensitive pool (17). Ca^{2+} imaging and immunofluorescence of IP_3 receptors were performed on the same duct cells in the preparations isolated from rat submandibular gland, and the results obtained showed that the strongest immunoreactivities for IP_3 receptor type 2 ($\text{IP}_3\text{R2}$ IR) were detected at the apical pole of duct cells, from which the carbachol (CCh)-induced Ca^{2+} oscillation initiated (18). In this study, however, the type of duct cells they showed $\text{IP}_3\text{R2}$ IR and CCh-induced Ca^{2+} oscillation was not identified.

The present study was thus carried out to obtain direct evidence that the duct cells exhibiting the strongest $\text{IP}_3\text{R2}$ IR are the granular duct cells, which contain CgA-like IR, and further that NAd caused secretion of CgA-like IR in correlation to dynamic subcellular redistribution of $\text{IP}_3\text{R2}$ IR and compound exocytosis in the granular duct cells.

MATERIALS AND METHODS

Isolation and perfusion of the submandibular gland. Male Wistar rats of 300–450 g were anaesthetized by intraperitoneal injection of pentobarbital sodium (Nembutal 50–70 mg/kg). The submandibular gland was isolated for perfusion by a method described by Compton *et al.* (1) and by Murakami *et al.* (10). The submandibular artery of the isolated gland was continuously perfused at a constant rate (2 mL min^{-1}) with the perfusing solution, which contained (mM): NaCl 120, KCl 4.3, MgCl_2 1.0, CaCl_2 1.0, Na_2HPO_4 1.0, NaHCO_3 25, glucose 5.0; and was bubbled with 95% O_2 –5% CO_2 . (5, 6). The secreted saliva was collected at an interval of 2 min. The animal was then killed by cutting the carotid arteries and vascular perfusion begun. The gland was isolated and transferred to an organ bath.

Immunoassay of chromogranin A. CgA-like IR was measured by a region-specific enzyme immunoassay (EIA) for rat CgA using anti-rat CgA(359–389) serum developed in our institute (11, 12). Typical standard curves ranged from 13.7 pM to 10 nM of CgA-like IR. In the present EIA, the curves for dilution

of rat saliva paralleled the standard curve for synthetic rat CgA(359–389). The range of the intra- and inter-assay coefficients of variation were from 12.33% to 14.18% and 13.51% to 15.63%, respectively.

Double immunostaining. The isolated submandibular gland was vascularly perfused with the perfusing solution (resting preparation), the solution containing $1 \mu\text{M}$ NAd and $0.1 \mu\text{M}$ acetylcholine (ACh) for 2 min (stimulated preparation), or the solution containing $100 \mu\text{M}$ 2APB for 2 min followed by $1 \mu\text{M}$ NAd and $0.1 \mu\text{M}$ ACh for further 2 min (inhibited preparation); and subsequently with 10% buffered formalin for 3 min, and immersed in the same fixative for an additional 12 h. After being dipped in 30% sucrose dissolved in 0.1 M phosphate buffer (pH 7.3), the tissues were snap-frozen in liquid nitrogen. Cryostat sections, $12 \mu\text{m}$ thick, were incubated with anti-rat CgA(359–389) serum followed by Cy3-labeled donkey anti-rabbit IgG (1 : 200, Jackson ImmunoResearch, West Grove, PA, USA), and then incubated with monoclonal antibody (mAb) against $\text{IP}_3\text{R2}$, KM1083, (14), followed by FITC-labeled donkey anti-mouse IgG (Jackson ImmunoResearch). The immunostained sections were obtained under a confocal laser scanning microscope (Fluoview, Olympus, Tokyo, Japan).

Preparation for electron microscopy. The isolated submandibular gland was vascularly perfused with the perfusing solution (control preparation) or the solution containing $1 \mu\text{M}$ NAd and $0.1 \mu\text{M}$ ACh for 2 min (stimulated preparation); and subsequently with 2.5% glutaraldehyde in 0.1 M phosphate buffer. The fixed tissue were dissected out into small pieces and immersed in the same fixative for an additional 6 h. They were post-fixed for 1.5 h in 1% OsO_4 dissolved in distilled water, dehydrated through a graded series of ethanol, and embedded in resin (Epon 812) according to the conventional method. Ultrathin sections were stained with uranyl acetate and citrate, and examined with a transmission electron microscope (Hitachi H-7000 TEM, Japan).

Statistical analysis. Values were expressed as mean $\pm$ S.E.M. Differences between individual means were then analyzed for statistical significance using Student's *t* test. Differences between means were considered to be significant at the level of $P < 0.05$.

RESULTS

Secretory responses to the combined stimulation with NAd and ACh.

The NAd-induced secretory responses (secretion of CgA-like IR and protein, and salivary flow) were examined in the isolated and perfused submandibular glands of rat. The 1 μ M NAd-induced secretion of highly concentrated CgA-like IR (~ 1 mM) was diluted and facilitated by adding 0.1 μ M ACh, which has been known to cause substantial increase in flow rate with indiscernible secretion of CgA-like IR and protein (6). The first combined stimulation induced significant maximum increases in secretory responses ($P < 0.01$ respectively): the rate of CgA-like IR secretion, the product of the CgA-like IR concentration and the salivary flow rate, increased from the resting zero level to $71.9 \text{ nmol min}^{-1} \text{ g}^{-1}$, the rate of protein secretion from the resting zero

level to $1.3 \text{ mg min}^{-1} \text{ g}^{-1}$, and the flow rate from the resting level ($1.3 \mu\text{L min}^{-1} \text{ g}^{-1}$) to $69.4 \mu\text{L min}^{-1} \text{ g}^{-1}$ (Fig. 1A). The secretory responses to the second stimulation were smaller than the corresponding first responses: the rate of CgA-like IR, the flow rate and the rate of protein secretion were 54%, 33%, and 44% of the corresponding first responses, respectively. The secretory responses to the third stimulation were much smaller than the corresponding first responses (Fig. 1A). The secretory responses to second combined stimulation in the presence of 100 μ M 2APB were significantly ($P < 0.05$ respectively) smaller than the corresponding responses. In the responses to the second combined stimulation, the rate of CgA-like IR secretion was $29.1 \text{ nmol min}^{-1} \text{ g}^{-1}$, the rate of protein secretion was $0.4 \text{ mg min}^{-1} \text{ g}^{-1}$, and the flow rate was $20.6 \mu\text{L min}^{-1} \text{ g}^{-1}$ (Fig. 1A), whereas, in the responses to the second stimulation in the presence of

Fig. 1 The effects of combined stimulation with 1 μ M NAd and 0.1 μ M ACh (A) and inhibition of the effects by 100 μ M 2APB (B) on the rate of CgA-like IR secretion, flow rate, and rate of protein secretion in the isolated and perfused submandibular gland of rat. Each value represents the mean $\pm$ S.E.M. of 2 min measurements from four (A) or the mean of five preparations (B). The transverse open bars indicate the period of perfusion with 1 μ M NAd and 0.1 μ M ACh, and the transverse stippled bar indicate the periods of adding 100 μ M 2APB.

2APB, the rate of CgA-like IR secretion was $2.5 \text{ nmols min}^{-1} \text{ g}^{-1}$, rate of protein secretion was $0.1 \text{ mg min}^{-1} \text{ g}^{-1}$, and the flow rate was $9.2 \mu\text{L min}^{-1} \text{ g}^{-1}$ (Fig. 1B).

Morphological evidence for compound exocytosis coincides with disappearance of apically concentrated IP₃R2 IR in granular duct cells

Double immunohistochemical staining was carried out by use of antibodies against CgA (359–389) (12) and IP₃R2 (14) on the same sections prepared from the isolated and perfused submandibular glands of rats. In resting state, an intense IP₃R2 IR appeared preferentially at the apical poles of several granulated duct cells, in which CgA-like IR distributed throughout the cytoplasm (Fig. 2a). Less intense IP₃R2 IR was observed at the luminal side in other type of duct cells containing weak or no CgA-like IR. Ultrastructural observation of the control preparation showed that the granular duct cells stored numerous membrane-bound granules in the apical cytoplasm (Fig. 2A). The luminal surface of the granular cells was smooth with no discernible evidence of exocytosis.

In marked contrast to the control preparation, the apically concentrated IP₃R2 IR disappeared and distributed diffusely throughout the apical half of the granular duct cells in the fluorescence micrograph of double immunostaining (Fig. 2b), which was prepared in the midst of the secretory response to the combined stimulation with $1 \mu\text{M}$ NAd and $0.1 \mu\text{M}$ ACh for 2 min. The disappearance of IP₃R2 IR may correspond to compound exocytosis in the electron micrograph of stimulated preparation: cytoplasmic granules adjacent to the apical cytoplasmic membrane were open to the lumen and vigorously fused to granules localized in the deeper region of cells, and the apical half of the stimulated granular cells was occupied by round or irregular-shaped empty spaces (Fig. 2B).

In the gland fixed during inhibition by $100 \mu\text{M}$ 2APB of secretory response to the compound stimulation, the compound exocytosis was hardly detected in the granular duct cells (data not shown). In double immunostaining (Fig. 2c), the localization of IP₃R2 IR and CgA-like IR in the inhibited preparation resembles that in the control preparation without stimulation.

DISCUSSION

The principal conclusion derived from the present study is that desensitization, a decay in the respon-

siveness of a signalling system, may involve subcellular redistribution of IP₃ receptor with the progress of compound exocytosis at the apical pole of granular duct cells composing the duct of rat submandibular gland. Support for this conclusion comes from the following results. First, vascular perfusion of isolated rat submandibular gland with a solution containing $1 \mu\text{M}$ NAd and $0.1 \mu\text{M}$ ACh caused rapid uprise of secretory response (secretion of CgA-like IR and protein, and flow) followed by steep decay (Fig. 1A). The decay in the responsiveness during the stimulation is, by definition, desensitization. The progressive reduction in the second and third response (Fig. 1A) may also be due to continuous desensitization. Second, the desensitization coincides with redistribution of subcellular IP₃ R2 IR. Double immunohistochemical staining by use of antibodies against CgA and IP₃R2 on the same sections demonstrated that, in the control preparation, the highly concentrated IP₃R2 IR localized preferentially at the apical pole of granular duct cells exhibiting the strongest CgA IR (Fig. 2a). Electron micrograph of the control preparation showed the granular duct cells contained numerous secretory granules in the apical cytoplasm (Fig. 2A). In the stimulated preparation fixed in the midst of maximum secretory response to the compound stimulation with $1 \mu\text{M}$ NAd and $0.1 \mu\text{M}$ ACh, the apically concentrated IP₃R2 IR disappeared (Fig. 2b), and the apical half of granular duct cell was occupied by compound exocytosis as shown in the electron micrograph (Fig. 2B). These morphological results may indicate that the apically convergent IP₃ R2 IR in the resting state may diverge to the remaining endoplasmic reticulum in concert with the progress of stimulus-induced compound exocytosis at the apical margin of the granular duct cell. The results harmonizes with a view that Ca^{2+} released from IP₃R-sensitive Ca^{2+} pool may play a cardinal role in the stimulus-secretion coupling in the granular cells secreting CgA-like IR (5). The divergence of IP₃R2 IR may result in decay of $[\text{Ca}^{2+}]_i$ due to divergence of IP₃-sensitive Ca^{2+} pool, and, in turn, in decay of secretory response during the continuous first stimulation as well as in the second and third response (Fig. 1A). Third, 2APB, an inhibitory modulator of IP₃-mediated Ca^{2+} release from intracellular Ca^{2+} stores (2, 5, 7, 8), inhibited the secretory response to the combined stimulation (Fig. 1B), and preserved the highly concentrated localization of IP₃R2 IR at the apical pole in the granulated duct cells containing CgA-like IR (Fig. 2c).

The principal conclusion corresponds a novel

paradigm that desensitization may involve subcellular redistribution of IP₃ receptor with the progress of compound exocytosis. The previously established paradigm for desensitization involves (i) receptor

phosphorylation by G protein-coupled receptor kinases, initiated by agonist-induced conformational changes in the receptor or by kinases activated by specific signaling pathways (13), and (ii) subcellular

Fig. 2 Fluorescence micrograph of double immunostaining for IP₃ R2 (green) and CgA (red) on granular duct cells (arrows indicate double-positive cells). The isolated submandibular glands were perfused for 2 min with the standard perfusing solution (a), the solution containing 1 μ M NAd and 0.1 μ M ACh (b), or the solution containing 100 μ M 2APB, 1 μ M NAd and 0.1 μ M ACh (c), and subsequently fixed. Time course of CgA-like IR secretion of the preparation examined by immunostaining is indicated in the graph immediately above the respective fluorescence micrograph. Electron micrographs show the ultrastructure of the granular duct cells of the rat submandibular gland in resting state (A) and secretory response to the combined stimulation (B). The luminal cell membrane is smooth in appearance (A). The combined stimulation with 1 μ M NAd and 0.1 μ M ACh induced irregular empty spaces at the apical cytoplasm, indicating degranulation by compound exocytosis (B). L; lumen.

redistribution of the cognate G proteins from plasma membranes to the light-vesicular membrane fractions, internalization from the cell surface into the cell interior and in transfer from the membrane to the soluble cell fraction (15).

Yamamoto-Hino *et al.* (18) performed both Ca^{2+} imaging and immunofluorescence on the same ducts, and detected the strongest immunostaining of $\text{IP}_3\text{R2}$ IR in the initiation sites of $[\text{Ca}^{2+}]_i$ oscillation in the pioneer cells in the rat submandibular gland. In this study, the type of pioneer cells was not identified. The present study provided the evidence that the cells exhibiting the strongest $\text{IP}_3\text{R2}$ IR at the apical pole are the granular duct cells, in which CgA-like IR was preferentially contained.

Muallem and Wilkie (9) proposed a model showing a direct interaction between the IP_3R in the endoplasmic reticulum and Ca^{2+} influx channel in basolateral portion of the plasma membrane in polarized cells. They have shown that the IP_3R interacts with and gates the store-operated human homologues of the drosophila TRP channels (hTRP3), and that N-terminal domain of the IP_3R is sufficient for activation of hTRP3, but full gating (activation upon depletion of Ca^{2+} stores and inhibition when the Ca^{2+} stores are full) requires full-length IP_3R . Their model, however, may not harmonized with the present result that the strongest immunostaining of $\text{IP}_3\text{R2}$ localized at the apical pole of the granular cells, and that the immunostaining of $\text{IP}_3\text{R2}$ IR may diverge throughout the remaining basal half in accordance with the stimulus-induced compound exocytosis at the apical margin of the cell. The present results show that the apically concentrated IP_3R redistributes dynamically with the progress of stimulus-induced exocytosis in the granular duct cells.

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