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Real-time Imaging of Inositol 1,4,5-trisphosphate Movement in Mouse Salivary Gland Cells

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Inositol 1,4,5-trisphosphate (IP_3) plays an important role in the release of Ca^{2+} from intracellular stores into the cytoplasm in a variety of cell types. IP_3 translocation dynamics have been studied in response to many types of cell signals. However, the dynamics of cytosolic IP_3 in salivary acinar cells are unclear. A green fluorescent protein (GFP)-tagged pleckstrin homology domain (PHD) was constructed and introduced into a phospholipase C δ1 (PLC δ1) transgenic mouse, and then the salivary acinar cells were isolated. GFP-PHD was heterogeneously localized at the plasma membrane and intracellular organelles in submandibular gland and parotid gland cells. Application of trypsin, a G protein-coupled receptor activator, to the two types of cells caused an increase in GFP fluorescence in the cell cytoplasm. The observed time course of trypsin-evoked IP_3 movement in acinar cells was independent of cell polarity, and the fluorescent label showed an immediate increase throughout the cells. These results suggest that GFP-PHD in many tissues of transgenic mice, including non-cultured primary cells, can be used as a model for examination of IP_3 intracellular dynamics.

Key words: inositol 1,4,5-trisphosphate imaging, green fluorescent protein, transgenic mouse, parotid gland, submandibular gland

Introduction

Inositol 1,4,5-trisphosphate (IP_3), a phosphoinositide (PI) produced by the phospholipase C (PLC)-dependent hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) in the plasma membrane, functions as an intracellular second messenger to induce Ca^{2+} sparks that mediate a range of cellular mechanisms, such as muscle contraction, secretion, neuronal excitability, proliferation, and etc (Berridge et al., 1998). The membrane-resident phospholipids phosphatidylinositol (PtdIns) is phosphorylated to produce distinct PIs, inducing various signal transduction events (Simonsen et al., 2001; Czech, 2000). PIs exist in the form of many derivatives, including phosphatidylinositol 3-phosphate (PtdIns(3)P), phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P), phosphatidylinositol 4-phosphate (PtdIns(4)P), phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P), phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P), and phosphatidylinositol 3,4,5-bisphosphate (PtdIns(3,4,5)P_3) (Simonsen et al., 2001). The turnover pathway of PIs and its derivatives suggest localization of the signaling molecules. The remarkable feature of PIs, which can be rapidly synthesized, degraded, and relocated through cell surface receptors for the extracellular domains or subnuclear structures, makes them as ideal integrators of many cell mechanisms (Payrastre, 2001).

Some studies reported that translocation of the pleckstrin homology domain (PHD)-PLCδ1 by agonist stimulation causes an increase in IP_3 and a decrease in PIP2, which contributes to the cytosolic redistribution of the probe. Hirose *et al* reported that the dynamics of IP_3 can be visualized in single living cells using a PHD-GFP probe. PHD-GFP binds to PIP2 at the plasma membrane and translocates to the cytoplasm via the increasing IP_3

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concentration in MDCK cells (Hirose et al., 1999). Based on the IP_3 binding domain of IP_3R, Tanimura et al developed fluorescent IP_3 sensors, nominally the luminous inositol trisphosphate-binding domain, for ratiometric analysis (Tanimura et al., 2004; Sato et al., 2005) using fluorescence resonance energy transfer. In addition, IP_3R-based IP_3 sensor-1 (IRIS-1) based on the IP_3 binding domain of mouse type 1 IP_3R (IP_3R1) was used to analyze the mechanism responsible for the generation of Ca^{2+} oscillations (Matsuura et al., 2006; Mikoshiba, 2007). Intracellular Ca^{2+} is triggered by IP_3 from the intracellular stores through the IP_3 receptors resulting in the activation of Ca^{2+}-motivated processes (Berridge et al., 1998; Simonsen et al., 2001). Therefore, the observation of the cytosolic IP_3 dynamics during Ca^{2+} spikes is considered to be a good tool for understanding the Ca^{2+} signal in salivary cells because several types of G protein-coupled receptors are related to the mechanism of salivary secretion (Kawabata et al., 2002; Kim et al., 2006;Chung et al., 2006). These fluorescent probes can be transfected into cancer cell lines and primary culture cells with the exception of non-cultured primary cells. Therefore, real time imaging of the cytosolic IP_3 dynamics in salivary acinar cells is needed.

Protease-activated receptor-2 (PAR-2) is expressed in the salivary glands and PAR-2 stimulates amylase secretion through the involvement of nitric oxide in vivo (Kawabata et al., 2002) and trypsin induces the secretion of amylase and mucin from salivary glands in vitro (Nishikawa, 2006). PAR-2 may involve the mechanism of exocrine dysfunctions, such as dry mouth. However, the intracellular mechanism of salivary secretion mediated by PAR-2 remains to be elucidated.

In this study, a transgenic mouse system, which included the vector expressing PIP2 by tagging the GFP-PH domain of PLC δ1, was constructed to visualize the dynamics of IP_3 *in vivo*. The simultaneous imaging of PAR-2-evoked IP_3 movement in the salivary acinar cells provided considerable information on the secretion mechanism.

Materials and Methods

Materials

Construct of PHD-GFP was a gift from Dr. Shmuel Muallem (University of Texas Southwestern, Dallas, USA). Collagenase P was purchased from Roche (Mannheim, Germany). Pure collagenase was purchased from Worthington (Lakewood, NJ, USA). Trypsin and all graded reagents were purchased from Sigma (St Louis, MO, USA).

Generation of PHD-GFP transgenic mouse

The PHD-GFP targeting construct was generated by inserting transprimer-1. Clones were injected into FVB inbred mice to produce PHD-GFP transgenic mice. Eggs surviving microinjection were transferred into the oviducts of pseudo-pregnant ICR females. The offspring were weaned at 4 weeks of age. Genomic DNA was extracted from their tails and integration of the transgene was screened by PCR analysis of PHD-GFP. The primer sets for PHD-GFP gene were follows; forward (5'-AGGACAT−TCAGGAGGTGCGA-3'), and reverse (5'-AGCTTGCC−GGTGGTGCAGAT-3'). cDNA was subjected to 35 cycles of PCR with a thermal cycler; 94 °C for 30 sec, 58 °C for 40 sec, and 72 °C for 30 sec. Mice were housed in a temperature-controlled room (24± 1 °C) and 55 % relative humidity under artificial illumination (06:00 h ~ 18:00 h) with free access to food and water. All animal protocols were performed according to institutional guidelines of Yonsei University College of Dentistry.

Preparation of parotid and submandibular acini

Acini were prepared from the parotid and submandibular glands of PHD-GFP transgenic mouse by limited collagenase digestion as previously described (Shin et al., 2001). After isolation, the acini were re-suspended in a physiologic salt solution (PSS) ([mM] 140 NaCl, 1 $MgCl_2$, 5 KCl, 1 $CaCl_2$, 10 glucose, 10 Hepes, pH 7.4 with NaOH, 310 mOsm), and store on ice until use. Doublet or triplet acinar cell clusters were obtained by incubating minced salivary cells in PSS including 0.025 % trypsin and 0.02 % EDTA solution for 5 min at 37 °C. After washing with PSS supplemented with 0.02 % soybean trypsin inhibitor and 0.1 % bovine serum albumin, acinus were liberated by 6 min incubation at 37 °C in the same solution that also contained 160 U/ml pure collagenase. The cells were washed with PSS and kept on ice until use.

Measurement of IP_3 movement

Cells were plated on glass coverslips that formed the bottom of a perfusion chamber. After 5 min of incubation, to allow cell attachment to the coverslip, the cells were continuously perfused with pre-warmed (37 °C) PSS. GFP fluorescence was measured at excitation wavelength of 488 nm and emission wavelength of long pass 505 nm using a confocal microscope image acquisition and analysis system (LSM510, Carl Zeiss, Germany). To remove the effect of GFP fluorescence beaching, all experiments were performed within 20 min.

Results

The subcellular distribution of GFP fluorescence in the mouse submandibular and parotid acinar cells was analyzed by confocal microscopy (Fig. 1, n = 6). GFP was expressed in the plasma membrane and intracellular organelles heterogeneously in both cell types. The fluorescent GFP located on the plasma membrane reflects the binding of PHD-PLC δ1 to PIP2. It was anticipated that PHD-GFP transgenic mice serve as a real time imaging tool for

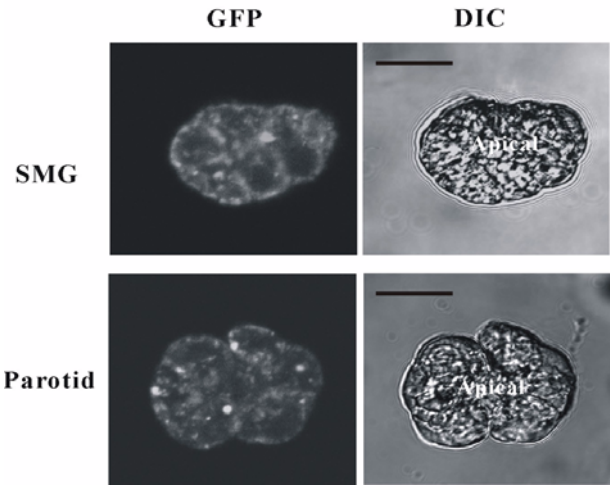

Fig. 1. Localization of GFP-tagged PH domain of PLC δ1 in salivary gland cells. Confocal images of isolated submandibular (SMG, *upper panel*) and parotid (*lower panel*) glands with GFP fluorescence (*left panel*) and bright (*right panel*) images, Original magnification × 400. The experiments were repeated at least six times and showed representative images. The scale bar in the DIC image of each tissue corresponds to 20 μm.

monitoring the movement of IP_3.

To induce IP_3 movement, trypsin, a PAR-2 activator, was applied to the submandibular and parotid acinar cells. Stimulation with trypsin caused an increase in GFP fluorescence in the cytosol (Fig. 2, n = 4). Simultaneous recording of the GFP images can trace the movement of IP_3. Both cell types showed an acute increase in fluorescence. Although submandibular and parotid cells have polarity regarding the direction of Ca^{2+} mobilization, the trypsin-stimulated movement of IP_3 in acinar cells was found to be independent of the polarity, and the fluorescent label showed an immediate increase.

Discussion

The mechanisms underlying the generation and dynamics of IP_3 have been studied extensively using a variety of theoretical models (Hirose et al., 1999; Tanimura et al., 2004; Sato et al., 2005; Matsu-ura et al., 2006; Mikoshiba, 2007; Varnai et al., 1998; Ashby et al., 2002). An experimental mouse model was constructed to be applied to an *in vivo* system in an attempt to address this issue. The real-time imaging of intracellular IP_3 dynamics in salivary acinar cells was performed using the PHD-GFP transgenic mouse system as the fluorescent IP_3 probe *in vivo*.

Translocation of the plasma membrane by agonist stimulation is important for initiating the signal transduction pathway (Fujii et al., 1999). As strong candidates, the PH domains, first described by Pleckstrin, are ~120 amino acids that bind to PIs in a specific manner with micromolar affinity (Harlan et al., 1994; Falasca et al., 1998). Ligands of the PH domains are revealed in the subunits of the heterotrimeric G proteins, protein kinase C, and PIP2 (Lemmon et al., 1995; Rameh et al., 1997). These PH domains, which are also found in a variety of signaling molecules, are associated with membrane surfaces and have been suggested to be involved in specific interactions between intracellular molecules (Ferguson et al., 1994). It was reported that the N-terminal site of PLC δ1 contains the PH domain (Musacchio et al., 1993). The N-terminus of the enzyme can bind to lipid vesicles that contain its substrate.

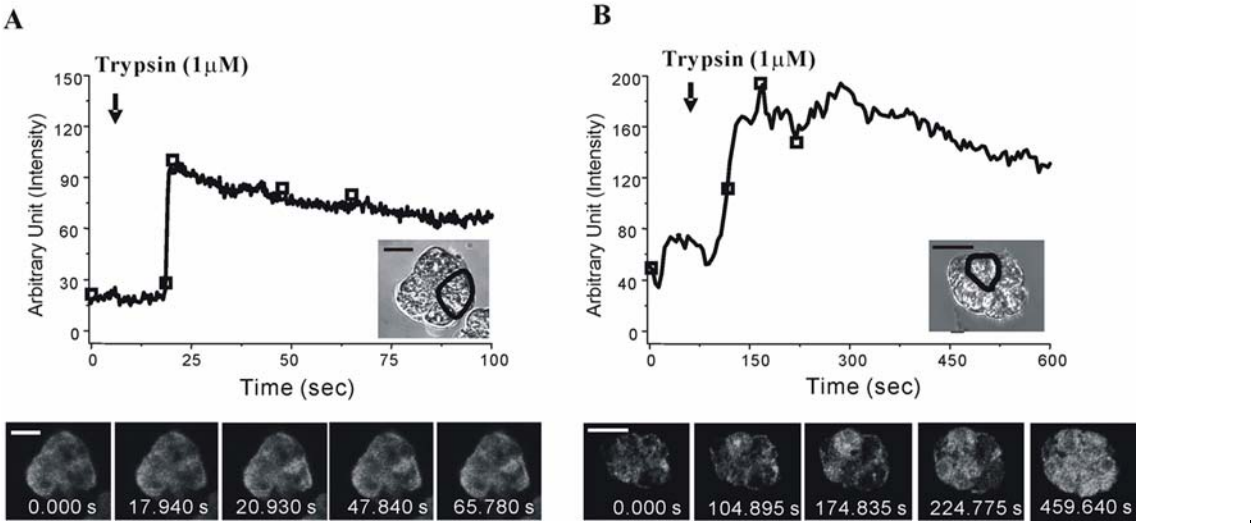

Fig. 2. Imaging of trypsin-evoked IP_3 mobilization in salivary gland cells. A, Submandibular gland cells, B, Parotid gland cells, isolated cells were stimulated with 1 μM Trypsin in PSS. The traces show the time courses of the GFP intensity in the region of interest (solid line). The images of fluorescence were selected at the times indicated by the open square dots in the traces. The experiments were repeated at least four times and representative images are shown. The scale bar in the left upper side of each tissue corresponds to 20 μm.

Many types of PH domains have relatively weak affinity and selectivity, whereas PHD-PLC δ1 can bind to PIP2 and IP_3 with high performance (Varnai et al., 1998; Harlan et al., 1994; Lemmon et al., 1995). Therefore, the stimulated PLC-coupled receptors are accompanied by the translocation of membrane fluorescence to the cytosol.

Polarized cells including salivary acinar cells produce polarized intracellular Ca^{2+} transients after agonist stimulation because they contain compartmentalized intracellular Ca^{2+} signaling molecules (Ashby et al., 2002). These coordinated Ca^{2+} signaling molecules are clustered in the microdomains of polarized cells, such as the synaptic ends in neurons and the apical side of secretary cells (Shin et al., 2003), and are involved in the propagation of Ca^{2+} ions, namely the Ca^{2+} wave, to the basal and lateral regions. The IP_3R and ryanodine receptor (RyR) are two types of Ca^{2+} release channels from the intracellular stores. IP_3Rs are expressed mainly in the apical pole, whereas RyRs have an unrestricted intracellular distribution (Nishikawa, 2006; Kawabata et al., 2001). Although there is a difference in its distribution, Ca^{2+}-induced Ca^{2+} release (CICR) depends on cooperation between the operational IP_3Rs and RyRs in pancreatic acinar cells (Ashby et al., 2002). However, the precise mechanism of the compartmentalized signaling protein required to regulate the fate of PIs is unclear. These results showed that an increase in IP_3 in the cells does not affect the polarity of IP_3 flow. Studies on the dynamics of IP_3 in salivary glands will help understand the relationship between IP_3Rs and RyRs in CICR.

PAR-2 is distributed abundantly in many tissues, such as the lung, gastrointestinal tract and exocrine cells (Kawabata et al., 2001, 2002, 2003; Cocks et al., 1999). The exocrine acinar cells including the parotid glands and submandibular glands are dependent on Ca^{2+} signaling, mediating fluid and enzyme secretion (Shin et al., 2001; Nishikawa, 2006; Kawabata et al., 2001). Kawabata *et al* reported that PAR-2 evoked *in vivo* amylase secretion through the involvement of nitric oxide (Kawabata et al., 2002). An in vitro study suggested that PAR-2 agonists, including endogenous PAR-2 activator trypsin induce the secretion of amylase and mucin from isolated rat parotid glands and sublingual glands, respectively (Nishikawa, 2006). The PAR-2-evoked exocrine secretion in salivary glands plays an important in pathophysiological events, such as mucosal cytoprotection (Kawabata et al., 2001).

Various intracellular membrane fractions in isolated parotid and submandibular acinar cells, including the plasma membrane, showed fluorescence. However, this mouse system cannot detect IP_3 oscillations but monitor the trypsin-induced IP_3 signal and sustained IP_3 in salivary cells. The real-time visualization system represented in this report offers a potential model for examining the dynamics of IP_3 in other tissues, including non-cultured primary cells.

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