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

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Inositol 1,4,5-trisphosphate (IP₃) plays an important role in the release of Ca²⁺ from intracellular stores into the cytoplasm in a variety of cell types. IP₃ translocation dynamics have been studied in response to many types of cell signals. However, the dynamics of cytosolic IP₃ in salivary acinar cells are unclear. A green fluorescent protein (GFP)-tagged pleckstrin homology domain (PHD) was constructed and introduced into a phospholipase Cδ₁ (PLCδ₁) 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 IP₃ 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 IP₃ 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 (IP₃), 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 Ca²⁺ 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) (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 IP₃ and a decrease in PIP₂, which contributes to the cytosolic redistribution of the probe. Hirose et al reported that the dynamics of IP₃ 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 IP₃.
concentration in MDCK cells (Hirose et al., 1999). Based on
the IP$_3$ binding domain of IP$_3$R, 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$_3$R-based IP$_3$
sensor-1 (IRIS-1) based on the IP$_3$ binding domain of mouse
type 1 IP$_3$R (IP$_3$R1) was used to analyze the mechanism
responsible for the generation of Ca$^{2+}$ oscillations (Matsu-
ura 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 dysfunc-
tions, 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$_\delta_1$, 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 Worth-
ington (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'-AGCTTGGC-
GGTGGTCAGAT-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 colla-
genase 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 Heps, 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 per-
formed 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$_\delta_1$ to IP$_3$. It was anticipated that PHD-GFP
transgenic mice serve as a real time imaging tool for
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 (Fuji 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$\delta$1 contains the PH domain (Musacchio et al., 1993). The N-terminus of the enzyme can bind to lipid vesicles that contain its substrate.
Many types of PH domains have relatively weak affinity and selectivity, whereas PHD-PLC δ1 can bind to PIP2 and IP3 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 Ca2+ transients after agonist stimulation because they contain compartmentalized intracellular Ca2+ signaling molecules (Ashby et al., 2002). These coordinated Ca2+ signaling molecules are clustered in the microdomains of polarized cells, such as the synaptic ends in neurons and the apical side of secretory cells (Shin et al., 2003), and are involved in the propagation of Ca2+ ions, namely the Ca2+ wave, to the basal and lateral regions. The IP3R and ryanodine receptor (RyR) are two types of Ca2+ release channels from the intracellular stores. IP3Rs 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, Ca2+-induced Ca2+ release (CICR) depends on cooperation between the operational IP3Rs 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 IP3 in the cells does not affect the polarity of IP3 flow. Studies on the dynamics of IP3 in salivary glands will help understand the relationship between IP3Rs 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 Ca2+ 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 IP3 oscillations but monitor the trypsin-induced IP3 signal and sustained IP3 in salivary cells. The real-time visualization system represented in this report offers a potential model for examining the dynamics of IP3 in other tissues, including non-cultured primary cells.

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References


