ELSEVIER

Cell Calcium 37 (2005) 349–357

cell calcium

www.elsevier.com/locate/ceca

Two-photon microscopic analysis of acetylcholine-induced mucus secretion in guinea pig nasal glands

Akihiro Oshima [a, b], Tatsuya Kojima [a], Kenji Dejima [b], Yasuo Hisa [b], Haruo Kasai [a, c], Tomomi Nemoto [a, d, *]

[a] *Department of Cell Physiology, National Institute for Physiological Sciences, Graduate University of Advanced Studies (SOKENDAI), 5-1 Higashiyama, Myodaiji-cho, Okazaki, Aichi 444-8787, Japan*

[b] *Department of Otolaryngology-Head and Neck Surgery, Kyoto Prefectural University of Medicine, 465 Kajii-cho Kawaramachi-Hirokouji, Kamigyo-ku, Kyoto 602-8566, Japan*

[c] *Center for Disease Biology and Integrative Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Tokyo 113-0033, Japan*

[d] *Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan*

Received 9 September 2004; received in revised form 10 October 2004; accepted 10 December 2004

Abstract

The spatiotemporal changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) as well as fluid secretion and exocytosis induced by acetylcholine (ACh) in intact acini of guinea pig nasal glands were investigated by two-photon excitation imaging. Cross-sectional images of acini loaded with the fluorescent Ca^{2+} indicator fura-2 revealed that the ACh-evoked increase in $[Ca^{2+}]_i$ was immediate and spread from the apical region (the secretory pole) of acinar cells to the basal region. Immersion of acini in a solution containing a fluorescent polar tracer, sulforhodamine B (SRB), revealed that fluid secretion, detected as a rapid disappearance of SRB fluorescence from the extracellular space, occurred exclusively in the luminal region and was accompanied by a reduction in acinar cell volume. Individual exocytic events were also visualized with SRB as the formation of Ω-shaped profiles at the apical membrane. In contrast to the rapidity of fluid secretion, exocytosis of secretory granules occurred with a delay of ∼70 s relative to the increase in $[Ca^{2+}]_i$. Exocytic events also occurred deep within the cytoplasm in a sequential manner with the latency of secondary exocytosis being greatly reduced compared with that of primary exocytosis. The delay in sequential compound exocytosis relative to fluid secretion may be important for release of the viscous contents of secretory granules into the nasal cavity.

Keywords: Mucus; Airway surface liquid; Multiphoton fluorescence microscopy; Nasal mucosa; Fura-2; Fluid phase polar tracer

1. Introduction

Mucus covering the nasal mucosa is an important contributor to host defense in the respiratory system of mammals [1–3]. In addition to its humidifying effect on inspired air, mucus contains a variety of substances—including glycoproteins, immunoglobulins, lysozyme, lactoferrin and antibacterial enzymes—that protect the airway from microorganisms and foreign chemicals. Most of these agents are stored in secretory granules of acinar cells in the nasal glands, and elucidation of the mechanism of mucus secretion by these cells is thus important for the development of new treatments for allergic responses, such as hypersecretion in the respiratory system [4–6].

The nasal glands are innervated by the parasympathetic nervous system [4,5,7–11]. As is the case for secretion from other exocrine cells, an increase in the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) has been implicated in mucus secretion induced by acetylcholine (ACh) in nasal gland acinar cells [12–14]. Cholinergic agonists induce activation both of

* Corresponding author. Tel.: +81 564 59 5873; fax: +81 564 59 5874.
E-mail address: tn@nips.ac.jp (T. Nemoto).

0143-4160/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ceca.2004.12.003

Cl^- channels in the apical membrane and of the Na^+–K^+–$2Cl^-$ cotransporter in the basolateral membrane of nasal gland acinar cells in a Ca^{2+}-dependent manner [12,15–20]. The consequent transepithelial Cl^- transport is thought to be the major driving force for fluid secretion in airway epithelia [21,22]. In addition, exocytosis of secretory granules induced by cholinergic agonists has been demonstrated in nasal gland acinar cells by electron microscopy [23,24] and by video enhanced contrast (VEC) microscopy [25]. However, previous studies have not examined $[Ca^{2+}]_i$, fluid secretion, and exocytosis in these cells simultaneously.

Imaging of intact nasal gland tissue by classical confocal microscopy is problematic, because this technique lacks the depth penetration necessary to visualize the fine organization of the apical plasma membrane within acini [26]. In contrast, two-photon excitation microscopy has the ability to penetrate deep into tissue [26,27] and allows simultaneous multicolor imaging with various combinations of fluorescent tracers [26,28,29]. Taking advantage of these attributes of two-photon microscopy, we have now investigated the increase in $[Ca^{2+}]_i$ as well as fluid secretion and exocytosis induced by ACh in intact acini of nasal glands.

2. Experimental procedures

2.1. Preparation of acini from guinea pig nasal glands

Acini were prepared basically as described previously [12]. In brief, 3- to 4-week-old guinea pigs were anesthetized by forced inhalation of diethyl ether and then killed by occipital dislocation. The nasal septum was rapidly removed and suspended in a Krebs–Ringer bicarbonate solution (KRB), consisting of 115.2 mM NaCl, 25 mM $NaHCO_3$, 0.96 mM HEPES-NaOH (pH 7.4), 5 mM NaH_2PO_4, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 11.37 mM glucose and 0.2% bovine serum albumin (fraction V; Sigma–Aldrich, St. Louis, MO), and equilibrated with a gas mixture of 95% O_2 and 5% CO_2. The septal mucosa was separated from the cartilage underlying the cartilaginous membrane and inverted in a dish containing KRB. After removal of the cartilaginous membrane, tissue containing a substantial number of nasal glands was dissected with the use of fine forceps, and ophthalmic scissors and without causing damage to the epithelial lining and was then minced in a tube. The tissue fragments were suspended in KRB containing collagenase (100 U ml^{-1}; fraction IV, Sigma–Aldrich) and incubated for 20 min at 37 °C in a shaking water bath. The digested tissue was dissociated by passage three times through a siliconized pipette. Clusters of acini so obtained were dispersed in a small chamber and superfused (1 ml min^{-1}) with KRB supplemented with 0.5% trypsin inhibitor (type I-S, Sigma–Aldrich), and an additional 0.2% bovine serum albumin. All experiments were performed at room temperature (22–25 °C), and, unless indicated otherwise, all chemicals were obtained from Nacalai Tesque (Kyoto, Japan). The study was approved by the Institutional Animal Care and Use Committee of Okazaki National Research Institutes.

2.2. Two-photon imaging of intact acini

Isolated clusters of acini were loaded with the Ca^{2+} indicator fura-2 by incubation for 30 min at room temperature with KRB containing the corresponding acetoxymethyl ester (Molecular Probes, Eugene, OR) at 20 μM. Acini in the recording chamber were continuously superfused with KRB during experiments. Two-photon excitation imaging of acinar cells was performed as described previously [26]. In brief, cells were imaged with an inverted microscope (IX70; Olympus, Tokyo, Japan) and a laser-scanning microscope (FV300, Olympus) equipped with a water-immersion objective lens (UPlanApo60× W/IR; numerical aperture, 1.2). A mode-locked Ti:Sapphire laser (Tsunami; Spectra Physics, Mountview, CA) with an original pulse duration of 70–100 fs was attached to the laser port of the laser-scanning microscope; the group velocity dispersion of the microscope was compensated for by a set of chirp compensation optics. The laser power at the specimen was 3–5 mW, and the excitation wavelength was 830 nm.

The superfusion medium was changed to KRB containing a fluorescent fluid-phase polar tracer, sulforhodamine B (SRB; Molecular Probes), at 0.5 mM before observations. Acetylcholine (Sigma–Aldrich) was dissolved in KRB containing SRB and applied to the cells through a glass pipette. The relative change in fura-2 fluorescence intensity induced by a change in $[Ca^{2+}]_i$ was calculated as $1 - F/F_0$, where F is the fluorescence intensity of fura-2 at a specific time, and F_0 is the average initial such intensity before agonist application (an increase in $[Ca^{2+}]_i$ results in a decrease in F) [26,29]. For simultaneous imaging of $[Ca^{2+}]_i$ and exocytosis, the fluorescence of SRB and that of fura-2 were measured at 570–650 and 400–550 nm, respectively. Fluorescence was detected by photomultiplier tubes (R7683; Hamamatsu Photonics, Hamamatsu, Japan) in the FV300 microscope, and fluorescence images were acquired every 0.5–2 s. The 12-bit images were analyzed and color-coded with "fall" and "gray" look-up tables of the image acquisition and analysis software, either Fluoview of the FV300 microscope or IPLab Spectrum (Scanalytics, Fairfax, VA).

The dose dependence of ACh action was examined by a non-linear least squares method based on the Levenberg–Marquardt algorithm with the use of the Kaleida Graph program (Synergy Software, Reading, PA). We assumed no apparent co-operativity of the applied ACh concentration (x) in measurements, and thus used an equation containing two free parameters, k (median effective concentration) and A (maximum value of measurement), for the fitting: $f(x) = Ax/(k + x)$. The two-tailed Student's t-test was used to evaluate the significance of differences between the average measurements of two groups; a P-value of <0.05