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Intracellular free Ca^{2+} dynamic changes to histamine are reduced in cystic fibrosis human tracheal gland cells

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Abstract This study documents a difference between cystic fibrosis human (CF-HTG) and normal human (HTG) tracheal gland cells: the ability of histamine to induce an increase of intracellular free calcium concentration $[Ca^{2+}]_i$ was abnormally reduced in CF-HTG cells. The magnitude of the $[Ca^{2+}]_i$ peak rise in response to histamine is smaller in CF-HTG cells than in HTG cells, and the percentage of CF-HTG cells that increase $[Ca^{2+}]_i$ is decreased compared with HTG cells. In contrast to histamine, the human neutrophil elastase (HNE) stimulation of both CF-HTG and HTG cells generated $[Ca^{2+}]_i$ asynchronous oscillations and the magnitude of the peak $[Ca^{2+}]_i$ response as well as the percentage of responding cells were similar for both groups. By videomicroscopy observations, the secretory response (exocytosis of secretion granules) of CF-HTG cells occurred with HNE, but not with histamine, thus suggesting that $[Ca^{2+}]_i$ asynchronous oscillations may be linked to the exocytosis process in human tracheal gland cells.

Key words: Cystic fibrosis; Human tracheal gland cell; Intracellular Ca^{2+}; Exocytosis

1. Introduction

The main cause of death among patients suffering from cystic fibrosis (CF) is complication associated with progressive blockage of airways by underhydrated and sticky mucus [1]. CF, the most common lethal genetic disease in Caucasians, is characterized by a defect in fluid transport and is caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) which has been shown to be a cAMP-activated Cl^- channel [2-4] and to regulate the activity of other channels [5,6]. In normal airway epithelial cells, both intracellular Ca^{2+} and cAMP have been implicated in Cl^- secretion. CF causes a decrease in cAMP-dependent but not Ca^{2+}-dependent regulation of Cl^- transport in airway surface epithelia [7]. However, it is not clear whether abnormal mucus present in airways of CF patients is due to defective regulation of airway submucosal gland secretory cells or to a secondary effect of altered surface epithelial cell ion transport [8].

The difficult procedures for isolating and culturing human tracheal submucosal gland cells have restricted the studies regarding receptors, ion channels and intracellular regulatory pathways in normal and CF submucosal gland cells. Several lines of evidence suggest, however, that submucosal gland cells are a major primary site of CF disease. A decreased Cl^- permeability [9] and a constitutive hypersecretion [10] have been reported in cultured CF tracheobronchial gland cells. Furthermore, a high expression of CFTR mRNA and protein [11-13] and of CFTR-like Cl^- channels [14] were demonstrated in normal human tracheobronchial glands. We recently showed [12] that CFTR was specifically associated with the membrane of serous cell secretory granules. In many exocrine gland cells, intracellular Ca^{2+} is an important second messenger in the process of exocytosis [15,16]. Whether or not $[Ca^{2+}]_i$ transients play a role in the exocytotic release of secretory granules in human tracheal gland cells remains unknown. Numerous inflammatory mediators, activating different signaling pathways, are known to initiate the exocytosis process in airway gland cells; among the most potent secretagogues, histamine, acetylcholine and arachidonate metabolites as well as neutrophil proteases, especially HNE, have been implicated in the pathogenesis of airway mucus hypersecretion [17,18]. In a recent study [19], we showed qualitative and quantitative differences in $[Ca^{2+}]_i$ mobilization in the human tracheal gland cell line MM39 after exposure to histamine and HNE. Data about $[Ca^{2+}]_i$ regulation in CF epithelial cells are contradictory and the exact role of dysregulation of $[Ca^{2+}]_i$ transients in CF is still unclear. Some authors observed a reduced $[Ca^{2+}]_i$ response of CF nasal surface epithelial cells to histamine [20] whereas others did not [21].

In the present study, we report the first investigations of the dynamic changes of $[Ca^{2+}]_i$ signals induced by histamine in cultured CF (CF-HTG) and normal (HTG) human tracheal gland cells. The $[Ca^{2+}]_i$ responses observed with histamine were compared to those obtained with HNE treatment in both CF-HTG and HTG cell groups. In parallel to $[Ca^{2+}]_i$ measurements performed by confocal UV laser microspectrofluorometry at the single-cell level, the study was complemented by an analysis of the secretory activity of CF-HTG cells, monitored by direct visualization of cells, using videomicroscopy.

2. Materials and methods

2.1. Solution and drugs

Histamine and calcium ionophore (4BrA23187) were purchased from Sigma Chemical Co. (St. Louis, MO). HNE isolated from human purulent sputum was purchased from Elastin Products (Pacific, MO). Indo-1/AM was obtained from Molecular Probes, Inc. (Eugene, OR).

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Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium ion concentration(s); HNE, human neutrophil elastase; cAMP, adenosine cyclic 3′,5′-monophosphate; Indo 1-AM, Indo-1 acetoxymethyl ester; CCD, charge coupled device

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2.2. Cell culture

Normal and CF tracheas were obtained during lung transplantations on CF patients. Isolation and culture of CF-HTG and HTG cells were performed as previously described [10,22]. The CF-HTG cells were homozygote for the ΔF 508 CFTR deletion (ΔF 508/ΔF 508). In brief, cells were isolated by enzymatic digestion from trachea mucosas and were grown onto type 1 collagen coated flasks in a Dulbecco's modified Eagle's medium/Ham's F12 mixture (50/50%, v/v) supplemented with 100 U/ml penicillin G and 100 μg/μl streptomycin as antibiotics and with 1% Ultroser G (IBF, Villeneuve la Garenne, France), 3 μM epinephrine, glucose and sodium pyruvate made up to 10 g/l and 0.33 g/l, respectively. In these culture conditions, cells proliferate and exhibit characteristics of epithelial and serous secretory cell type [10,22].

2.3. $[Ca^{2+}]_i$ measurements by UV microspectrofluorometry

Normal HTG and CF-HTG cells were plated on type 1 collagen coated glass coverslips and maintained in an Ultroser G-free, hormonally defined cell culture medium for 3 days prior to $[Ca^{2+}]_i$ measurements. Cells were loaded with 2 μM Indo-1/AM in red phenol-free RPMI 1640 medium for 30 min at 37°C, washed three times and covered with 1 ml of the same medium. Dynamic changes of $[Ca^{2+}]_i$ in individual cells in monolayer culture were measured using a new procedure as recently described [19,23]. Fields of 6–8 adjacent cells were visualized on an optical microscope (Olympus BH2) equipped with a water immersion objective lens (100×, N.A. 0.95) (State Optical Institute of St. Petersburg, Russia) which has been specially developed for the total transmission of UV radiation down to 300 nm and corrected for axial chromatic aberrations. Fluorescent emission spectra within each selected cell were recorded using a UV confocal microspectrofluorometer (XY model, Dilor, Lille, France). The 351 nm laser line (Ar+, 2065A model, Spectra Physics) was focused on the sample with a measured power of 0.5 μW. The fluorescent emission in the 360–560 nm range was spectrally dispersed by diffraction grating, and was detected with an optical multichannel analyzer consisting of a cooled 1024 diode array. Dynamic changes of $[Ca^{2+}]_i$ were measured as follows: an (X,Y) motorized stage (Märzhäuser, model MCL-2 with increments of 0.1 μm), coupled with a computer, allowed the storage of the (X,Y) positions of several (up to 10) chosen points in different locations either from one living individual cell or from different living cells. The elementary duration of measurement for each point was fixed to 1 s, so that $[Ca^{2+}]_i$ could be measured in each point every 6 s, when 6 different adjacent cells were analyzed. Basal fluorescence emission was recorded for the first 5 measurements in each cell prior to addition of secretagogue. Cells were then monitored for about 10 min after secretagogue addition at room temperature, and successive measurements were stored in the computer.

2.4. Videomicroscopy

Video images obtained during $[Ca^{2+}]_i$ measurements were recorded using a CCD (4910 model, Cohu, Inc.) camera and analyzed using a custom-made computer program that allowed us to record the time of each individual process of degranulation within a particular cell. Temporal analyses in spatially restricted areas were thus obtained throughout the experiments.

2.5. Statistics

The differences ($\Delta[Ca^{2+}]_i$) between the mean basal level and the maximum peak $[Ca^{2+}]_i$ increase after exposure to secretagogues were calculated. As a low level of spontaneous noise in the fluorescence signals could possibly have been interpreted as an alteration in $[Ca^{2+}]_i$, a positive response to an applied agonist was defined as a $[Ca^{2+}]_i$ increase of at least 25 nM. Data are reported as means ± S.D. Statistical significance was determined by an unpaired Student's test for two group comparisons. Values of $P<0.01$ were considered significant.

3. Results

Examples of the dynamic change of $[Ca^{2+}]_i$ in HTG and CF-HTG cells in monolayer culture are shown in Fig. 1. The addition of 100 μM histamine to HTG cells causes a rapid $[Ca^{2+}]_i$ peak rise, followed by a smooth decay back to baseline in a synchronous fashion in all cells (Fig. 1A). In contrast to HTG cells in which 100% of cells responded, a significantly lower percentage of CF-HTG cells (Fig. 1B) responded to histamine (46%, $n=26$, Table 1). The magnitude of the mean peak increase of $[Ca^{2+}]_i$ in responding CF-HTG cells (i.e. with $[Ca^{2+}]_i$ increases ≥25 nM) was significantly smaller than that of HTG cells (Fig. 2). After a 100 μM histamine stimulation, the $[Ca^{2+}]_i$ increases were 112 ± 103 nM ($n=26$) and 240 ± 127 nM ($n=40$) in CF-HTG and HTG cells, respectively (Table 1). Of note were the low and heterogeneous changes in $[Ca^{2+}]_i$ of CF-HTG cells in response to histamine in which the $[Ca^{2+}]_i$ increases were higher than 100 nM in only 2 out of 26 cells (Fig. 2A). Basal $[Ca^{2+}]_i$ was not different from HTG (95 ± 38 nM, $n=70$) and CF-HTG (106 ± 37 nM, $n=67$) cells and CF-HTG cells showed a rapid $[Ca^{2+}]_i$ increase after a 1.0 μM 4BrA23187 calcium ionophore treatment (Fig. 2B), suggesting that the lower $\Delta[Ca^{2+}]_i$ responses to histamine did not result from a lower viability of the CF-HTG cells. Exposure of CF-HTG cells to 100 μM histamine did not affect the $[Ca^{2+}]_i$ responses to the subsequent stimulation with 1.0 μM 4BrA23187 calcium ionophore (Fig. 2B). Note that, in HTG cells, the magnitude of the $[Ca^{2+}]_i$ peak rise to sequential addition of histamine followed by 4BrA23187 calcium ionophore appeared to be significantly correlated from cell to cell analysis ($r=0.71$, $P<0.01$, $n=26$, Fig. 2B). As illustrated in Fig. 3, upon the calcium ionophore stimulation, the spatio-temporal aspects of the Ca^{2+} signal within a single CF-HTG cell displayed significant differences between the $[Ca^{2+}]_i$ transient patterns in the nucleoplasm and the cytosol. No differences in the spatio-temporal patterns of the Ca^{2+} signal through the cell between HTG and CF-HTG cells were observed after the calcium ionophore stimulation (data not shown).

Cultured HTG and CF-HTG cells were also studied after exposure to a biologically relevant concentration of HNE (1.0 μM). The latter has been reported to be the most potent secretagogue described so far, for airway submucosal gland cells in culture as well as in tracheal tissue explants for various species including human [18]. After exposure to HNE (Fig. 4A), the HTG cells exhibited asynchronous $[Ca^{2+}]_i$ oscillations with or without a delay time (up to 180 s). At 1.0 μM HNE, the $[Ca^{2+}]_i$ increase was 109 ± 52 nM ($n=45$, 52% of cells responding). A similar response of asynchronous

Table 1
Differences in $[Ca^{2+}]_i$ increase between HTG and CF-HTG cells

	Histamine 100 μM			HNE 1.0 μM		
	$[Ca^{2+}]_i$ increase (nM)	Responding cells (%)	n	$[Ca^{2+}]_i$ increase (nM)	Responding cells (%)	n
HTG cells	240 ± 127*	100**	40	109 ± 52	52	45
CF-HTG cells	112 ± 103*	46**	26	120 ± 101	56	41

Data are expressed as means ± SD; n is the number of cells studied.
*$P<0.01$, **$P<0.001$.

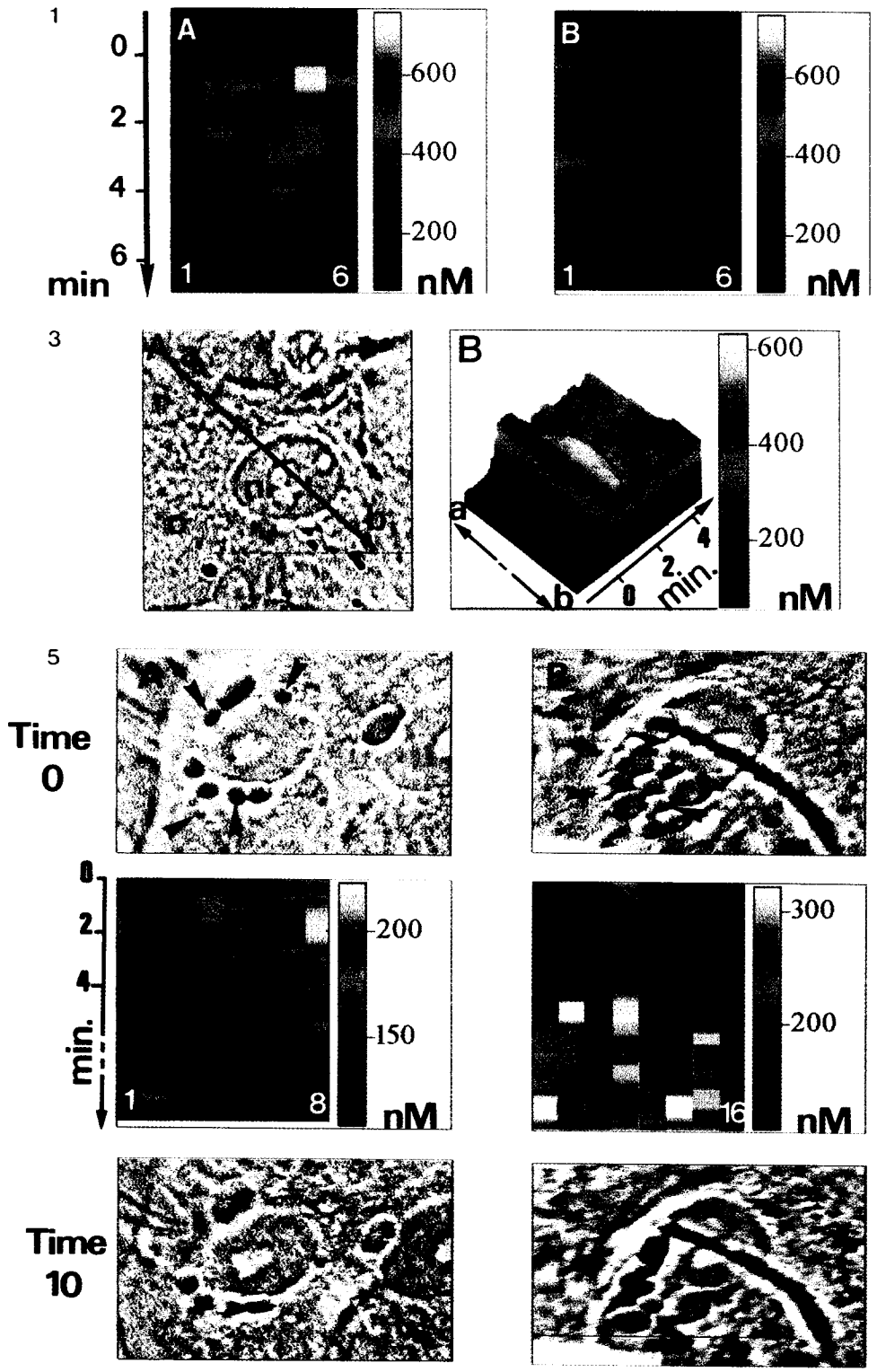

Fig. 1. Histamine induced $[Ca^{2+}]_i$ increases in HTG (A) and CF-HTG (B) cells. Time courses of $[Ca^{2+}]_i$ transients were recorded in 6 adjacent HTG (A) and CF-HTG (B) cells (columns 1–6), before and after addition of 100 μM histamine (time 0). In each column, $[Ca^{2+}]_i$ is measured during 1 s, every 6 s.

Fig. 3. Spatio-temporal $[Ca^{2+}]_i$ changes in an individual CF-HTG cell in response to the addition of 1.0 μM A23187 calcium ionophore (time 0). (A) Optical microscopy videoimage of the cell in which the $[Ca^{2+}]_i$ transients were recorded along the line [a–b] with an acquisition time of 1 s. (B) Three-dimensional map of the $[Ca^{2+}]_i$ levels along the line [a–b] for the first 5 min-period according to the pseudocolor Ca^{2+} scale. c, cytoplasm; n, nucleus.

Fig. 5. HNE-induced secretion granules movements and exocytosis. Video microscopic images and dynamic changes of $[Ca^{2+}]_i$ were simultaneously recorded in 2 (A, B) isolated CF-HTG cells exposed to 1.0 μM HNE for a 10 min period. Time 0: resting cell prior to HNE treatment. Numerous secretory granules predominate near the cell center. Time 10: after a 10 min exposure to HNE, many secretion granules (4 in cell A; 2 in cell B; see arrows) have presumably exocytosed from the cell. Columns 1–8 and columns 9–16 correspond to the $[Ca^{2+}]_i$ transient changes in 8 different locations of the cytoplasm from 2 CF-HTG cells during the 10 min period.

$[Ca^{2+}]_i$ oscillations was obtained from CF-HTG cells (Fig. 4B) with a mean peak increase of $[Ca^{2+}]_i$ of 120 ± 101 nM (n=41, 56% of cells responding). Therefore, in contrast to the differences found in $[Ca^{2+}]_i$ regulation between CF-HTG and HTG cells with histamine, the $[Ca^{2+}]_i$ response to HNE was similar for both cell groups (Table 1). During the $[Ca^{2+}]_i$ measurement period, exocytosis of individual secretion granules from CF-HTG cells was visualized as the disappearance of granules from the cell. As shown in Fig. 5, several (2–4) degranulations were observed in two different CF-HTG cells in which repetitive $[Ca^{2+}]_i$ asynchronous oscillations were generated by a 1.0 μM HNE stimulation for a 10 min period. When CF-HTG cells were exposed to 100 μM histamine, neither $[Ca^{2+}]_i$ asynchronous oscillations nor degranulation (data not shown) but only a decreased transient rise in $[Ca^{2+}]_i$ (Figs. 1 and 2A) occurred over a 10 min treatment period.

4. Discussion

Numerous recent reports agree on the fact that the temporal patterns of $[Ca^{2+}]_i$ transients referred to as Ca^{2+} spiking [15,16,25] play a fundamental role in exocytosis, i.e. the last transport step in the secretory pathway, a process that involves the specific interaction and fusion of secretory granules with plasma membrane. The regulation of exocytosis appears to vary depending on the cell type studied, and marked differences in the sensitivity of $[Ca^{2+}]_i$, Ca^{2+} binding proteins and protein phosphorylation have been reported [26,27]. In CF, several aspects of $[Ca^{2+}]_i$ regulation have been suggested as being abnormal such as, decreased secretagogue induced $[Ca^{2+}]_i$ increase in epithelial cells [20,28] and a higher basal $[Ca^{2+}]_i$ in blood cells [29]. As yet, no data have been reported on dynamic changes of $[Ca^{2+}]_i$ transients in CF human airway submucosal gland serous cells at single-cell level in response to external secretagogues. For a better understanding of the relationship between the CF defect and mucus hypersecretion, we monitored simultaneously the $[Ca^{2+}]_i$ transient changes and exocytosis of secretion granules of CF-HTG cells (carrying the ΔF 508 deletion), measured from cell to cell in response to histamine and HNE exposure. Our observation of

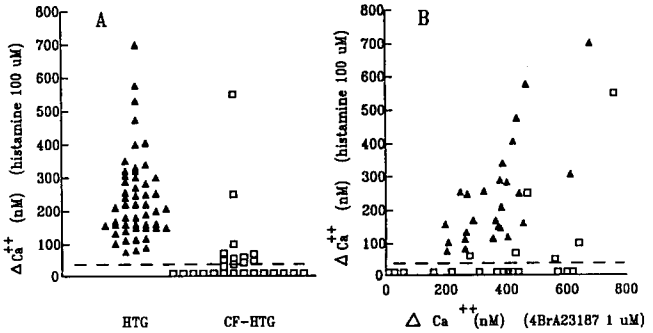

Fig. 2. Cell-to-cell variation of $\Delta[Ca^{2+}]_i$ response to histamine and A23187 calcium ionophore in HTG (▲) and CF-HTG (□) cells. (A) After a 100 μM histamine stimulation. (B) After a sequential addition of 100 μM histamine followed by 1.0 μM A23187 calcium ionophore. The increase of $[Ca^{2+}]_i$ was calculated after the addition of histamine (time 0). After the decay back to basal $[Ca^{2+}]_i$ levels, the response of $[Ca^{2+}]_i$ to the addition of A23187 calcium ionophore (time 6 min) was calculated in each cell. The dotted line represents a $\Delta[Ca^{2+}]_i$ of 25 nM, considered as a positive response to agonist.

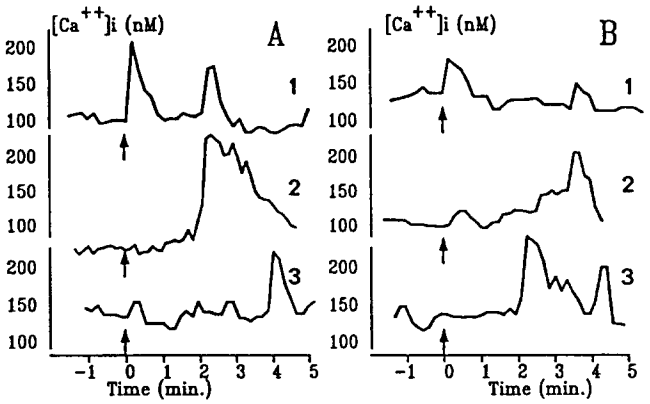

Fig. 4. Response of $[Ca^{2+}]_i$ changes in HTG (A) and CF-HTG (B) cells to 1.0 μM HNE treatment. The HNE addition (indicated by arrows) induced dynamics changes in $[Ca^{2+}]_i$ according to 3 different patterns (1–3) in both HTG and CF-HTG cells. Recordings are results obtained by analyzing more than 10 fields of adherent cells under similar conditions.

a significant inhibition of the $[Ca^{2+}]_i$ peak rise of CF-HTG cells and the lack of secretory response to histamine is in agreement with previous studies showing: (a) a defective response of $[Ca^{2+}]_i$ increase to histamine and prostaglandin E1 in CF nasal epithelial cells [20] and (b) that chelation of Ca^{2+} within submandibular gland cells mimics the decreased β-adrenergic stimulation of glycoprotein secretion demonstrated in CF human [28] and CF mice [30] submandibular glands. A decreased secretory response to stimuli which increases total cAMP levels has been described in all epithelial cell types affected in CF, indicating that the CF defect concerns a pathway distal to cAMP formation [28]. This is an indication that intracellular Ca^{2+} is an essential requirement at a point in the intracellular signaling pathways, distal to the action of cAMP in triggering secretion. The relationship between the decreased $[Ca^{2+}]_i$ response to histamine, the mislocalization and misfolding of CFTR protein in the ΔF 508 deletion of CF cells [31] and the regulation of exocytosis process in airway gland serous cells remains an open question. Given that the endoplasmic reticulum is thought to function as a major intracellular Ca^{2+} reservoir [25], we propose that abnormal CFTR protein accumulation in endoplasmic reticulum described in CF epithelial cells [24] might result in the defect in Ca^{2+} handling of certain Ca^{2+} stores. Taken together, these results suggest that, in addition to apical Cl^- channel function [2-4], CFTR may also play an intracellular role in the Ca^{2+} mobilization associated to the exocytosis process in human airway gland cells.

In contrast to the decreased $[Ca^{2+}]_i$ peak rise in CF-HTG cells to histamine compared to HTG cells, a biological relevant concentration of HNE (1.0 μM) generated $[Ca^{2+}]_i$ asynchronous oscillations (i.e. repetitive spikes) to approximately the same extent in CF-HTG cells as in HTG cells (Fig. 4, Table 1). The secretory response (degranulation) to HNE exposure was intact in CF-HTG cells (Fig. 5), suggesting that this response is mediated by a cAMP-independent pathway, possibly $[Ca^{2+}]_i$ oscillations, since ion transport studies in airway epithelial cells have shown the Ca^{2+} mobilization to be unaffected in CF [7]. Studies on salivary [32] and sweat glands [33] showed a normal fluid secretion in response to Ca^{2+} mediated stimuli in CF. In HTG and CF-HTG cells, the exact mechanisms of the different magnitudes of $[Ca^{2+}]_i$ response

and patterns of $[Ca^{2+}]_i$ oscillations to histamine and HNE remain to be elucidated. An alternative explanation is that differences either in the degree of receptor expression or in the transduction machinery of HTG and CF-HTG cells may give access to different intracellular Ca^{2+} storage pools of varying sizes. Another possibility is that second messengers for Ca^{2+} mobilization by the secretagogues may differ and produce different effects in the ability to mobilize Ca^{2+}. Our finding has particular relevance to the understanding of the pathogenesis of the hypersecretion in CF airways. In vivo, as airway submucosal gland cells are often stimulated by HNE to secrete [17,18], it is highly plausible that asynchronous $[Ca^{2+}]_i$ oscillations may mediate the secretory responses and initiate stimulus-secretion coupling leading to exocytotic granule release into the airway lumen. Repetitive $[Ca^{2+}]_i$ oscillations may be signals of biological significance and high safety factor in comparison to a continuous elevation of $[Ca^{2+}]_i$, in avoiding cell damage [34] due to excess increase in $[Ca^{2+}]_i$. Future studies may help to define whether different patterns of $[Ca^{2+}]_i$ oscillations in airway submucosal gland cells in relation to the localization patterns of CFTR are involved in the exocytosis regulation and abnormalities in CF gland hypersecretion.

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