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Actin cytoskeleton controls movement of intracellular organelles in pancreatic duct epithelial cells

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Abstract

In most eukaryotic cells, microtubules and filamentous actin (F-actin) provide tracks on which intracellular organelles move using molecular motors. Here we report that cytoplasmic movement of both mitochondria and lysosomes is slowed by F-actin meshwork formation in pancreatic duct epithelial cells (PDEC). Mitochondria and lysosomes were labeled with fluorescent MitoTracker Red CMXRos and LysoTracker Red DND-99, respectively, and their movements were monitored using epi-fluorescence and confocal microscopy. Mitochondria and lysosomes moving actively at rest stopped rapidly within several seconds after an intracellular Ca^{2+} rise induced by activation of P2Y_2 purinergic receptors. The ‘freezing’ of the organelles was inhibited by blocking the Ca^{2+} rise or by pretreatment with latrunculin B, an inhibitor of F-actin formation. Indeed, this freezing effect on the organelles was accompanied by the formation of F-actin in the whole cytoplasm as stained with Alexa 488-phalloidin in fixed PDEC. For real-time monitoring of F-actin formation in live cells, we expressed sGFP-fimbrin actin binding domain2 (fABD2) in PDEC. Rapid recruitment of the fluorescent probe near the nucleus and lysosomes suggested dense F-actin formation around intracellular structures. The development of F-actin paralleled that of organelle freezing. We conclude that rapid Ca^{2+} -dependent F-actin formation physically restrains intracellular organelles and reduces their mobility non-selectively in PDEC.

Keywords

calcium; mitochondria; lysosomes; F-actin; sGFP-fABD2

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Conflict of interest

There is no conflict of interest.

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1. INTRODUCTION

In addition to its role as the cytoskeleton, filamentous actin (F-actin) serves as a track for movement of intracellular organelles such as mitochondria and secretory granules [1]. F-actin is dynamically rearranged by actin binding proteins or severing enzymes. Activity of those proteins is controlled by intracellular signals such as small GTPases. Previous studies emphasized Ca^{2+} as a regulator of F-actin disassembly. For example, in neutrophils, Ca^{2+} activates severing enzymes of F-actin but did not affect F-actin formation [2,3]. Gelsolin is a well-known Ca^{2+} -dependent severing protein for F-actin in several cell types [4]. It promotes exocytosis by eliminating the actin cortex for better access of secretory granules to the plasma membrane [4,5,6,7,8]. Here we demonstrate, in contrast, that Ca^{2+} triggers the formation of a dense F-actin meshwork in epithelia and thereby restrains the movement of intracellular organelles.

In pancreatic duct epithelial cells (PDEC), an increase of intracellular Ca^{2+} stimulates exocytotic mucin secretion [9,10,11,12,13,14]. Because secretory granules are not predocked to the plasma membrane in epithelial cells [15], granule movement in the cytoplasm is critical during exocytosis. Previously we have shown a novel phenomenon of fast immobilization of secretory granules by a rapid formation of F-actin in PDEC [12]. We called this “freezing”. The granule freezing occurred within ~5 s after intracellular Ca^{2+} rise, and granule movement recovered at least 20 times slower than the onset. The granule freezing was mediated by Ca^{2+} -dependent F-actin formation in the whole cytoplasm. The freezing of secretory granules retarded exocytosis upon a moderate $[\text{Ca}^{2+}]_i$ increase (e.g. < 1 μM) but did not hinder the exocytosis triggered by the cAMP/PKA pathway [13].

This study addresses whether the F-actin meshwork also restrains other intracellular organelles such as lysosomes or mitochondria and measures the kinetics of F-actin formation in real time using a fluorescent variant of actin-binding domain2 of fimbrin (sGFP-fABD2). Our results support the notion that Ca^{2+} -dependent formation of F-actin negatively and non-selectively controls the movement of intracellular organelles in exocrine PDEC.

2. MATERIALS AND METHODS

2.1 Cell culture

Non-transformed PDEC derived from a main pancreatic duct of the dog in 1996 were cultured on Transwell inserts (Corning Costar, Acton, MA) coated with Vitrogen (Inamed Biomaterials, Fremont, CA) over a confluent feeder layer of human gallbladder myofibroblasts as previously described [15]. For single-cell experiments, PDEC were cultured on small Vitrogen-coated glass chips in a medium conditioned by the myofibroblasts. Isolated unpolarized single cells were used 1 to 3 days after plating [11,12].

2.2 Solutions and chemicals

Extracellular normal saline solution contained (in mM): 137.5 NaCl, 2.5 KCl, 1 MgCl_2 , 2 CaCl_2 , 10 glucose, 10 HEPES (pH adjusted to 7.3 with NaOH). Ca^{2+} -free solution included (mM): 137.5 NaCl, 2.5 KCl, 1 MgCl_2 , 0.1 EGTA, 10 glucose, 10 HEPES (pH adjusted to 7.3 with NaOH). Stocks of Mitotracker Red CMXRos (1 mM), LysoTracker Red DND-99 (1 mM), fura-2 AM (1 mM), sulfinpyrazone (500 mM), thapsigargin (5 mM), and latrunculin B (Lat B, 10 mM) were dissolved in dimethyl sulfoxide and Alexa Fluo-568 Phalloidin (200 units/ml) in methanol. Mitotracker Red CMXRos, LysoTracker Red DND-99, Alexa Fluo-568 Phalloidin, and fura-2 AM was purchased from Invitrogen (Carlsbad, CA, USA) and Lat B from EMD biosciences (San Diego, CA, USA). All experiments were performed at room temperature (22–24°C).

2.3 Single-cell Ca^{2+} imaging

Intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was measured using fura-2 following preincubation of the PDEC for 30 min with 2 μM of the AM form of the dye, 0.01% pluronic F-127, and 1 mM sulfinpyrazone in saline solution. The dye was excited at 340 and 380 nm, and fluorescence signals were recorded at > 470 nm every 1 s using a CCD camera (Pixelfly QE, Cooke). Background fluorescence measured in a cell-free region was subtracted. $[\text{Ca}^{2+}]_i$ was calculated as $[\text{Ca}^{2+}]_i = K_d^* \times (R - R_{\min}) / (R_{\max} - R)$, where K_d^* is the effective dissociation constant of fura-2, R is the ratio of fluorescence obtained at 340 nm and 380 nm (F_{340}/F_{380}), and $R_{\min}$ and $R_{\max}$ are the ratios for Ca^{2+} -free and Ca^{2+} -bound dye, respectively [16]. $R_{\min}$, $R_{\max}$, and K_d^* were determined to be 0.44, 8.55, and, 2.432 μM , respectively ($N = 3-7$ cells for each value), using cells incubated for at least 10 min with saline solutions containing 20 μM ionomycin plus 20 mM EGTA ($R_{\min}$) or 15 mM Ca^{2+} ($R_{\max}$), or 20 mM EGTA and 15 mM Ca^{2+} (K_d^*).

2.4 Visualization of mitochondria and lysosomes

To label mitochondria, cells were incubated with 0.05–0.2 μM Mitotracker Red CMXRos for 10–15 min in conditioned culture medium at 37°C, and labeled mitochondria were monitored in dye-free saline solution using an epi-fluorescence microscope (TE2000, Nikon, Japan) with 100 $\times$ oil/1.3 N.A. objective at room temperature. The dye was excited at 550 nm using a Polychrome IV monochromator (TILL, Germany), and the signal was observed at 590 nm every 2 s using a CCD camera (Pixelfly). For labeling of acidic compartments such as lysosomes, cells were loaded with 0.2 μM LysoTracker Red DND-99 as for the Mitotracker dye and observed with the same excitation and emission wavelengths every 1.2 s.

2.5 Visualization of F-actin

The sGFP-fABD2 construct was made by inserting fABD2 into the 326GFP-3G vector (CLONTECH Laboratories, Palo Alto, CA) using the SalI sites. Then the sGFP-fABD2 region of p326GFP 3G vector was transferred into pcDNA3.1 (Invitrogen) by restriction digestion with Blunt end (BamHI) and XhoI. For real-time monitoring of F-actin, PDEC were transfected with sGFP-fABD2 (2.2 $\mu\text{g}/\text{ml}$) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were used for experiments after one day of overexpression. For dual recording of sGFP-fABD2 and lysosomes we used a confocal microscope (LSM Meta 510, Carl Zeiss, Germany) equipped with a 63 $\times$ oil/1.4 N.A. lens. The sGFP-fABD2 and LysoTracker Red DND-99 were excited with 488 nm and 561 nm lasers, respectively. Fluorescence was observed over wavelength ranges of 505–530 nm for sGFP-fABD2 and 570–630 nm for LysoTracker Red DND-99.

For F-actin distribution at a specific time point, cells were fixed with 3.7% formaldehyde for 30 min, permeabilized with 0.1% Triton X-100 for 10 min, and stained with Alexa Fluo-568 Phalloidin (5 units/ml) in a phosphate-buffered saline solution.

2.6 Analysis of organelle mobility

Acidic compartments labeled with LysoTracker Red DND-99 appeared round and their location in subsequent images was analyzed using an autotracing function implemented in Metamorph software (Universal imaging). The autotracing algorithm first identified the boundaries of granular lysosomes using a threshold method and then tracked their x–y centers in a series of images. Mobility was highly variable between lysosomes, and even that of a single lysosome fluctuated in time. Therefore we present lysosomal mobilities as average values of multiple lysosomes (typically $n = 39$ to 48) from several cells. We randomly selected only the lysosomes that did not disappear from the focused Z-plane or

collide with other lysosomes during the control period (~1 min). Since such problems occurred most frequently with a minor fraction of lysosomes that travel over a long range, the average speed may be slightly underestimated. The speed of lysosomes, defined as (distance traveled)/(time), was calculated for each image frame (1.2 s). To reduce the fluctuation, each value presented is the mean of two adjacent points (Fig. 2). Mean-square displacement (MSD) for the n th time interval was calculated using the following equation [17,18]

$$\text{MSD}(n\delta t) = 1/(N-n) \sum_{j=1}^{N-n} ([x(j\delta t+n\delta t)-x(j\delta t)]^2 + [y(j\delta t+n\delta t)-y(j\delta t)]^2), \quad (\text{equation 1})$$

where δt is the time interval (1.2 s) at which images were taken, $x(t)$ and $y(t)$ are the coordinates of a structure at time t , and N is the total number of analyzed images. Both n and j are positive integers with $n = 1, 2, \dots, N-1$.

Mitochondria labeled with Mitotracker Red CMXRos displayed continuous network structures. Their mobility was estimated using an image subtraction function in Image J (NIH) (Fig. 3). The difference of two images resulted in bright pixels as a result of lateral movement of the organelles. Total pixel intensity in the difference images was measured after addition of a constant chosen to avoid reporting any negative pixel values. To remove cell-to-cell variation, the intensity changes were normalized by the pre-UTP value and averaged.

2.7 Fluorescence recovery after photobleaching (FRAP)

Real-time FRAP imaging experiments were conducted using confocal microscopy (LSM Meta 510) 1 day after the transfection of the sGFP-fABD2 probe in PDEC. The confocal pinhole was set to produce approximately 2 μm optical sections in the z-axis. A 30 mW argon laser (488 nm) set at 60% power with 100% transmission was used to photobleach sGFP-fABD2 within a circular region of interest (~4.2 μm diameter). Prebleach and recovery images were acquired at a rate of 1 image per 2 s using 2–3% of transmission with the same laser power. The pixel size of the images in the x–y plane was 0.28 μm .

2.8 Statistical analysis

All numerical values in the text and figures are given as mean $\pm$ SEM. n and N denote the number of analyzed lysosomes and cells, respectively. Statistical significance was determined by Student's t -test and $P < 0.05$ was considered significant. Otherwise, we noted the statistical significance.

3. RESULTS

3.1 Dynamics of lysosomes in PDEC

We labeled PDEC with Lysotracker Red DND-99 (named Lysotracker hereafter) that accumulates within acidic compartments such as lysosomes and secretory granules [19] (Fig. 1, Fig. S1, and Movie 1). To estimate the fraction of labeled lysosomes relative to secretory granules, we co-stained PDEC with Lysotracker and FM 1–43 (Fig. S2). Previously we showed that most FM 1–43-labeled round structures contained mucin [12]. Therefore granular structures that co-labeled with both dyes may be mature, acidic mucin granules, whereas the ones labeled only with Lysotracker can be regarded as lysosomes. About 5% of structures showed co-localization of the two dyes, suggesting that the majority of Lysotracker-labeled granules are lysosomes, not secretory granules in PDEC. Hence we regarded the structures labeled with Lysotracker as lysosomes for convenience.

Tracking of individual lysosomes revealed different levels of mobility. In Figure 1 we chose and analyzed three lysosomes with high ('1'), moderate ('2'), and low ('3') mobility. The lysosomes tended to continue to make multiple steps in one direction before they turned in another direction (Fig. 1B). The speed of each lysosome, defined as step distance traveled per 1.2 s, fluctuated in time mainly due to stop-and-go movements (Fig. 1C). Mobility of lysosomes can be visualized in an alternative way as cumulative distance traveled (Fig. 1D). Steeper slopes indicate higher speed of granules. In order to characterize the overall nature of lysosomal movement, we analyzed the mean-square displacement (MSD) as a function of time (Fig. 1E, See Methods). The MSD plot should rise linearly in time for a random walk, sub-linearly for caged motion or restricted motion, or supra-linearly for a directed motion [12,17]. For example, lysosome 1 shown in Figure 1B had a convex MSD curve due to its stop-and-go motion. The initial slope of an MSD curve corresponds to the apparent diffusion constant of the lysosome.

These active motions of lysosomes were drastically slowed when $[Ca^{2+}]_i$ was increased by the activation of endogenous P2Y₂ purinergic receptors in PDEC with 100 μ M UTP [11, 20. Refer to Fig. 2A for $[Ca^{2+}]_i$ rise]. The treatment reduced mobility of all lysosomes quickly (< 5 s) (Movie 1) as reflected by the reduction of speed (Fig. 1C), cumulative distance traveled (Fig. 1D), and MSD (Fig. 1E). These changes indicate that all lysosomes were frozen during the UTP treatment as we had previously shown for secretory granules labeled with FM 1-43 [12].

3.2 Control of lysosomal mobility by Ca^{2+}

Next we addressed whether the freezing of lysosomes was induced by the Ca^{2+} signal generated by UTP treatment. Figure 2A summarizes the time course of average speed of lysosomes in response to 100 μ M UTP and the accompanying rise in $[Ca^{2+}]_i$ as measured with fura-2 dye. The $[Ca^{2+}]_i$ rise and the initial freezing of lysosomes were nearly synchronous; freezing occurred within a few seconds (time constant, $\tau = 1.8$ s). The mobility of lysosomes returned very slowly during UTP treatment, suggesting a slow adaptation mechanism. In contrast to the rapid onset, the recovery time constant from lysosomal freezing after the removal of UTP was 26 s and the mobility was not fully recovered to the basal level even after 80 s. Similar onset time and recovery from freezing were observed previously for FM 1-43-labeled secretory granules [12]. In comparison, $[Ca^{2+}]_i$ rose abruptly, then decayed to an elevated steady level in the presence of UTP, and returned quickly to the basal level after UTP washout ($\tau = 9.7$ s). A lower concentration of UTP (2 μ M) also evoked the freezing of lysosomes but to a lesser extent when compared to that with 100 μ M UTP (Fig. S3). Since the Ca^{2+} signal elicited by 2 μ M UTP is oscillatory and the peak $[Ca^{2+}]_i$ values were similar in both concentrations (1.6 ± 0.24 μ M ($N = 5$) with 2 μ M UTP versus 1.9 ± 0.17 μ M with 100 μ M UTP ($N = 34$)), we speculate that sustained Ca^{2+} rise is more efficient to reduce lysosomal movement.

To test whether the freezing required a $[Ca^{2+}]_i$ rise, we applied 100 μ M UTP under conditions that do not elicit a Ca^{2+} rise, as follows. Cells were pretreated with 1 μ M thapsigargin, a blocker of sarco- and endoplasmic reticulum Ca^{2+} ATPase, to deplete internal Ca^{2+} stores in a Ca^{2+} -free external solution for at least 5 min before the recording. Application of UTP this time elicited neither $[Ca^{2+}]_i$ increase nor freezing of lysosomes (Fig. 2C and Table 1). Thus a $[Ca^{2+}]_i$ rise appears to be key for freezing. When the thapsigargin-treated cells were returned to an external solution containing 2 mM Ca^{2+} , $[Ca^{2+}]_i$ rose slowly to near 0.22 ± 0.03 μ M ($N = 11$) due to Ca^{2+} influx through store-operated Ca^{2+} (SOC) channels endogenously expressed in PDEC [12,14]. This decreased the speed of the lysosomes, suggesting that even small $[Ca^{2+}]_i$ rises (~ 50 nM) can initiate lysosomal freezing.

Finally, we checked whether the freezing of lysosomes correlated with the assembly of F-actin (Fig. 2E). Cells were pretreated with 10 μ M latrunculin B (Lat B), a sponge toxin that blocks actin polymerization by binding to actin monomers. Lat B did not change the mean speed of lysosomes before addition of UTP but it significantly attenuated freezing of lysosomes by 100 μ M UTP despite preservation of strong $[Ca^{2+}]_i$ rises up to a few micromolar (Fig. 2E and Table 1). This suggests that new F-actin formation is a significant factor for the lysosomal freezing. Application of UTP still reduced the speed of lysosomes but only very slowly, as if some remaining slow mechanisms could contribute to Ca^{2+} -dependent inhibition of lysosomal mobility.

The same conclusions can be drawn by analyzing the distribution of step speeds of lysosomes (Fig. 2B, D, and F). The histograms show that individual lysosomal movements during 1.2 s intervals occur at a wide range of speeds. The peaks of the histograms are located around 20–40 nm/s. Short-range movements of granules (<20 nm/s) can be driven by thermal agitation and partially by lysosomal motors, while longer-range movements, solely by motors. As expected, upon $[Ca^{2+}]_i$ rise by UTP, long-range movements (> 50 nm/s) decreased whereas slower events (< 50 nm/s) increased (Fig. 2B). The speed distribution of lysosomes did not change when $[Ca^{2+}]_i$ rises were suppressed by thapsigargin pretreatment (Fig. 2D). After a $[Ca^{2+}]_i$ rise through SOC, the distribution shifted toward slower speeds. Fig. 2F demonstrates the contribution of F-actin to lysosomal movement. The disruption of the existing actin cytoskeleton by Lat B treatment had little effect on the basal mobility of lysosomes since the 'Lat B' distribution was similar to the 'Control' in Fig. 2B. The 'UTP+Lat B' distribution suggests a reduction of mobility but still has some high speed events (> 50 nm/s) when compared to the 'UTP' alone in Fig. 2B.

In summary, our results suggest that a $[Ca^{2+}]_i$ increase accelerates the formation of F-actin to inhibit lysosomal movement as we had observed with secretory granules, organelles of comparable size [12].

3.3 Control of mitochondrial mobility by Ca^{2+} rise

We studied the effect of Ca^{2+} rise on mobility of another organelle, the mitochondria, which are larger than lysosomes. Our 'F-actin meshwork' hypothesis predicts the same freezing effect for mitochondria. When the cells were preincubated with Mitotracker Red CMXRos, typical intermingled worm-like structures were visible (Fig. 3A-a and Fig. S4; Movie 2).

Since mitochondria did not have a simple defined structure, the tracking method was not applicable. As a first approximation, we compared distribution of mitochondria in two images that were 30 frames apart. For visual inspection, the first and 30th images were colored red and green, respectively, and overlaid (Fig. 3A-c). The overlaid images obtained in control conditions did show some red and green pixels at different sites of the cell, indicating that mitochondria had moved to different places during the 58 s time interval. In contrast, the overlaid image during 100 μ M UTP treatment exhibited more yellow pixels and fewer red and green pixels, suggesting that the mobility of mitochondria was reduced (Fig. 3A-g). An alternative way to visualize mobility of mitochondria was to calculate the difference of two images (See Materials and Methods for details of this subtraction method and reference 12). A difference image obtained in the control period (F30-F1) contained several bright pixels (dark in the inverted image of Fig. 3A-d) due to the significant change of mitochondrial positions. The difference image obtained during UTP (F65-F36) showed fewer bright pixels (Fig. 3A-h), indicating significantly decreased mitochondrial movements. Hence we draw the same conclusion as for Figures 3A-c and 3A-g. For the time course of mitochondrial mobility we subtracted pairs of images with 5-frame intervals instead of 29-frame intervals (Fig. 3B). The mobility decreased quickly within 10 s after onset of UTP treatment, close to the limited time resolution of the subtraction method. The

immediate drop of mitochondrial mobility (by about 50%) and the slow recovery during UTP are reminiscent of the time course of lysosomal freezing. The freezing of mitochondria was tightly correlated with the Ca^{2+} signal since it was inhibited after elimination of the $[\text{Ca}^{2+}]_i$ rise by thapsigargin pretreatment (Fig. 3C, '0 Ca^{2+} ' trace). Lat B pretreatment retarded the mitochondrial freezing as observed with lysosomes (Fig. 2E and F) and secretory granules [12], suggesting that nonspecific hindrance by formation of F-actin is key for freezing of intracellular organelles. A lower concentration of UTP (2 μM) also froze mitochondria, but the effect was less than with 100 μM UTP (Fig. S5) as observed with lysosomes.

3.4 F-actin dynamics in PDEC

Previously, using Alexa 488-phalloidin, we observed a dense F-actin meshwork formed in the whole cytoplasm after $[\text{Ca}^{2+}]_i$ rise [12]. We now monitor intracellular F-actin formation in real-time using a new fluorescent probe, sGFP-tagged actin binding domain2 from fimbrin (sGFP-fABD2). This probe translocates to cell domains enriched with F-actin [21,22]. We first investigated its binding specificity to F-actin and its redistribution time within the cell.

To verify that the sGFP-fABD2 probe binds to F-actin, we fixed cells-expressing sGFP-fABD2 and then further labeled their F-actin with Alexa 568-phalloidin (Fig. 4). The distribution of sGFP-fABD2 overlapped well with Alexa 568-phalloidin labeling both in the cytoplasm and in the actin cortex (Fig. 4B–D). An intensity-correlation graph illustrates that the two probes were colocalized (Fig. 4E); image pixels with a bright Alexa 568-phalloidin signal have high sGFP-fABD2 intensity. The average correlation coefficient was 0.75. Then we performed fluorescence recovery after photobleaching (FRAP) experiments to estimate the time scale for sGFP-fABD2 probe redistribution after a sudden perturbation (Fig. 5). In Figure 5A, four different regions of interest (ROIs) in a control cell were bleached with an argon laser (488 nm) for 10 s. Fluorescence in the ROIs decreased abruptly by 50–80% and slowly recovered over several hundred seconds (Fig. 5B). Recovery fell short of the original level because a significant fraction (~20%) of the sGFP fluorophores had been bleached irreversibly as could be seen from the decay of fluorescence in unbleached ROIs. It is conventional to analyze such recoveries by fitting several exponential components. In these terms, control cells showed 39% recovery by a fast time constant (15 s) and 61% recovery by a slower time constant (101 s), and UTP-treated cells (with more F-actin) showed little recovery (7%) by a fast time constant (7 s) and 93% recovery by a slower time constant (57 s). These numbers give an order-of-magnitude estimate of the reporting speed we can expect for sGFP-fABD2 as a probe. In a control experiment, we bleached the same area twice sequentially in the absence of UTP (Fig. S6). In this case, we did not observe significant change of recovery kinetics between the first and second bleachings, suggesting F-actins and mechanisms involved in FRAP are not damaged by the laser bleaching.

Previously, using fixed PDEC and fluorescently labeled phalloidin, we had observed extensive F-actin formation in the whole cytoplasm, especially near the nucleus and on unidentified small and round organelles [12]. We expected to observe translocation of the sGFP-fABD2 probe to such areas of new F-actin formation. To address whether F-actin was being formed around intracellular organelles and the kinetics in live cells, we monitored F-actin dynamics and lysosomes simultaneously (Fig. 6 and Movie 3). PDEC were transiently transfected with the sGFP-fABD2 construct for one day, labeled with Lysotracker-Red for 15 min, and observed by confocal microscopy. sGFP-fABD2 fluorescence was seen as patches in the cytoplasm (Fig. 6Aa) similar to phalloidin staining [12]. After UTP treatment, the sGFP-fABD2 fluorescence redistributed, increasing in some regions including near the nucleus (red ROIs in Fig. 6Ac and Af) and decreasing in others (white ROIs). The average intensity in the randomly chosen ROIs that showed fluorescence increases (Fig. 6A) doubled

within 10 s (Fig. 6C) followed by a slower, prolonged increase, whereas the signal in other ROIs decreased (Fig. 6C), suggesting a dynamic reequilibration. Monitoring of lysosomes labeled with LysoTracker-Red in the same cell revealed a quick freezing of the organelles at the time when F-actin formation was first detected with the sGFP-fABD2 probe (Fig. 6Ab and C). In addition, we saw that some lysosomes marked by arrow heads already had some sGFP-fABD2 surrounding them, suggesting that a small amount of F-actin ('F-actin patches') existed around the lysosomes at rest (Fig. 6B). The quantity of F-actin near the lysosomes increased after UTP treatment. The rising time course for fluorescence in red ROIs after UTP resembled that of FRAP recovery after a bleach, suggesting that, although the time resolution of the probe is limited, the actual formation of new F-actin might take only a few seconds.

4. DISCUSSION

Together this work and our previous one [12] demonstrate that movements of intracellular organelles including lysosomes, mitochondria, and secretory granules in epithelial cells can be slowed down quickly after a Ca^{2+} rise. This general slowing is due to the formation of a cytoplasmic F-actin meshwork in response to Ca^{2+} . A new fluorescent probe, sGFP-fABD2, suggests that F-actin patches present around lysosomes are augmented after a Ca^{2+} rise, and new F-actin accumulates around intracellular organelles. This dense F-actin enrichment on the organelles results in effective and rapid retardation of their movement through the cytoplasm. Finally, the frozen organelles can be translocated toward the plasma membrane by a myosin II-dependent mechanism (See Fig. 7 and reference 12).

4.1 Cytoskeleton-dependent control of lysosomes and mitochondria in PDEC and other cell types

Intracellular organelles move in the cytoplasm by virtue of molecular motor proteins attached to them [1]. Both microtubules and F-actin, serve as cytoskeletal tracks for these movements. In PDEC, secretory granules (SGs) exhibited fast long-range movements that could be disrupted by depolymerizing microtubules with nocodazole [12]. On the other hand, short-range movement of SGs seemed to use F-actin because a slow caged motion of granules seen by MSD analysis was inhibited by Lat B. In the present study, the long-range movement of lysosomes was little affected by Lat B. The speed of individual lysosomes was highly variable and even that of single lysosomes fluctuated. The average speed of lysosomes before UTP treatment (62 nm/s) was similar to that for SGs in PDEC (82 nm/s) [12]. Others have reported faster rates of movement of organelles at 37°C versus our 22°C, e.g. lysosomes in mouse hepatoma cells (~450 nm/s) [23] or endosomes in hippocampal neurons (~850 nm/s) [24]. Such faster rates would be consistent with ours if we note that a standard Q_{10} of 3 would predict a 5-fold speeding on going from 22°C to 37°C.

Mitochondria in neurons, especially in axons, move along microtubules anterograde (from perinuclear to cell periphery) using kinesin and retrograde using dynein [25]. In those long tracks, movement of individual mitochondria can be resolved. In comparison, mitochondrial morphology in PDEC was not round but dispersed as worm-like tubular structures and, unlike in neuron, their motion was not obviously directional.

F-actin acts as a negative regulator of organelle movements in PDEC during $[\text{Ca}^{2+}]_i$ rises in this study and our previous one [12] as evidenced by the following observations: Both F-actin formation and the freezing of several organelles (1) are mediated by $[\text{Ca}^{2+}]_i$ rise, (2) overlap in onset kinetics, quickly (<10 s) after a $[\text{Ca}^{2+}]_i$ rise, and (3) are inhibited by Lat. B. Further we observed a very dense F-actin meshwork in the cytoplasm with SG-sized voids in phalloidin staining of F-actin. Use of our live-cell F-actin probe also indicated formation of F-actin around lysosomes. Thus, we hypothesize that organelles larger than 300 nm become entrapped non-selectively within a dense F-actin meshwork (Fig. 7). In astrocytes,

the movement of endosomes and lysosomes is also negatively controlled by Ca^{2+} rise after ionomycin and ATP treatments (26). When the formation of intermediate filaments is hindered, the freezing effect was reduced, suggesting that intermediate filaments in astrocytes may have similar role in organelle movement as F-actin does in PDEC.

The Hajnoczky group reported that mitochondrial movement on microtubules was strongly inhibited after a $[\text{Ca}^{2+}]_i$ rise in myoblasts [27] and that Miro, a Ca^{2+} sensor protein, is responsible for mitochondrial freezing in this cell type [28]. Similarly in neurons, Miro interrupts interaction between kinesin-1 and microtubules in a Ca^{2+} -dependent manner [29]. In myelinated nerves, high level of electrical activity and depletion of ATP to restore ion gradients reduced mitochondria mobility at the node of Ranvier [30]. This mechanism for mitochondrial freezing in muscle and neurons appears to be different from the F-actin-mediated inhibition seen in exocrine PDEC. Recovery from freezing of mitochondria is quite quick in the excitable cells but very slow in PDEC. It is not known whether Miro might be involved in the control of organelle movements in PDEC as well.

4.2 Ca^{2+} -dependent rapid formation of F-actin in PDEC

F-actin remodeling is critical for many cell functions such as cell motility [31,32] and exocytosis of secretory granules [7,12,33,34,35]. Several cellular signals including PIP_2 , Ca^{2+} , and small GTPases trigger F-actin assembly [36,37,38]. F-actin formation triggered by Ca^{2+} in PDEC is exceptionally fast compared to that triggered by lysophosphatidic acid in Swiss 3T3 fibroblasts, which takes minutes [39].

We have previously reported dynamic changes of F-actin after $[\text{Ca}^{2+}]_i$ rise in cells expressing EYFP-actin and in cells labeled with Alexa 488-phalloidin [12]. In live cells, the EYFP-actin probe redistributed in the cell during a $[\text{Ca}^{2+}]_i$ rise, and in cells fixed after a $[\text{Ca}^{2+}]_i$ rise, we observed a higher density of F-actin (phalloidin) near the nucleus compared to the rest of the cytoplasm. The time constant of EYFP fluorescence change in specific ROIs after UTP treatment was about 5 seconds, paralleling SG freezing.

The sGFP-fABD2 probe we used here provides higher contrast because it highlights locations of high F-actin density better than EYFP-actin, which creates an obscuring high background of fluorescent monomeric actin. At the same time, the sGFP-fABD2 probe is slow. If we interpreted the time constants of redistribution after bleaching as a measure of mobility, we would get effective diffusion coefficients as small as $0.03\text{--}0.2\ \mu\text{m}^2/\text{s}$ compared with $15\text{--}40\ \mu\text{m}^2/\text{s}$ for GFP in cytoplasm [40]. Since free sGFP-fABD2 ought to diffuse almost as well as GFP, we can conclude that its slow redistribution merely indicates that $>99\%$ of the probe is bound to F-actin and other targets so that its effective diffusion is slowed by repeated binding. The new F-actin probe has extended our understanding of F-actin dynamics in PDEC: The redistribution of sGFP-fABD2 fluorescence in the cytoplasm upon UTP treatment shows more clearly new fast F-actin formation and its location. Some lysosomes appeared coated more densely with F-actin than other regions in the cytoplasm, suggesting more localized F-actin polymerization around lysosomes. New F-actin formation may be initiated from the surface of the organelles and propagate into the cytoplasm, efficiently retarding movement of the organelles. An analogous rapid formation of F-actin on the surface of zymogen granules has been reported in pancreatic acinar cells [41].

4.3 Physiological role for control of mitochondrial movement in PDEC

What is the physiological function of mitochondrial arrest after Ca^{2+} rises in PDEC? It has been shown in many cell types that mitochondria take up Ca^{2+} using their high negative membrane potential and Ca^{2+} uniporters in the inner membrane [42]. Ca^{2+} accumulation in mitochondria is temporary and the ion is eventually transported back to the cytoplasm for

final clearance by other mechanisms. Some studies indicated that mitochondria interact physically with the ER, giving a close proximity that allows Ca^{2+} released from mitochondria to be harvested by the ER [43]. In myoblasts, freezing of mitochondria on microtubules upon Ca^{2+} rise facilitated local Ca^{2+} clearance [27]. The authors suggested that restriction of mitochondrial mobility promotes recruitment of mitochondria to enhance local Ca^{2+} buffering. In line with that notion, the peak level of global Ca^{2+} during UTP treatment became larger in Lat B-treated PDEC (where mitochondrial freezing is inhibited) than that in control cells (Figure 2A and E). In addition to providing a transiently localized Ca^{2+} sink, frozen mitochondria might also provide a localized enhanced source of ATP. Cytoplasmic Ca^{2+} elevation predicts a forthcoming need for cytoplasmic ATP to fuel Ca^{2+} -triggered processes such as Ca^{2+} clearance by plasma membrane and sarco/endoplasmic reticulum Ca^{2+} pumps. Local uptake of Ca^{2+} into frozen mitochondria serves as a signal that accelerates mitochondrial dehydrogenases and will enhance local mitochondrial ATP production [42].

In conclusion, we have now demonstrated in several ways that stimulation of purinergic receptors leads to the assembly of a dense F-actin network in the PDEC cytoplasm. The network impedes the motions of each kind of cytoplasmic organelle that we have been able to monitor. This polymerization of actin is initiated by elevation of cytoplasmic Ca^{2+} . A few outstanding questions remain for the future studies: Does this Ca^{2+} -mediated F-actin formation and reduction of organelle mobility occur in other cell types? What are the unique Ca^{2+} -dependent steps that initiate this major actin polymerization? What are the physiological consequences of mitochondrial and lysosomal freezing?

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

sGFP-fABD2	sGFP-fimbrin actin binding domain2
Mitotracker	Mitotracker Red DMXRos
Lysotracker	Lysotracker Red DND-99
FRAP	Fluorescence recovery after photobleaching

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Figure 1.

Dynamics of Lysotracker-labeled lysosomes in PDEC. (A) An image taken from Lysotracker-loaded PDEC. The scale bar is 5 μm . (B) Trajectories of three lysosomes marked in A are displayed in the x-y plane. Black, red, and gray lines are the trajectories at 1.2 s intervals of the organelles before, during, and after UTP (100 μM) treatment, respectively. Scale bars in μm . Speed (C), cumulative traveled distance (D), and mean-square displacement (MSD) (E) of the three labeled structures in A. Seven points successive are plotted right before UTP treatment (black circles and black Π mark in D) or 10 frames later, after UTP treatment (red circles and red Π mark in D). Red bar and symbols indicate their behaviors during 100 μM UTP.

Figure 2.

$[Ca^{2+}]_i$ rise decreases the mobility of lysosomes. (A, C, E) Average speed of the organelles (filled circles) and $[Ca^{2+}]_i$ rise (red dotted line) during 100 μ M UTP application in the absence (A) or presence of Lat B (E). The cells treated with Lat B were preincubated for 15 min before experiments. Some PDEC were pretreated with 1 μ M thapsigargin in Ca^{2+} -free solution for 5 min before the recording and challenged with UTP (C). Switching to an external solution containing 2 mM Ca^{2+} caused a slow $[Ca^{2+}]_i$ rise and lysosomal freezing. Note the different scales for $[Ca^{2+}]_i$ in A, C, and E. For comparison, speed of lysosomes in A without any drug treatment is replicated as blue dotted lines in C and E. $**P < 0.005$; Significantly different compared to before UTP or 2 Ca^{2+} treatment (gray bar) in C. $##P < 0.005$; Significantly different compared with UTP alone (blue bar) in E. (B, D, F) Histogram of speed of lysosomes in different conditions as obtained from A, C, and E, respectively. Black and red lines indicate speed of lysosomes before or during UTP treatment in the absence (B) or presence of Lat B (F). Black and red lines in D indicate speed of lysosomes before and during UTP treatment, respectively, without intracellular Ca^{2+} rise and the blue line indicates the speed during perfusion of 2 mM Ca^{2+} in the presence of UTP.

Figure 3.

Effect of $[Ca^{2+}]_i$ rise and F-actin on mitochondrial mobility. (A) Images were obtained from Mitotracker Red-labeled PDEC every 2 s. The scale bar is 4 μ m. (A-a and A-b) Two images before UTP treatment. F1 (1st) and F30 (30th) image were taken at time zero and 58 s, respectively. The two images were superposed (A-c) or subtracted (A-d). Similarly, during UTP treatment, images F36 (A-e) and F65 (A-f) were superposed (A-g) or subtracted (A-h). (B) Mitochondrial mobility calculated by subtracting each image from that 5 images before it ('subtraction' method as shown in A-d and A-h). Total pixel intensity from the same cell in A was measured and normalized to the average value before UTP treatment. (C) Averaged mitochondrial mobility under different conditions. For better visualization of three traces, we omitted error bars for each line. The data points indicated by gray bar were significantly different compared to UTP alone ($P < 0.005$, ##). Cells were stimulated with 100 μ M UTP without pretreatment ('Control'), with 1 μ M thapsigargin pretreatment in Ca^{2+} -free solution for 10 min ('0 Ca^{2+} '), or with 10 μ M Lat B for 15 min ('Lat B').

Figure 4.

Colocalization of sGFP-fABD2 and Alexa 568-phalloidin. (A) Bright field image. The scale bar indicates 28 μm . (B) sGFP-fABD2 expression in PDEC. (C) F-actin staining with Alexa 568-phalloidin in the same cell. (D) Merged image of sGFP-fABD2 and Alexa 568-phalloidin fluorescences. (E) Frequency plot of sGFP-fABD2 intensity *versus* Alexa 568-phalloidin intensity. The frequency was color-coded as scaled in the inset.

Figure 5.

Redistribution of the sGFP-fABD2 probe using FRAP analysis. (A) Confocal images of a PDEC expressing sGFP-fABD2. Four round ROIs of 4.2 μm in the cytoplasm were photobleached at time 0 s. Scale bars are 10 μm . FRAP measurement were repeated in the same cells after treatment of UTP for 1 min. (B) Summary results for FRAP experiments with ('+UTP', N = 4) and without ('Control', N = 4) UTP-treatment. The intensity from unbleached region was from control cells.

Figure 6.

F-actin reorganization monitored with sGFP-fABD2 after UTP-treatment. (A) Time-lapse confocal images of sGFP-fABD2 (Green, Aa and Ad) and lysosomal labeling with LysoTracker Red (Red, A-b and A-e) before (0 s) and at 40 s after UTP. Merged images are shown in A-c and A-f. Scale bar: 10 μ m. White and red circular ROIs were drawn at the regions showing decrease and increase of sGFP-fABD2 fluorescence, respectively. (B) A cytoplasmic region indicated with a rectangular box in A-c is expanded at different time points during UTP treatment. Arrow heads indicate LysoTracker labeled lysosomes with colocalized F-actin. (C) Average speed of LysoTracker labeled organelles (red dots). Normalized intensity changes of sGFP-fABD2 from white and red ROIs shown in A are shown with open and closed green circles, respectively. The ROIs were randomly chosen. Time 0 s and 40 s after UTP treatment are indicated by asterisks in the graph.

Figure 7.

Schematic diagram of the freezing of intracellular organelles in PDEC. At rest, lysosomes (LS, round orange), mitochondria (elongated red), and secretory granules (SG, round black) are mobile in the cytoplasm along microtubules. Some F-actin patches (green on SG or LS) exist around the organelles. Ca²⁺-dependent formation of F-actin restrains movements of the organelles nonspecifically in the cytoplasm. Based on the results from this study and reference 12.

Table 1[Ca²⁺]_i and speed of lysosomes

	Averaged [Ca ²⁺] _i during UTP treatment (μM)	Speed before UTP (nm/s)	Speed during UTP (nm/s)
UTP	0.91 ± 0.063 (N = 34)	62 ± 6.6 (n = 39)	21 ± 1.7 (n = 39) **
UTP+Lat B	1.4 ± 0.14 (N = 12) ##	57 ± 5.5 (n = 47)	42 ± 4 (n = 47) * ##
UTP+0 Ca ²⁺	0.084 ± 0.008 (N = 11) ##	52 ± 7.2 (n = 48)	57 ± 6.4 (n = 48)

* P < 0.05,

** P < 0.005; Significantly different compared to before UTP treatment at each condition.

N and n indicate the number of cells and lysosomes, respectively.

P < 0.005; Significantly different compared with UTP alone.

The basal [Ca²⁺]_i level after 0 Ca²⁺ treatment was 0.092 ± 0.009 μM (N = 11). The Ca²⁺ rise was averaged during UTP treatment. Speed of lysosomes was measured in two or three cells for each condition (N = 2 – 3).