Myosin 2 Maintains an Open Exocytic Fusion Pore in Secretory Epithelial Cells

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Many studies have implicated F-actin and myosin 2 in the control of regulated secretion. Most recently, evidence suggests a role for the microfilament network in regulating the postfusion events of vesicle dynamics. This is of potential importance as postfusion behavior can influence the loss of vesicle content and may provide a new target for drug therapy. We have investigated the role of myosin 2 in regulating exocytosis in secretory epithelial cells by using novel assays to determine the behavior of the fusion pore in individual granules. We immunolocalize myosin 2A to the apical region of pancreatic acinar cells, suggesting it is this isoform that plays a role in granule exocytosis. We further show myosin 2 phosphorylation increased on cell stimulation, consistent with a regulatory role in secretion. Importantly, in a single-cell, single-granule secretion assay, neither the myosin 2 inhibitor (–)-blebbistatin nor the myosin light chain kinase inhibitor ML-9 had any effect on the numbers of granules stimulated to fuse after cell stimulation. These data indicate that myosin 2, if it has any action on secretion, must be targeting postfusion granule behavior. This interpretation is supported by direct study of fusion pore opening in which we show that (–)-blebbistatin and ML-9 promote fusion pore closure and decrease fusion pore lifetimes. Our work now adds to a growing body of evidence showing that myosin 2 is an essential regulator of postfusion granule behavior. In particular, in the case of the secretory epithelial cells, myosin 2 activity is necessary to maintain fusion pore opening.

INTRODUCTION

Recent developments have led to exciting breakthroughs in our understanding of the core molecular components used in vesicle membrane fusion with the cell membrane (Rettig and Neher, 2002; Sudhof, 2004). But although vesicle fusion is the point where content release is initiated it is now recognized that postfusion events can be critical in regulating the kinetics and amount of released vesicle content (Albillos *et al.*, 1997; Graham *et al.*, 2002; Perrais *et al.*, 2004; Vardjan *et al.*, 2007). The mechanisms that control postfusion vesicle behavior are not well understood, but given that they control the release of content they may provide excellent therapeutic targets to up or down-regulate secretory responses in the treatment of disease (Fernandez-Peruchena *et al.*, 2005).

F-Actin and Myosin Involvement

Long-standing observations show that cortical F-actin can influence secretory processes (Burgoyne and Cheek, 1987; Trifaro and Vitale, 1993), but its exact role is not clear (Muallem *et al.*, 1995; Malacombe *et al.*, 2006). For example, it has been suggested that subplasmalemmal F-actin has to be cleared out the way to allow vesicles to dock with the

plasma membrane (Giner *et al.*, 2005). It is also possible that F-actin remodelling in some way moves vesicles from within the cell into a position where they might then fuse with the cell surface membrane (Lang *et al.*, 2000) or F-actin may form part of the poststimulation recovery of vesicles (Shupliakov *et al.*, 2002).

The findings of F-actin involvement in secretion further suggest that myosins, a family of molecular motors that bind to actin, may also play a role. There are many classes of mammalian myosins and in conjunction with the F-actin cytoskeleton all use ATP hydrolysis to promote movement or generate force (Hodge and Cope, 2000). Additional studies imply myosin Va (Varadi *et al.*, 2005; Desnos *et al.*, 2007) and myosin VI (Buss *et al.*, 2002) involvement in vesicle movement, but here we focus on the possible role of myosin 2. There is extensive evidence that myosin 2 plays a role in the secretory processes of a variety of cells, including: mast cells (Choi *et al.*, 1994; Ludowyke *et al.*, 2006), natural killer cells (Andzelm *et al.*, 2007), hippocampal (Ryan, 1999) and sensory neurons (Mochida *et al.*, 1994), chromaffin cells (Neco *et al.*, 2004), β cells (Iida *et al.*, 1997; Wilson *et al.*, 2001), exocrine cells (Segawa and Yamashina, 1989; Torgerson and McNiven, 2000; Jerdeva *et al.*, 2005), and oocytes (Becker and Hart, 1999). However, most of these studies rely on measurement of secretory output and therefore lack evidence to show the site(s) of action of F-actin and myosin 2. Also some of these studies use 2,3-butanedione monoxime, as a non-specific myosin inhibitor, and the clear and dramatic effects of this drug on cell calcium responses (Turvey *et al.*, 2003) cloud interpretation of these data.

More recently, the methods that have been developed to resolve the sequential steps of granule fusion and fission have been applied to the study of the regulation of these processes. With these techniques, accumulating evidence indicates a role for F-actin and myosin 2 in regulating the

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Abbreviations used: ACh, acetylcholine; CCK, cholecystokinin; FITC, fluorescein isothiocyanate; MLCK, myosin light chain kinase; TMRE tetramethylrhodamine ethyl ester; SRB, sulforhodamine B.

postfusion behavior of vesicles. In eggs and pancreas, F-actin coats individual vesicles immediately postfusion (Valentijn *et al.*, 2000; Sokac *et al.*, 2003; Turvey and Thorn, 2004; Nemoto *et al.*, 2004; Yu and Bement, 2007). In the pancreas, eggs, and chromaffin cells, pharmacological block of actin polymerization influences postfusion vesicle behavior (Sokac *et al.*, 2003; Neco *et al.*, 2004; Nemoto *et al.*, 2004; Larina *et al.*, 2007; Yu and Bement 2007; Doreian *et al.*, 2008) and vesicle content loss (Felmy, 2007). Likewise, inhibition of myosin 2 affects postfusion events, slowing the opening of the fusion pore (Neco *et al.*, 2008; Doreian *et al.*, 2008) and playing a role in promoting vesicle full fusion (Yu and Bement, 2007; Doreian *et al.*, 2008).

Pancreas

Methods developed to visualize single vesicles during the secretory cycle have shown postfusion F-actin coating of zymogen granules (Nemoto *et al.*, 2004; Turvey and Thorn, 2004). Treatment with the F-actin depolymerizing agent latrunculin decreases granule lifetimes (Nemoto *et al.*, 2004) and closes the fusion pore (Larina *et al.*, 2007). In this article, we show that myosin 2A is located in the apical region and is phosphorylated during cell stimulation. We further show that myosin 2 inhibition does not regulate the number of granule fusion events stimulated by agonist but instead affects the postfusion behavior of the granules, inhibition leading to closure of the fusion pore (here we use the term fusion pore to describe the structural link between the cell membrane and the granule during fusion—some other authors use this term to strictly define the small, nanometer-size pore that forms on initial fusion). Our work is consistent with recent findings in other cell types (Yu and Bement, 2007; Doreian *et al.*, 2008; Neco *et al.*, 2008) and points to myosin 2 as a key player in what is likely to be the complex regulation of postfusion granule behavior that may be important in regulating the release of vesicle content.

MATERIALS AND METHODS

Cell Preparation

Mice were humanely killed according to local animal ethics procedures. Isolated mouse pancreatic tissue was prepared by a collagenase digestion method in normal NaCl-rich extracellular solution (Thorn *et al.*, 1993), modified to reduce the time in collagenase and limit mechanical trituration. The resultant preparation was composed mainly of pancreatic lobules and fragments (50–100 cells), which were plated onto poly-L-lysine-coated glass coverslips and used within 3 h of isolation from the animal.

Confocal Imaging

Fixed specimens were imaged using a Zeiss LSM 510 Axioscope confocal laser scanning microscope, with a 63 $\times$ objective lens (numerical aperture [NA] 1.3), and an optical slice depth of $\sim 1\ \mu\text{m}$. Images were collected with the appropriate filters and captured in sequential tracks to minimize cross-talk to $<2\%$. Fluorescent probes were from Invitrogen (Carlsbad, CA). All other compounds were from (Sigma-Aldrich, Sydney, Australia). All experiments were performed at least three times. We used MetaMorph software (Molecular Devices, Sunnyvale, CA) for analysis of LSM images and Adobe Photoshop (Adobe Systems, Mountain View, CA) for image processing and presentation.

Indirect Immunofluorescence

Cell preparations were bathed in 2 mg/ml lysine-fixable, fluorescein isothiocyanate (FITC) or tetramethylrhodamine ethyl ester (TMRE) conjugated 3 kDa dextran dyes (Invitrogen, Molecular Probes), stimulated for 5 min with 10 pM cholecystokinin (CCK), fixed in freshly prepared 3.4% paraformaldehyde in PBS for 30 min, and permeabilized with 1% Triton-X 100 in PBS for 30 min. Myosin subtypes were detected using goat anti-myosin 2A or 2B antibodies (Sigma-Aldrich) and visualized with rabbit anti-goat Alexa 633 secondary antibody (Invitrogen, Molecular probes). Phospho-myosin distribution in cells was imaged using rabbit anti-phospho-myosin 2 (Ser19) antibody (Cell Signal Technology), and mouse anti-rabbit Alexa 633. F-actin was visualized in fixed and permeabilized tissue with phalloidin Alexa 633 (Invitrogen, Molecular probes), or TRITC-phalloidin (Sigma-Aldrich).

Western Blotting

Pancreatic tissue fragments were treated with myosin inhibitors for 20 min at room temperature before stimulation with acetylcholine (ACh) 10 μM for 1 min followed by atropine (100 μM). Cells were rapidly frozen in liquid nitrogen 4 min later and lysed by 3 freeze-thaw cycles. For time-course studies, cells were not drug treated, but stimulated with acetylcholine (ACh) and atropine, and harvested at various times after this. The lysate was centrifuged to isolate the cytosolic fraction. Samples were boiled in Laemmli buffer and electrophoresed on Mini-Protein II apparatus (Bio-Rad, Hercules, CA) through a 13% polyacrylamide reducing gel before transfer to Hybond-C nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) by using a Trans Blot semidry transfer cell (Bio-Rad) for Western blotting. The membrane was blocked in 5% bovine serum albumin and then probed with rabbit anti-phospho-myosin 2 antibody, or mouse anti- β -actin as loading control, at 4°C overnight. Bands were detected using anti-rabbit Alexa 680 and anti-mouse Alexa 800 and visualized on an Odyssey 2 fluorescence plate reader (Li-Cor Biosciences, Lincoln, NE). Bands were quantified using MetaMorph and expressed as a ratio of β -actin, and as a percentage of control.

Assessment of Fusion Pore Dynamics (Figure 5 and 6)

Freshly prepared cells were placed on poly-L-lysine-coated coverslips and incubated with lysine-fixable FITC-dextran (2 mg/ml) at 37°C. Lysine-fixable TMRE-dextran (4 mg/ml) was added either along with the FITC-dextran or at various times after the start of the ACh stimulation. After stimulation, cells were fixed in fresh 3.4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. To determine the TMRE/FITC (red/green) ratio, fluorescence intensity was measured in a region of interest (diameter, 0.5 μm) in granule and luminal areas. The TMRE/FITC ratio in the granules were normalized to the ratios within the adjacent lumens (see Larina *et al.*, 2007 for details). All graphs were produced in Excel (Microsoft, Redmond, WA).

Live-Cell Two-Photon Imaging (Figure 7)

We used a custom-made, video-rate, two-photon microscope using a Sapphire-Ti laser (Thorn *et al.*, 2004), with a 60 $\times$ oil immersion objective (NA 1.42; Olympus, Tokyo, Japan), providing an axial resolution (full width, half maximum) of $\sim 1\ \mu\text{m}$. We imaged exocytic events by using sulforhodamine B (SRB, 20 $\mu\text{g ml}^{-1}$; Sigma-Aldrich) and FITC as a membrane-impermeant fluorescent extracellular markers excited by femtosecond laser pulses at 800 nm, with fluorescence emission detected at 450–510 nm (FITC) and 550–700 nm (SRB).

Images (resolution of 10 pixels/ μm ; average of 15 video frames) were analyzed with MetaMorph. An epifluorescent mercury light source provided high-intensity light to photobleach the FITC extracellular dye in an $\sim 30\text{-}\mu\text{m}$ diameter field at the image plane. Exocytic event kinetics was measured from regions of interest (0.78 μm^2 ; 100 pixels) over granules. Traces were rejected if extensive movement was observed. All data are shown as mean $\pm$ SEM.

Neurite Outgrowth Assay

We tested the efficacy of Y27632 in an independent assay to validate our results on the epithelial cells. Y27632 has been well characterized to stimulate differentiation and neurite outgrowth in PC12 cells (Zhang *et al.*, 2005). PC12 cells were plated onto six-well plates at 10 cells/well and allowed to adhere. Cells were cultured with 0, 10, 30, or 50 μM Y27632 or 100 ng ml^{-1} of neurite growth factor for 48 h. Cells were then imaged by light microscopy, and the number of cells with greater than one neurite was counted. Y27632 caused neurite growth in PC12 cells in a dose-related manner, comparable at 50 μM with neurite growth factor (see Supplemental Data).

RESULTS

Location and Agonist-induced Activation of Myosin 2 in Native Secretory Epithelial Cells

Previous work has shown that myosin 2 is distributed in the apical domain of secretory epithelial cells (Torgerson and McNiven, 2000). Here, we wanted to examine the specific distribution of myosin 2 isoforms. The myosin 2 isoforms A and B were immunolocalized in isolated mouse pancreatic acinar cell clusters that were costained with phalloidin to reveal the F-actin distribution. In addition, the cells were bathed in lysine-fixable fluorescent dye (FITC), which, after fixation, labels the acinar lumen (Larina and Thorn 2005). Figure 1 shows the dotted appearance of myosin 2B in the perinuclear and basal domain but not present in the apical region. In contrast myosin 2A (Figure 1) colocalized extensively with both the luminal FITC marker and with phalloi-

Figure 1. Immunofluorescence localizes myosin 2A to the apical domain of pancreatic acinar cells. Paraformaldehyde-fixed and permeabilized pancreatic acinar clusters retain the polarized enrichment of the F-actin cytoskeleton in the subapical domain as shown with phalloidin staining. Extracellular lysine-fixable FITC dye is retained in the acinar lumens after fixation and is surrounded by the phalloidin staining as expected for the luminal structure. Myosin 2B immunostaining (MY2B) is not found close to the luminal markers instead it is found in the basal part of the cell. Myosin 2A (MY2A) in contrast is located in a narrow band along the lumen (see enlarged images at bottom of the figure).

din staining indicating that it was predominantly, although not exclusively, localized to the apical region. Our work now shows that the apical myosin isoform is likely myosin 2A and is therefore in a position to regulate the exocytic release of digestive enzymes because this occurs exclusively at the apical plasma membrane.

Myosin 2 is activated by phosphorylation of its regulatory light chain. We wished to explore what effect stimulation of secretion would have on the expression of phosphorylated myosin 2 and used Western blot techniques by using a serine-19 anti-phospho-myosin light chain 2 antibody. We examined phospho-myosin expression over time after cells were stimulated with a short application of acetylcholine (10 μ M for 1 min followed by 100 μ M atropine; unpublished data in live cells shows rapid recovery back to resting calcium levels) (Figure 2). Myosin 2 phosphorylation increases rapidly after stimulation, continuing to increase even up to 4 min later, and still maintaining an elevated level of phosphorylation 11 min after stimulation. As shown previously (Burnham *et al.*, 1988, Torgerson and McNiven 2000), physiological levels of stimulation in these cells results in a rapid and prolonged increase in myosin 2 light chain phosphorylation. The persistence of myosin phosphorylation, despite the transient stimulation, suggests that phosphatase activity in this system may be weak.

Pharmacological Manipulation of Phospho-myosin Activation

To study the pathways of myosin 2 phosphorylation and the effectiveness of drug action, we examined phospho-myosin

Figure 2. Phospho-myosin levels increase rapidly after stimulation and are maintained for a protracted time even after stimulus removal. In these experiments, cell clusters were stimulated with ACh (10 μ M) and atropine (100 μ M) applied 1 min later to stop stimulation. Then, at the indicated times after the start of stimulation, the cell cluster suspension was rapidly frozen and the tissue processed for Western blotting. Phospho-myosin band densities (A), normalized to the β -actin loading controls, showed that phospho-myosin levels increased after stimulation, reaching maximal levels by 4 min, and maintaining a high expression level even at 11 min post-ACh stimulation (B).

levels by using a Western blot assay. We inhibited myosin 2 activity with (–)-blebbistatin, a direct inhibitor of the ATPase activity of myosin (Kovacs *et al.*, 2004) and investigated its upstream regulation by using ML-9, an inhibitor of myosin light chain kinase (MLCK), the major regulator of myosin phosphorylation, or Y27632, a Rho-kinase inhibitor. The latter is expected to affect phosphorylation of myosin either directly through Rho-kinase or indirectly *via* an enhancement of phosphatase activity (Amano *et al.*, 1996). Cells were drug treated for 20 min and then stimulated for 1 min with 10 μ M ACh followed by 100 μ M atropine. Cells were lysed by rapid freeze-thaw and processed for SDS-polyacrylamide gel electrophoresis and Western blotting by using ser-19 phospho-myosin 2 antibody and β -actin loading control. Bands were quantified and corrected for β -actin and expressed as a percentage of no-drug controls. Figure 3 shows that ML-9 treatment (30 μ M) significantly decreased myosin phosphorylation to 82.6% compared with untreated controls ($p < 0.05$). In contrast, neither (–)-blebbistatin nor Y27632 (50 μ M) significantly changed phospho-myosin levels. For Y27632, we were expecting to see a change due to its direct or indirect action. To confirm that the compound was bioactive, we used Y27632 in a neurite growth assay in PC12 cells (Zhang *et al.*, 2005). Here, it has been shown that Y27632 is as effective as neurite growth factor in inducing neurite outgrowth (Zhang *et al.*, 2005), a finding we confirm (see Supplemental Data). We conclude that the negative effects of Y27632 on myosin 2 phosphorylation in the pancreatic acinar cells are truly indicative of low levels of Rho-kinase activity.

Myosin 2 Does Not Prevent Granule Fusion

To determine whether myosin 2A affected secretion we directly counted the numbers of granule fusion events per cell,

Figure 3. Drug actions on phospho-myosin levels. Cell clusters, treated with drugs for 20 min, at room temperatures were then ACh stimulated (10 μ M) for 1 min before atropine (100 μ M) was added. At 4 min after stimulation, cells were rapidly frozen and lysed. Quantitation of bands (shown in A) normalized for β -actin loading controls are expressed as a percentage of control (B). ML-9 (30 μ M) significantly decreased phospho-myosin levels in ACh stimulated cell clusters ($p < 0.05$), whereas blebbistatin (50 μ M) and Y27632 (50 μ M) had no significant effect (data from 5 replicate experiments).

stimulated by 10 pM CCK over a 5-min period. Cells bathed in a lysine-fixable fluorescent dye were treated with CCK, stimulating granule fusion with the plasma membrane and allowing the extracellular dye to enter the granule; 5 min later, cells were fixed with fresh paraformaldehyde. These fixed cells were then imaged with confocal microscopy, and the number of fluorescent granules per cell was counted. This method gives a reliable determination of granule numbers in these particular cells because fused granules stay at the cell membrane for many minutes (Thorn *et al.*, 2004). Figure 4 shows that without CCK there is a "spontaneous" count of approximately one granule per cell; something we have observed before and presumably due to neuronal and cell-to-cell regulatory mechanisms still present in the tissue fragments (Thorn *et al.*, 2004). CCK stimulation leads to a threefold increase in the numbers of fused granules per cell. This increase is not affected by either the myosin inhibitor, (–)-blebbistatin (50 μ M) or by the myosin light chain kinase inhibitor ML-9 (30 μ M). Thus, in acinar cells, inhibition of myosin 2 does not alter the number of granules secreted per cell in response to CCK stimulation. We conclude that the exocytic fusion process itself is not affected by myosin 2 inhibition. Because studies have shown that myosin inhibition can significantly reduce the amount of enzyme secreted from acinar cells (Mizuno *et al.*, 2000; Torgerson and McNiven, 2000), our data place myosin 2 as a postfusion regulator of secretion possibly through a control of the granule behavior at the plasma membrane.

Inhibition of Myosin 2 Results in Fusion Pore Closure

To study postfusion granule behavior we used a method of time-delayed sequential application of two fluorescent dyes

Figure 4. The numbers of agonist-stimulated granule fusion events are not affected by myosin 2 inhibitors. The histogram shows that cell stimulation (with 10 pM CCK) increases granule fusion events measured per cell in the 5-min period after stimulation. Neither 50 μ M blebbistatin nor 30 μ M ML-9 pretreatment 20 min before CCK addition had any significant effect on the numbers of fusion events ($n = 15$ in each group; $p = 0.9$, $p = 0.7$, respectively, compared with no drug controls).

during stimulated exocytosis (Larina *et al.*, 2007). Adding a second dye, 5 min after stimulation probes whether the fusion pore is open: if open, the dye enters then the granules are labeled; if closed, the second dye cannot enter and the first dye is trapped. Quantification of the ratio of second (TMRE) to first dye (FITC) therefore gives us a measure of fusion pore behavior, which we have used previously to show that granule fusion is often transient (Larina *et al.*, 2007). Figure 5A shows that pretreatment with (–)-blebbistatin or ML-9 before cell stimulation (with 10 pM CCK) increased the prevalence of granules with a low second to first dye ratio. To analyze the data, the TMRE/FITC fluorescence intensity within a region drawn over each granule, normalized to the dye ratio in the lumen, was calculated. If the fusion pore is open, then there will be almost equal amounts of FITC and TMRE present, and their ratio is nearly 1. Values < 0.4 indicated that less than half of the amount of dye 2 compared with dye 1 was in the granule, and the fusion pore was deemed closed. Analysis of > 50 cell clusters from 15 of these images from six experiments showed that untreated cells had a fusion pore closure rate of under 20% at 5 min after stimulation (Figure 5B). Treatment with the myosin 2 inhibitor blebbistatin, however, resulted in 58% closed granules 5 min after stimulation ($p < 0.001$, compared with controls; $n = 3$ preparations). The (+)-blebbistatin inactive enantiomer was used as a control in this experiment (with no effect). We conclude that myosin 2 inhibition leads to a marked increase in the number of closed fusion pores at 5 min after stimulation, suggesting early fusion pore closure. We wanted to examine the functional upstream regulation of myosin 2 by MLCK because in Western blot experiments (Figure 3), we show that ML-9 decreases phosphorylation. Cell fragments were treated with ML-9 and examined for fusion pore closure at 5 min after stimulation using the two-dye fixed cell technique as described. As with (–)-blebbistatin treatment, there was a marked increase in the number of granules with closed fusion pores in ML-9-treated cells (Figure 6). In similar experiments, pretreatment of tissue fragments with the Rho-kinase inhibitor Y27632 had no significant effect on the number of closed fusion pores at 5 min after stimulation (data not shown). These data suggest the Ca^{2+} /calmodulin MLCK pathway primarily regulates myosin 2 phosphorylation in these cells, with little role for Rho-kinase.

Figure 5. Blebbistatin increases the numbers of closed fusion pores. (A) Representative images of CCK stimulated clusters showing the lumens and fused granules labeled with extracellular dyes (FITC, green; TMRE, red). The FITC was present throughout the experiment and the TMRE added 5 min after stimulation with CCK (10 pM). FITC-only labeling of a granule indicates a closed fusion pore. Pretreatment with (–)-blebbistatin (50 μM) resulted in increased number of closed fusion pores. (B) The ratio of TMRE/FITC in the granules was calculated. As a value of 1.00 is consistent with the fusion pore being open and the two dyes equilibrating, lower ratios (arbitrarily set as <0.4) indicate a closed fusion pore preventing complete intragranular equilibration with extracellular TMRE. The number of these granules is significantly higher after blebbistatin treatment ($n = 201$) compared with untreated ($n = 251$) resulting in a left-shift of the frequency graph ($p < 0.0001$). The (+)-blebbistatin enantiomer was used as a negative control and showed no significant difference from no drug ($n = 183$).

Inhibition of Myosin 2 Decreases Fusion Pore Lifetimes

We further examined the behavior of the fusion pore in real-time by using live cells. We have developed a method to determine fusion pore closure using multiphoton microscopy and targeted photobleaching (Larina *et al.*, 2007). Tissue fragments bathed in extracellular FITC dye were stimulated with CCK (10 pM), allowing dye to enter exocytic cells. Then, at each cycle of photobleaching, the fluorescence of the dye within the granule decreases, with fluorescence recovery indicating that the fusion pore is open and fresh dye can enter, whereas lack of recovery indicating fusion pore closure. Photobleaching CCK-stimulated cells every 55 s, we observed fluorescence recovery in most granules ($n = 33$) over a period of 10 min after fusion (mean pore lifetime 10.44 ± 0.44 min) (Figure 7A). In contrast, after 20-min blebbistatin pretreatment (50 μM; $n = 16$) followed by CCK stimulation, fluorescence recovery in fused granules was seen in the first few cycles of photobleaching but at later cycles recovery was not seen, indicating premature closure of the fusion pore (mean pore lifetime 4.86 ± 0.43 min) (Figure 7B). To analyze the data we arbitrarily defined a

Figure 6. ML-9 dose-dependently increases the numbers of closed fusion pores. As in Figure 5, at two-dye technique was used to identify granules with closed fusion pores. ML-9 pretreatment ($n = 264$) resulted in a dose-dependent increase in the numbers of granules that predominantly are single-labeled with FITC (green). (A) Highlights of a single green granule, whereas the majority of granules are yellow in control. In contrast, after ML-9 (30 μM) treatment the majority of granules are green. (B) Quantification of these data showed that the graph of the normalized dye ratios within each granule shifted to the left indicative of an increased prevalence of closed granules ($p < 0.001$; $n = 85$ –100 granules in each dose).

slope (fitted in Excel by a simple linear regression) of <5% fluorescence recovery per minute as indicative of lack of recovery. The summarized data (Figure 7C) show that the modal values for closure of the fusion pore in control cells is 11 min compared with 4 min after blebbistatin ($p < 0.001$). We conclude that these methods of probing fusion pore behavior show that inhibition of myosin 2 decreases the fusion pore lifetimes.

Myosin 2 Does Not Affect Actin Coating of the Granules

One possible way that myosin 2 inhibition could lead to fusion pore closure is through a destabilization of the F-actin coating that is seen to cover granules immediately after fusion (Sokac *et al.*, 2003; Turvey and Thorn, 2004; Nemoto *et al.*, 2004). Phalloidin staining of the cell clusters treated with (–)-blebbistatin (50 μM), ML-9 (30 μM), or Y27632 (30 μM), however, did not affect the appearance of F-actin-coated granules (Figure 8A), nor the number of actin-coated granules (Figure 8B). There were $n > 200$ granules per group, and the difference between no drug and the treatment groups did not reach statistical significance. This data are consistent with the hypothesis that granule F-actin coating is initiated by actin nucleation probably under the control of Cdc42 (Nemoto *et al.*, 2004; Yu and Bement, 2007) and suggests that myosin 2 is specifically necessary to maintain fusion pore opening.

Figure 7. Myosin 2 inhibition decreases fusion pore lifetimes. Live cells were imaged during entry of extracellular dye into fused granules, and local photobleaching was applied to a region drawn around an individual granule. (A) Shows a typical record. The fluorescence signal increases as it enters the granule, and is bleached down in the first cycle. The granule recovers as fresh unbleached dye from the lumen enters the granule through an open fusion pore. In the controls, sequential cycles of photobleaching showed ongoing granule recovery. The short straight lines imposed on the fluorescence trace show the lines fitted by linear regression to the recovery slopes. (B) After pretreatment with 50 μ M (-)-blebbistatin we observed failure of fluorescence recovery sooner (on the fifth cycle in the example shown, shown by the asterisk [*]), indicating earlier closure of the fusion pore. (C) Frequency histogram of data from multiple experiments, indicating the times where fluorescence recovery first failed (i.e., the time of no fluorescence recovery when the fusion pore closed). The average time to fusion pore closure in control cells ($n = 33$, black bars) was 10.44 min (SEM 0.41), compared with blebbistatin-treated cells ($n = 16$, gray bars) 4.86 min (SEM 0.43), indicating significantly earlier granule closing with inhibition of myosin 2 ($p < 0.0001$).

DISCUSSION

The major finding presented is that myosin 2, probably 2A, acts on postfusion granules to maintain an open fusion pore. In independent assays, myosin and MLCK inhibition leads to an increase in the numbers of granules with closed fusion pores and a decrease in fusion pore lifetimes. Consistent with these functional assays for myosin activity, we show that agonist stimulation leads to rapid myosin phosphorylation that is maintained over many minutes. The inhibitory

Figure 8. Myosin 2 inhibitors do not affect F-actin coating of post-fusion secretory granules. The images (A) show cells stimulated for 5 min with CCK (10 pM) in the presence of an extracellular FITC dye (green) and labeled (after fixation and permeabilization) with phalloidin staining F-actin (red). Inhibition of myosin 2 or its upstream regulatory elements did not affect the pattern of coating of membrane-fused granules with F-actin, which occur as rings surrounding the granule. (B) Data from multiple separate experiments examining >200 granules in each treatment group shows that the number of F-actin-coated granules in stimulated acinar cells did not change with treatment with myosin 2 inhibitors (50 μ M (-)-blebbistatin, 50 μ M Y27632, and 30 μ M ML-9; $p = ns$ compared with controls). Bar, 5 μ m.

action of ML-9 indicates this phosphorylation is dependent on the activation of MLCK. The lack of effect of Y27632 and the maintained phosphorylation of myosin after stimulation suggests little dependence on Rho-kinase or Rho-dependent phosphatase activity. We conclude that MLCK activation leads to myosin 2 phosphorylation and, in consort with F-actin this supports a structural change in the microfilament organization that maintains fusion pore opening.

F-Actin and Myosin 2 Play a Postfusion Role during Exocytosis

The exact role of F-actin postfusion is still not clear, but evidence suggests there are two distinct sites of action: one at the fusion pore and the other in coating fused granules. In the first, F-actin dynamics regulate the behavior of the fusion pore. We have directly shown that F-actin disruption with latrunculin closes the fusion pore (Larina *et al.*, 2007). Consistent with this, Felmy (2007) shows that the expression of actin and actin associated proteins, expected to affect F-actin dynamics, influence the release of granule content. And finally, granule collapse, presumably reflecting loss of fusion pore integrity can be enhanced by F-actin disruption (Sokac *et al.*, 2003; Doreian *et al.*, 2008). The second site of actin involvement leads to the F-actin coating of fused granules in eggs and acinar cells (Segawa and Yamashina, 1989, Nemoto *et al.*, 2004; Sokac *et al.*, 2003; Turvey and Thorn, 2004). This

coating might be specific to these cell types or it might reflect difficulties in visualizing the smaller secretory granules in other cell types. Previous findings show that this coating requires actin nucleation (Sokac *et al.*, 2003; Nemoto *et al.*, 2004) and our work, showing myosin 2 activity is not part of the mechanism of F-actin coating of the granule (Figure 8), is consistent with this. The functional significance of F-actin coating is not known (Malacombe *et al.*, 2006). It is apparently not specifically required to maintain granule integrity because fused granules are still observed after drug treatments that specifically prevent F-actin coating (Nemoto *et al.*, 2004; Yu and Bement, 2007). Yu and Bement (2007) have evidence that it might be part of a contractile process, squeezing content out, an idea first put forward by Segawa and Yamashina (1989). Furthermore, F-actin coating might be an early step in the endocytic recovery of granule membrane as suggested from work in neurons (Shupliakov *et al.*, 2002).

There are some contradictions in this work on F-actin that probably reflect differences in the consequences of drug actions in different cell types, as well as possible different functions for the preexisting cortical F-actin and newly formed F-actin. For example, in some studies treatment with agents to disrupt F-actin formation destabilizes the fused granules as measured by a favoring of granule collapse (Sokac *et al.*, 2004; Doreian *et al.*, 2008) and an increase in the measured quantal size of granule content release (Doreian *et al.*, 2008). In contrast, Yu and Bement (2007) show that mild F-actin disruption (with cytochalasin D) “traps” fused granules at the plasma membrane. Consistent with this, in acinar cells that are known to have a particularly robust preexisting F-actin cytoskeleton, resistant to drug disruption (Turvey *et al.*, 2005), latrunculin treatment leads to the long-term retention of fused granules (Nemoto *et al.*, 2004). We might tentatively conclude that treatments that disrupt the preexisting F-actin cytoskeleton destabilize granules leading to collapse, whereas with more subtle treatments, or in cells with a particularly extensive F-actin cortex, granule integrity is maintained but behaviors such as fusion pore opening are affected.

Our data place myosin 2 as necessary for the maintained opening of the fusion pore. This is consistent with other reports of myosin 2 inhibition leading to a slowing of fusion pore expansion (Doreian *et al.*, 2008; Neco *et al.*, 2008) and, of course, does not exclude an additional role for myosin in providing a contractile force for expulsion of granule content (Segawa and Yamashina, 1989; Yu and Bement, 2007). To maintain fusion pore opening myosin 2 is likely to be part of a machinery that is linked to the pore membrane and linked to the surrounding F-actin cytoskeleton providing forces to pull (or maintain) pore opening.

Other Potential Regulators of the Fusion Pore

The indication that myosin 2 is a regulator of the fusion pore, places it as potential component in a fusion pore macromolecular complex. A range of other factors such as dynamin (Graham *et al.*, 2002; Tsuboi *et al.*, 2004), complexin (Archer *et al.*, 2002), calcium (Alés *et al.*, 1999; Haller *et al.*, 2001; Elhamdani *et al.*, 2006; Llobet *et al.*, 2008), syntaxin (Wang *et al.*, 2003), and amphiphysin (Llobet *et al.*, 2008) have also been shown to affect pore dynamics. Although a coherent view of the composition and regulation of the fusion pore complex has yet to emerge, it is likely, given this diversity of possible control pathways, that its regulation may subserve genuine physiological functions.

Possible Role of Fusion Pore Dynamics in Regulating Granule Content Release

The regulation of granule content release is an exciting possible function for the control of fusion pore behavior. It has been shown that different exogenous content can be differentially released from granules (Michael *et al.*, 2004; Felmy 2007). In dense-core granules, ATP content has been shown to be released rapidly and preferentially compared with peptide content (Obermuller *et al.*, 2005). Bauer *et al.* (2004) and Perrais *et al.* (2004) show that protein content can be retained after a cycle of fusion and fission and recycled back into the cell. These studies are consistent with either fusion pore size or dynamics regulating content release. Furthermore, Felmy (2007) shows that molecular weight is not the sole determinant of content release kinetics, indicating that, in addition to pore size and dynamics as potential regulators of release, the time course of intragranule matrix dissociation may also play a role.

Role of F-Actin and Myosin 2 in the Physiology of Secretion in Acinar Cells

Torgerson and McNiven (2000) originally described a decrease in enzyme secretion from acinar cells treated with myosin inhibitors. Our work now suggests that at least in part this is due to the premature closure of the fusion pore. We might imagine this would have two consequences. First, it could directly limit the release of content from the fused granule. This seems unlikely because we have shown that content release is very rapid, with the majority of content lost within a few seconds (Thorn and Parker, 2005). Second, and more likely, the closure of the fusion pore could prevent the release of content from secondary granules, fused to the first as a result of compound exocytosis (Nemoto *et al.*, 2001). In this way, granule content would equilibrate within the fused granules but could not escape to the outside.

CONCLUSIONS

We conclude that F-actin and myosin 2 play a role in maintaining an open fusion pore in pancreatic acinar cells. Our work adds to a growing body of evidence supporting the idea that microfilament network dynamics influence postfusion granule behavior.

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