Cortical F-Actin, the Exocytic Mode, and Neuropeptide Release in Mouse Chromaffin Cells Is Regulated by Myristoylated Alanine-rich C-Kinase Substrate and Myosin II

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Submitted March 10, 2009; Revised April 16, 2009; Accepted April 29, 2009

Monitoring Editor: Adam Linstedt

Adrenal medullary chromaffin cells are innervated by the sympathetic splanchnic nerve and translate graded sympathetic firing into a differential hormonal exocytosis. Basal sympathetic firing elicits a transient kiss-and-run mode of exocytosis and modest catecholamine release, whereas elevated firing under the sympathetic stress response results in full granule collapse to release catecholamine and peptide transmitters into the circulation. Previous studies have shown that rearrangement of the cell actin cortex regulates the mode of exocytosis. An intact cortex favors kiss-and-run exocytosis, whereas disrupting the cortex favors the full granule collapse mode. Here, we investigate the specific roles of two actin-associated proteins, myosin II and myristoylated alanine-rich C-kinase substrate (MARCKS) in this process. Our data demonstrate that MARCKS phosphorylation under elevated cell firing is required for cortical actin disruption but is not sufficient to elicit peptide transmitter exocytosis. Our data also demonstrate that myosin II is phospho-activated under high stimulation conditions. Inhibiting myosin II activity prevented disruption of the actin cortex, full granule collapse, and peptide transmitter release. These results suggest that phosphorylation of both MARCKS and myosin II lead to disruption of the actin cortex. However, myosin II, but not MARCKS, is required for the activity-dependent exocytosis of the peptide transmitters.

INTRODUCTION

Chromaffin cells of the adrenal medulla are a major output of the sympathetic nervous system responsible for secretion of catecholamines and vaso- and neuroactive peptide transmitters. Both classes of transmitter, catecholamines and peptides, are packaged in the same secretory organelle (Winkler, 1976; Trifaro, 1977; O'Connor and Frigon, 1984). Sympathetic stimulation evokes secretory granule fusion to the cell surface and release of its contents into the circulation. Previous studies have shown that catecholamines and peptide transmitters are differentially released in an activity-dependent manner. Light electrical stimulation, which mimics input under basal sympathetic tone, causes chromaffin cells to selectively release freely soluble catecholamines through a narrow fusion pore characteristic of Ω -form kiss-and-run exocytosis (Elhamdani *et al.*, 2006; Fulop and Smith, 2006). In contrast, under elevated electrical stimulation, mimicking input under the acute stress response (Brandt *et al.*, 1976; Kidokoro and Ritchie, 1980), the fusion pore dilates leading to full granule collapse. This mode of exocytosis maximizes quantal catecholamine release and evokes the release of

peptide transmitters from the dense granule core (Rahamimoff and Fernandez, 1997; Fulop *et al.*, 2005). Therefore, regulation of fusion pore dilation defines differential catecholamine and peptide transmitter release, an essential component of the physiological stress response. Misregulation of this process can lead to pathological conditions, including hypertension, diabetes mellitus, and depression (Habib *et al.*, 2001).

Accumulating experimental evidence has indicated a potential regulatory role for filamentous actin (F-actin) and cytoskeletal proteins in controlling the activity-dependent transition from kiss-and-run exocytosis to full granule collapse exocytosis. In resting conditions, chromaffin cells exhibit a dense subplasmalemmal F-actin cortical network that is thought to act as a physical barrier to granule recruitment to the plasma membrane during sustained stimulation (Vitale *et al.*, 1995). High stimulation leads to a partial dissolution of the F-actin cortex, a process generally interpreted as necessary to facilitate granule recruitment under sustained stimulation (Vitale *et al.*, 1991). Recently, our laboratory showed that under light stimulation and kiss-and-run exocytosis, the actin cortex remained intact (Doreian *et al.*, 2008). Furthermore, the kiss-and-run mode of exocytosis does not require dissolution of the cortical actin network. Rather, an intact cortex is required to stabilize the Ω -form fusion transient. Pharmacological disruption of the actin cortex subsequently leads to the full collapse exocytic mode independently of stimulation intensity. Investigating the mechanisms responsible for activity-dependent dissolution of the actin cortex, and thus the transition in secretory mode, is essential to understanding the physiological sympatho-adrenal stress response. The goal of this study

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E09-03-0197>) on May 6, 2009.

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Abbreviations used: APe, action potential equivalent; CgA/B, chromogranins A/B; MLCK, myosin light chain kinase; MPSP, myristoylated alanine-rich C-kinase substrate PKC substrate domain; RLC, regulatory light chain.

was to investigate the stimulus-mediated role of myosin II and MARCKS in the control of the actin cortex and to determine their role in the downstream control of catecholamine and peptide transmitter release.

In its unphosphorylated form, MARCKS is bound to and stabilizes F-actin. Phosphorylated MARCKS dissociates from F-actin to favor actin depolymerization (Cuchillo-Ibanez *et al.*, 2004). Increased cytosolic Ca^{2+} , as experienced under elevated stimulation, leads to an activation of conventional isoforms of protein kinase C (PKC; Smith *et al.*, 1998; Fulop and Smith, 2006). In this manner, elevated cell firing provides a pathway for the activation of PKC-dependent signaling events. Thus, MARCKS may play a stimulus-dependent role in regulating the actin cortex and the mode of granule fusion. Moreover, it has been shown that nonmuscle myosin II, which associates with F-actin, plays a role in regulating secretory granule fusion (Neco *et al.*, 2002). On the single granule level, inhibition of myosin II decreased the quantal size of catecholamine exocytosis (Neco *et al.*, 2004). More recent studies showed that inhibition of myosin II phospho-activation by blocking myosin light chain kinase (MLCK), prevented full granule collapse (Doreian *et al.*, 2008). Last, direct measurements of fusion pore conductance showed that block of myosin II activity slowed fusion pore dilation (Neco *et al.*, 2008; Berberian *et al.*, 2009). Together, these studies point to a potential role for both MARCKS and myosin II in regulating the actin cortex and the activity-dependent shift in exocytic mode and peptide transmitter release.

Here, we show that phosphorylation of MARCKS led to disruption of the actin cortex. Blocking this step inhibited dissolution of the actin cortex and also reduced catecholamine quantal size. However, blocking MARCKS phosphorylation did not prevent the release of the peptide transmitters pan-chromogranin A/B (CgA/B). We also show that myosin II activity under high-frequency stimulation was required to disrupt the actin cortex. Preventing myosin II activity blocked disruption of the actin cortex, reduced catecholamine quantal size, and blocked CgA/B release normally observed under high-frequency stimulation. Together, these data assign roles for activity-mediated functions of both MARCKS and myosin II in effecting full granule collapse under elevated native electrical stimulation. These data also provide insight into the respective sites of action for MARCKS and myosin II. Although both molecules control the activity-dependent disruption of the actin cortex and subsequent increase in catecholamine quantal size, only myosin II controlled the release of the peptide transmitters, whereas MARCKS phosphorylation did not. Therefore, these data define a specific order of the molecular signaling cascade responsible for the sympatho-adrenal contribution to the acute stress response.

MATERIALS AND METHODS

All experiments were performed using chromaffin cells isolated from the adrenal medullae of adult (8-wk-old) C57/BL6 mice (The Jackson Laboratory, Bar Harbor, ME). All anesthesia and euthanasia protocols were reviewed and approved by the institutional animal care and use committee of Case Western Reserve University (Cleveland, OH), an accredited oversight body (federal animal welfare assurance A3145-01). Animals were deeply anesthetized by isoflurane inhalation and killed by decapitation. All chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise noted in the text.

Cell Preparation

Adrenal glands were removed immediately after animal sacrifice and placed in an ice-cold dissociation solution containing 80 mM Na glutamate, 55 mM NaCl, 6 mM KCl, 1 mM MgCl_2 , 10 mM HEPES, and 10 mM glucose, pH 7.0,

and osmolality was adjusted to 280 mOsm. Glands were trimmed of fat, and the adrenal cortex was dissected from the medulla. Cells were isolated as described previously (Fulop *et al.*, 2005), plated on 25-mm round coverglasses, and cultured in ~3 ml of DMEM supplemented with ITS-X artificial serum substitute (1×; Invitrogen, Carlsbad, CA) and penicillin/streptomycin (20 U/ml each). The cells were incubated at 35°C in 10% CO_2 , and the experiments were carried out at ~25°C 24–48 h after the initial cell plating.

Electrophysiological Recordings

All electrophysiological recordings were performed in the perforated-patch configuration (Korn and Horn, 1989) as described previously (Chan and Smith, 2001), with some modifications. For cell electrophysiology measurements, patch pipettes were pulled from borosilicate glass. They were partially coated with molten dental wax and fire polished. The internal pipette solution contained the following composition: 135 mM Cs glutamate, 10 mM HEPES-H, 9.5 mM NaCl, 0.5 mM tetraethylammonium-Cl, and 0.53 mM amphotericin B, pH 7.2, and osmolality was 320 mOsm. Amphotericin B was prepared as a 100× stock solution in dimethyl sulfoxide daily and diluted into the standard internal solution. The electrical stimulus protocols were delivered by either an EPC-9 or EPC-10 amplifier (HEKA Elektronik, Lambrecht, Germany) controlled by Pulse, version 8.8 (HEKA Elektronik). Cell capacitance was measured by imposing a 635-Hz sine wave 25 mV in amplitude superimposed on a holding potential of -80 mV, and cell capacitance and conductance were determined by the Sine+D.C. method (Gillis, 1995) implemented through the built-in lock-in module in Pulse. Cells were stimulated with action potential equivalent (APe) waveforms embedded in the sine wave holding potential at either 0.5 or 15 Hz as described previously (Doreian *et al.*, 2008). Voltage-dependent sodium and calcium influx, elicited catecholamine release, and capacitance jumps in response to APe stimulation have been shown to be statistically identical to native AP waveforms (Chan and Smith, 2001). During all recordings, the cells were constantly superfused at a rate of ~1 ml/min with a Ringer's solution of the following composition: 150 mM NaCl, 10 mM HEPES-H, 10 mM glucose, 2.8 mM CaCl_2 , 2.8 mM KCl, and 2 mM MgCl_2 . The osmolality was adjusted to 320 mOsm with mannitol, and the pH was adjusted to 7.2. The junction potential for this internal/Ringer's solution set was measured to be approximately -13 mV, and all potentials were adjusted accordingly. For high potassium stimulation, KCl was increased to either 8 or 30 mM, and NaCl was reduced to maintain osmolality (Fulop and Smith, 2007). Cell capacitance-noise-analysis detection of Ω -form kiss-and-run granule fusion was performed as described previously (Fulop and Smith, 2006). To compare data across frequencies, stimulation protocols were balanced to evoke the fusion and release of a similar amount of catecholamine in each condition (Fulop *et al.*, 2005).

Amperometric Recordings

Amperometric recordings were also performed as described previously (Fulop *et al.*, 2005). Commercially available 5- μm -diameter carbon fiber electrodes (ALA Scientific, Westbury, NY) were used for catecholamine detection. The carbon fiber tip was cut before each recording, and the fiber was held at +650 mV. The fiber tip was positioned close to the cell membrane to minimize diffusion distance from the cell membrane. Amperometric current was recorded using a dedicated amplifier (VA-10x; ALA Scientific) with a modified head stage containing a 1-G Ω feedback resistor to minimize noise. Recorded signals were passed through a four-pole analogue Bessel filter at a cut-off frequency of 1.3 kHz and sampled at 20 kHz through an ITC-1600 (Instrutech, Port Washington, NY) into IGOR Pro (WaveMetrics, Lake Oswego, OR).

Peptide Transfection

The MPSD and MPSD-Ala peptides were synthesized by the Molecular Biotechnology Core at the Learner Research Institute (Cleveland, OH) after a previously published sequence (Trifaro *et al.*, 2000). To determine transfection efficacy, peptides were tagged with a fluorescein tag on the N terminus to provide an initial fluorescence-based report of cell transfection; however, all experiments were carried out using peptides lacking the tag to prevent interference with secondary antibody labeling. Cells were acutely transfected using the Chariot (Active Motif, Carlsbad, CA) transfection method with some modifications. The suggested transfection protocol was modified to increase efficiency in adrenal chromaffin cells as described previously (Chan and Smith, 2003). Briefly, 2 ng of Chariot reagent was dissolved in 50 μl of distilled water, whereas the peptide was prepared as a solution of 500 ng of protein in 50 μl of normal Ringer's solution. Solutions were sonicated for 30 min and kept at room temperature for 30 min to allow the Chariot-peptide complex to complete. The Chariot-peptide complex was then brought to a final volume of 5 ml in DMEM. Isolated chromaffin cells were incubated with the Chariot-peptide solution for 1 h before recording.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis for Myosin II Isoforms

Total RNA was isolated from mouse chromaffin cells at the Gene Expression and Genotyping Facility at Case Western Reserve University. RT-PCR was performed with TaqMan gene expression assays against Myh9

(Mm00502575_m1) for IIA, Myh10 (Mm00805131_m1) for IIB, and Myh14 (Mm00651358_m1) for IIC.

Imaging

Wide field fluorescence images were acquired on an IX-81 inverted microscope (Olympus, Melville, NY) with a 100 $\times$ oil immersion objective (numerical aperture = 1.3). Cells were superfused with the above described Ringer's solution. The Ringer's solution additionally contained 10 μ M aminated styryl dye AM1-43 (Biotium, Hayward, CA) when indicated in the text. Excitation illumination was provided by a Polychromator IV (TILL Photonics, Pleasanton, CA) under the control of SlideBook 4.1 image acquisition software (Intelligent Imaging, Denver, CO). Images were collected with a cooled charge-coupled device camera (Retiga EXi; QImaging Corporation, Burnaby, BC, Canada) at a set exposure time and camera gain consistent throughout a protocol data set to allow for comparison of signal magnitude between cells and to minimize photobleach during the illumination. Deconvolution was performed by the constrained iterative method built into SlideBook by using a measured point spread function.

Immunocytochemical Staining

First, cells were stimulated as described in the text. After stimulation, cells were perfused with phosphate-buffered saline containing 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 25 min. After being washed three times with 1 $\times$ phosphate-buffered saline (PBS), cells were bathed in 1 $\times$ PBS for 5 min. Cells were permeabilized using 1.5 ml of 0.15% Triton superfused into the dish and left for 30 min. Triton was removed by three washes in 1 $\times$ PBS. Next, cells were incubated for 1 h in 1% bovine serum albumin. For chromogranin A/B labeling, cells were incubated for 1 h in PBS containing a CgA/B antibody (11422; MP Biomedicals, Irvine, CA). The cells were then washed for 5 min in 1 $\times$ PBS and labeled with an Alexa Fluor-594 immunoglobulin G (IgG) secondary antibody (Invitrogen) for 1.5 h to visualize CgA/B. For MARCKS staining, cells were incubated for 2 h in PBS containing a primary goat MARCKS antibody at a 1/50 dilution (Santa Cruz Biotechnology, Santa Cruz, CA) that recognizes the C-terminal region of MARCKS. The cells were then washed for 5 min in 1 $\times$ PBS and labeled with an Alexa Fluor-488 donkey anti-goat IgG secondary antibody (Invitrogen). For phospho-MARCKS staining, cells were incubated for 2 h in PBS containing a primary rabbit MARCKS antibody (Santa Cruz Biotechnology) that recognizes the phospho-Ser159/163 sites of MARCKS at a 1/50 dilution. The cells were then washed for 5 min in 1 $\times$ PBS and labeled with an Alexa Fluor-598 donkey anti-rabbit IgG secondary antibody (Invitrogen). Phosphorylated myosin II was labeled by incubating cells for 2 h in 1 $\times$ PBS containing monoclonal antibody (mAb) that only detects phosphorylation at serine 19 of the MLC (Cell Signaling Technology, Danvers, MA). The cells were then washed for 5 min in 1 $\times$ PBS and labeled with an Alexa Fluor-488 secondary antibody (Invitrogen). Detection of myosin IIA and IIB isoforms was performed using affinity-isolated antibodies developed in rabbits by using synthetic peptides corresponding to amino acids 1950–1961 of heavy chain IIA and amino acids 1965–1976 of heavy chain IIB (Sigma-Aldrich). Actin labeling and quantification of filamentous actin (F-actin) were performed as follows: cells were incubated with 5 μ M (200 U/1.5 ml; Invitrogen) of rhodamine-conjugated phalloidin in 1.5 ml of 1 $\times$ PBS for 30 min before imaging.

Pharmacological Agents

Cells were treated with 10 μ M ML7 (Calbiochem, San Diego, CA), 25 μ M blebbistatin (BIOMOL Research Laboratories, Plymouth Meeting, PA), or 100 nM Gö 6983 (Calbiochem) as indicated in the text. All preincubations were carried out by addition of the agent to the culture medium and incubation at 35°C to facilitate reagent uptake. This preincubation was 10 min for all reagents. After pretreatment, cells were then immediately transferred to the recording chamber, superfused with the appropriate reagent-containing recording Ringer's solution and used for experimentation.

Data Analysis

Image and data analysis of fluorescence images were performed using custom-written macros in IGOR Pro (WaveMetrics). Amperometric records were analyzed on a single spike basis in IGOR Pro with a modified peak detection routine based on the "Spike" macro initially described by the Borges group (Gomez *et al.*, 2002). The original macro was modified to incorporate analysis of foot currents. Nonparametric Mann–Whitney statistical analysis of medians was performed using MINITAB, version 15 (Minitab, State College, PA). Statistical significance was tested at 99.999% ($p < 0.001$) confidence level. Statistical significance for each category plot of each mean parameter was determined by Student's *t* test. Statistical significance was tested at 95% ($p < 0.05$) confidence level. Data are expressed as mean $\pm$ SE of the mean.

RESULTS

We initiated a series of experiments designed to test the activity dependence of myosin II and MARCKS phosphor-

ylation on cortical F-actin dynamics, exocytic mode, and transmitter release in isolated chromaffin cells. Cells were held in the perforated-patch voltage-clamp configuration. Electrical stimuli consisted of voltage templates designed to quantitatively match native action potentials (APe; Chan and Smith, 2001). Stimulus trains were delivered at either 0.5 Hz to mimic input under sympathetic tone or at 15 Hz to mimic input under the sympathetic stress response (Brandt *et al.*, 1976; Kidokoro and Ritchie, 1980). Stimulation trains were balanced to evoke an equal amount of transmitter release for each frequency (Fulop *et al.*, 2005). When performing imaging experiments, we stimulated cells with graded potassium concentrations calibrated to quantitatively deliver equivalent calcium influx and evoke quantitatively equivalent secretion kinetics seen with 0.5 and 15 Hz APe stimulation, respectively (Fulop and Smith, 2007). We manipulated PKC, myosin II, MLCK, and MARCKS activities with pharmacological reagents and acute peptide transfection. Cortical F-actin, myosin II and MARCKS phosphorylation, quantal catecholamine release, and CgA/B release under low- and high-frequency stimulation were monitored.

Myosin II and MARCKS Are Necessary for F-Actin Reorganization under High Stimulation

The literature has outlined a potential role for an actin cytoskeleton and several associated molecules in the regulation of exocytosis in adrenal chromaffin and other cells. Studies have shown that strong stimulation results in the disruption of the dense actin cortex in chromaffin cells (Trifaro *et al.*, 1985, 2008; Vitale *et al.*, 1995). These studies indicated that disruption of the actin cortex removed a physical barrier for granule recruitment to the cell surface to support sustained secretion. Work from our lab showed that secretion persists under high-frequency stimulation even when the actin cortex was preserved by jasplakinolide treatment (Doreian *et al.*, 2008). This finding indicates that disruption of the cortex is not the only requirement for granule recruitment to support sustained secretion. However, stabilization of the cortex was able to shift secretory behavior to the kiss-and-run exocytic mode under conditions that would normally otherwise result in full granule collapse.

Recent studies showed that myosin II acts to regulate the exocytic fusion event on the single granule level (Doreian *et al.*, 2008; Neco *et al.*, 2008). Myosin II is a contractile motor protein that generates movement with respect to actin and has been shown to alter F-actin structures in other cell types (Cai *et al.*, 2006; Urven *et al.*, 2006; Yu and Bement, 2007). Calcium-mediated PKC-dependent activation of MLCK leads to phosphorylation of the regulatory light chain (RLC) at residue Ser19 and stimulates homomeric self-assembly and ATPase activity (Ludowyke *et al.*, 1996; Bresnick, 1999; Wilson *et al.*, 1999). The increased calcium levels observed under high electrical stimulation, coinciding with fusion pore dilation and granule collapse, have been shown to be sufficient to initiate this signaling cascade (Chan *et al.*, 2003; Fulop and Smith, 2006). Given this potential mechanism, we asked whether myosin II activity is directly affecting cortical actin and subsequently the exocytic mode and peptide release under elevated stimulation conditions.

Nonmuscle myosin II is known to have three isoforms (A, B, and C), each a separate gene product that has been shown to be differentially expressed in a number of tissues (Golomb *et al.*, 2004). We tested for both message and protein expression profiles in the adrenal medulla. We performed real-time PCR for each isoform and determined that message for all three isoforms was present in the adrenal medulla but that only the A and B isoforms were present at significant levels

Figure 1. Isoform identification and activity-dependent phosphorylation of myosin II RLC on a single cell level. (A) Images of single chromaffin cells after being fixed, permeabilized, and stained with rhodamine phalloidin to provide a fluorescence-based index of F-actin (red). Myosin IIA heavy chain (left) or myosin IIB (right) immunoreactivity (green) is also shown. Overlays of the two signals (yellow) are shown in the third image. (B) Fixed and permeabilized cells were stained for F-actin as described in A (red). In the same cells, phosphorylated myosin II regulatory light chain was probed with a mAb against an epitope containing phospho-Ser19 (second row; green). The overlay of the two signals is shown in the third row (yellow). (C) Quantified cortical staining (cell periphery and $1 \mu\text{m}$ under periphery) along with paired phospho-specific myosin II RLC immunoreactivity are represented as mean $\pm$ SEM ($n \geq 15$ for each condition). * or #, $p < 0.05$ compared with unstimulated control for myosin II or F-actin, respectively (Student's t test).

(data not shown). Next, we probed for protein expression of the isoforms with the highest message by immunostaining. Fluorescence staining for IIA and IIB show the same protein expression trend as reported for message levels by RT-PCR (Figure 1A).

We then probed for activity-dependent phosphorylation status of the RLC. Phosphorylation of the RLC is expected to activate all isoforms of myosin II. Unstimulated cells and cells stimulated with low and high potassium-containing Ringer's solution were stained with a phospho-specific an-

tibody to detect Ser19 phosphorylation of the RLC (Figure 1B). Cells were fixed and permeabilized for staining immediately after stimulation as described in *Materials and Methods*. F-actin was stained with rhodamine-conjugated phalloidin (Wieland, 1986) to track cortical disruption as a function of myosin RLC phosphorylation. Sample images from this protocol are shown (Figure 1B). No phospho-RLC staining was observed in unstimulated cells or under low potassium stimulation. Cortical F-actin remained continuous in each of these conditions. However, elevated potassium stimulation

resulted in both disruption of the actin cortex as well as phosphorylation of myosin II RLC. This effect was mimicked by pretreating cells with phorbol 12-myristate 13-acetate (PMA), a phorbol ester that activates PKC; phospho-myosin II RLC staining increased and cortical F-actin staining decreased. Pretreatment with the pharmacological MLCK inhibitor ML7 prevented phosphorylation of the RLC in PMA-treated cells, indicating that PMA effects are dependent on MLCK activity. The same cocktail (PMA + ML7) prevented the decrease in cortical F-actin staining seen in cells treated with PMA alone. Pretreatment with the PKC inhibitor Gö 6983 before high stimulation also reduced phosphorylation of RLC and prevented disruption of the cortex. Last, the data demonstrate an inverse relationship between myosin II RLC phosphorylation and F-actin staining. Together, these data indicate activity-dependent phosphorylation of myosin II RLC occurs through a PKC-mediated MLCK activity and leads to disruption of the actin cortex.

MARCKS is an F-actin cross-linking protein that aids in stabilizing F-actin networks. MARCKS is also a phosphorylation substrate for PKC and thus is expected to be phosphorylated under elevated stimulation conditions used in this study. Phospho-MARCKS has a decreased affinity for F-actin and dissociates from actin networks, resulting in destabilization of the F-actin filaments (Hartwig *et al.*, 1992; Rose *et al.*, 2001). We tested for MARCKS phosphorylation under low and high potassium stimulation protocols in our cell type. We used a polyclonal antibody to bind total MARCKS and a phospho-specific mAb to bind phospho-MARCKS at the Ser159 and Ser163 PKC phosphorylation sites in the same cell. Each antibody was raised in a different host, allowing for differential staining using different fluorescently labeled secondary antibodies. This configuration allowed us to measure fractional MARCKS phosphorylation under different stimulation conditions. In unstimulated cells, little phospho-MARCKS fluorescence was detected (Figure 2A). Similarly, with low stimulation, MARCKS phospho-specific staining remained low (Figure 2A). High stimulation or PMA treatment conversely resulted in increased phospho-MARCKS staining. Pretreatment of cells with the PKC blocker Gö 6983 prevented the increased phospho-MARCKS staining seen in untreated cells under high stimulation (Figure 2A). Phospho-MARCKS staining and total MARCKS staining were measured in all cells. Proportional phospho-staining was calculated for each cell, pooled and these data are presented in Figure 2B. These data show that strong stimulation results in PKC-dependent MARCKS phosphorylation.

Next, we tested whether perturbing normal myosin II and MARCKS function alters cortical F-actin under high stimulation. Cells were acutely transfected (see *Materials and Methods*) with a peptide inhibitor for MARCKS phosphorylation (MPSD) (Hartwig *et al.*, 1992; Trifaro *et al.*, 2000). The phospho-incompetent MPSD-Ala peptide served as a negative control (Rose *et al.*, 2001). Cells were stimulated with low- and high-intensity potassium-containing Ringer's solutions. F-actin was again stained and visualized with rhodamine phalloidin. Single cell intensity plots (Figure 3A) as well as quantified phalloidin staining (Figure 3B) show that low potassium stimulation had no effect on the distribution of the actin staining or the quantified intensity compared with unstimulated cells. Cells stimulated with high potassium Ringer's solution exhibited a dispersed punctuate actin staining and lower total fluorescence, indicating a significantly decreased amount of polymerized F-actin at the periphery. PMA treatment caused a similar F-actin staining pattern in the absence of stimulation. MPSD transfection blocked

Figure 2. Activity and PKC-dependent phosphorylation of MARCKS at the single cell level. (A) Cells were stimulated as labeled, fixed, stained with a polyclonal MARCKS antibody and counterstained with an Alexa Fluor-488 secondary antibody to detect total MARCKS (left column). Phospho-MARCKS was detected with a polyclonal antibody raised to an epitope containing phospho-Ser159/163 and counterstained with an AlexaFluor-598 secondary antibody (right column). (B) Pooled data of the ratio of phospho-MARCKS to total MARCKS staining was calculated for each cell and is represented as mean $\pm$ SEM ($n \geq 15$ cells for each condition). * $p < 0.05$ compared with control (Student's *t* test).

F-actin disruption normally observed under elevated stimulation, whereas the phospho-incompetent MPSD-Ala (alanine substituted for the serine PKC substrate residues) peptide had no effect (data not shown). Next, the effect of myosin II inhibition on activity-dependent F-actin disruption was tested. We pretreated cells with blebbistatin to prevent myosin II function. Blebbistatin blocks ATP hydrolysis and motor activity (Allingham *et al.*, 2005). Cells were stimulated as described above, and F-actin was stained with phalloidin. Resulting images showed that pretreatment with blebbistatin blocked disruption of cortical F-actin to an even greater level than in the MPSD-transfected cells.

MARCKS and Myosin II Affect the Kinetics of Catecholamine Release

Carbon fiber amperometry is a high-resolution electrochemical method for detecting catecholamine release from single secretory granules (Wightman *et al.*, 1991; Chow *et al.*, 1992). This sensitive technique has shown that quantal size, the amount of catecholamine released from a single granule, increases in a stimulus-dependent manner (Elhamdani *et al.*, 2001) probably due to a transition from a diffusion limiting restricted fusion pore under kiss-and-run exocytosis to the dilated pore under full granule collapse (Fulop *et al.*, 2005; Fulop and Smith, 2006). Representative amperometric recordings from single cells are shown (Figure 4A) from cells electrically stimulated with either 0.5- or 15-Hz APe trains. From such experiments, we analyzed single spike parameters that correlate to fusion pore dilation and thus overall exocytic mode. Spike amplitude and spike charge (an index of total catecholamine content) have been shown to increase with dilation of the fusion pore (Alvarez de Toledo *et al.*,

Figure 3. Cortical actin remains intact when myosin II or MARCKS is inhibited. Chromaffin cells were treated with inhibitors for MARCKS or myosin II and with PMA as indicated. Cells were then stimulated as labeled, fixed, permeabilized, and stained with rhodamine-conjugated phalloidin to provide a fluorescence-based index of F-actin. (A) Intensity plots from equatorial images of representative cells for each condition are provided. (B) Cortical staining was measured as in Figure 1, and data were pooled and plotted as mean $\pm$ SEM ($n \geq 15$ cells for each condition). * or •, $p < 0.05$ compared with high stimulation or PMA controls, respectively (Student's t test).

1993; Albillos *et al.*, 1997; Lindau and Alvarez de Toledo, 2003; Fulop and Smith, 2006). Cumulative probability plots for spike amplitude, the spike parameter traditionally used to quantify quantal size in the literature (Moser and Neher, 1997; Elhamdani *et al.*, 2001; Mosharov and Sulzer, 2005), are provided for each condition in Figure 4B. Measured amplitudes did not fall into a normal distribution; rather, they formed a skewed distribution. We present statistics for each data set as box-and-whisker plots for the median and range values (inset in each panel) (Fulop and Smith, 2007). Non-parametric Mann-Whitney median tests indicate that spike amplitude is significantly smaller under 0.5- versus 15-Hz APe stimulation. Table 1 contains median spike amplitude and charge values for each condition. Cells were then treated with PMA to activate PKC, and stimulated with 0.5-Hz APe trains. Single spikes were again quantified and

the cumulative probability plots for their amplitudes are provided (Figure 4C). The solid line indicates the probability plot for 15 Hz in untreated control cells and shows that PMA converts amperometric spike amplitude to the larger size observed under elevated stimulation. A significant increase also was observed for spike charge with PMA treatment at 0.5-Hz stimulation (Table 1). Catecholamine secretion evoked by 15-Hz electrical APe stimulation or by activation of PKC is more rapid than that measured under 0.5-Hz stimulation. This behavior pattern is consistent with a larger fusion pore diameter under higher stimulation intensity (Fulop and Smith, 2007). Conversely, cells treated with the PKC blocker Gö 6983 and stimulated at 15 Hz displayed statistically smaller spikes and charges compared with control (Figure 4D). The solid line indicates the probability plot for 0.5 Hz in untreated control cells and shows that blocking PKC activation converts spikes to smaller amplitude normally observed under lower frequency stimulation.

Next, we tested the effect of inhibiting the phosphorylation of MARCKS on catecholamine release. We transfected cells with the MARCKS phosphorylation inhibitor MPD and stimulated with conditions that normally evoke the larger amperometric spikes (15-Hz APe stimulation or 0.5-Hz APe stimulation in the presence of PMA). Cumulative probability plots generated from amperometric data collected under these conditions are shown along with paired control data collected from untransfected control cells (Figure 5, A and B). These data show that blocking MARCKS phosphorylation in all cases causes a decrease in catecholamine quantal size. The inset box-and-whisker plots indicate that the median spike amplitude was significantly decreased with MPD treatment compared with control cells. MPD also significantly decreased spike charge and had no effect under 0.5-Hz stimulation in the absence of PMA (Supplemental Table S1). As a further control, we used the MPD-Ala peptide, which had no effect at either 15 Hz or 0.5 Hz (Supplemental Table S1). We next isolated the role of myosin II in a similar manner by blebbistatin treatment. Cells were stimulated at either 15 or 0.5 Hz after PMA pretreatment as described above. Single amperometric spike amplitude and charge were measured again as described above. As indicated by both the cumulative probability plot and box-and-whisker plot, spike amplitudes were significantly decreased compared with control (Figure 5, C and D). Inhibiting the phosphorylation of myosin II or MARCKS both decreased quantal size evoked by 15-Hz electrical APe stimulation compared with untreated cells. Disruption of myosin II or MARCKS function favored smaller and slower spikes characteristic of kiss-and-run exocytosis. Moreover, MARCKS or myosin II inhibition had no effect on prespike foot currents (Supplemental Table S1), a parameter that correlates to formation of the initial fusion pore (Chow *et al.*, 1992; Mosharov and Sulzer, 2005). Thus, we conclude that MARCKS and myosin II do not alter the initial fusion event or the formation of the fusion pore. Rather their role is in regulation of the pore dilation after initial fusion occurs.

Variance Analysis Indicates Myosin II and MARCKS Regulate Fusion Pore Dilation

Electrical cell capacitance is proportional to cell surface area. Time-resolved capacitance measurements have long been used to provide an index of total exocytosis (increase in cell surface area) and endocytosis (decrease in surface area) in chromaffin cells (Neher and Marty, 1982). Variance of frequency-domain capacitance signals is proportional to the resistive elements of the cell equivalent circuit (Chen and Gillis, 2000). Any addition or accumulation of resistive ele-

Figure 4. PKC activation is necessary for the characteristic kinetics of catecholamine release under 15-Hz stimulation. Chromaffin cells were held at -80 mV in the perforated-patch voltage-clamp configuration and stimulated with trains of Ape at 0.5 or 15 Hz. (A) Representative traces are provided (note the difference in scale). (B) Pooled cumulative probability plots for spike amplitude are provided. Insets show box-and-whisker plots for each data set. Parameters

Table 1. Summary of amperometric data

Condition	$I_{\text{amplitude}}$ (pA)	Spike charge (pC)	Samples (cells/preparation)
0.5 Hz	1.48	0.04	14/5
15 Hz	4.58	0.25	13/5
0.5 Hz			
MPSD	1.49	0.05	10/3
MPSD-Ala	1.52	0.04	8/3
Bleb	1.56	0.07	9/3
Gö	1.71	0.07	13/4
0.5 Hz + PMA	4.63	0.20	11/3
MPSD	2.73	0.09	12/3
MPSD-Ala	4.57	0.21	11/3
Bleb	2.27	0.08	10/3
MPSD + Bleb	2.04	0.07	12/4
Gö	2.30	0.12	11/3
15 Hz			
MPSD	2.27	0.10	12/3
MPSD-Ala	4.75	0.23	9/4
Bleb	2.17	0.08	14/3
MPSD + Bleb	1.97	0.06	13/3
Gö	2.96	0.13	14/4

Single spike amplitude ($I_{\text{amplitude}}$) and spike charge (pC) were measured for each secretory event. Median values are provided. Experimental values statistically different from their controls are indicated in bold ($p < 0.001$; nonparametric Mann-Whitney median test).

ments therefore increases capacitance variance. Accumulation of Ω -figures during kiss-and-run exocytosis increases capacitance variance by an amount proportional to the total number of fusion events. During activity-dependent transition to full collapse exocytosis, electrical conductance through the Ω -figure fusion pore increases as it dilates to the point that the Ω -figure becomes indistinguishable from the cell surface, removing its contribution to the total capacitance variance. In this manner, kiss-and-run exocytosis elevates capacitance variance, whereas full collapse exocytosis does not. Thus, this parameter allows the detection of Ω -form fusion transients as an increase in capacitance variance (Fulop and Smith, 2006). We used this technique to determine whether inhibiting MARCKS and myosin II function under 15-Hz stimulation or under 0.5-Hz stimulation after PMA treat-

for the box-and-whisker plots are as follows (from left to right); smallest nonoutlier, first quartile, median, third quartile, and largest nonoutlier observation. Statistical significance for median values was determined by a Mann-Whitney nonparametric median analysis. * $p < 0.001$, $n = 14$ and 13 recordings for 0.5 and 15 Hz, respectively. (C) Cells were pretreated by bath application of 100 nM PMA (as an addition to the normal recording Ringer's solution) for 10 min and stimulated with trains of Ape at 0.5 Hz. The cumulative probability plot for spike amplitude of PMA-treated cells is shown. The solid line shows control data from untreated cells stimulated at 15 Hz for comparison (replotted from B). The inset box-and-whisker plot shows that PMA treatment acted to increase the median spike amplitude compared with control. * $p < 0.001$ compared with untreated control, $n = 11$. (D) Cells were treated with the pan-PKC inhibitor Gö 6983 and stimulated with trains of Ape at 15 Hz. The cumulative probability plot for spike amplitude of Gö 6983-treated cells is shown. The solid line shows control data from untreated cells stimulated at 0.5 Hz for comparison (replotted from B). The inset box-and-whisker plot shows that Gö treatment acted to decrease the median spike amplitude compared with control. * $p < 0.001$ compared with untreated control, $n = 11$.

Figure 5. Inhibiting MARCKS or myosin II effect the kinetics of catecholamine release under 15-Hz stimulation or with PKC activation. Chromaffin cells were voltage-clamped and stimulated as in the previous figure. (A and B) Cells were transfected with the MPSD peptide to block MARCKS activation and stimulated at 15 Hz ($n = 12$ in A) or at 0.5 Hz after PMA treatment ($n = 12$ in B). Single

ment would prevent the full granule collapse typically observed under high-stimulation conditions.

Chromaffin cells were patched and stimulated at 0.5 or 15 Hz. The SD of the capacitance signal was measured in response to stimulation (Figure 6A, δ). Variance is then determined simply as the square of the SD. A cartoon illustrating the equivalent electrical circuit of a patch-clamped cell is provided to show the additional electrical components resulting from the fused granule (Figure 6B). Mean capacitance variance was calculated, and data were pooled for each condition (Figure 6C). As expected, action potential stimulation at 0.5 Hz resulted in relatively high-capacitance variance, indicating the accumulation of Ω -figures during kiss-and-run exocytosis. Despite eliciting robust granule fusion and catecholamine release, 15-Hz stimulation did not increase capacitance variance above unstimulated control values, indicating full collapse exocytosis (Figure 6C). The role of MARCKS and myosin II activity on normal variance behavior were determined next. Chromaffin cells were again pretreated with blebbistatin or Gö 6983 and transfected with either MPSD or MPSD-Ala. We stimulated cells at 15 Hz and measured variance. Our data show that preventing the function of myosin II with blebbistatin or preventing the phosphorylation of MARCKS with MPSD kept variance levels elevated compared with untreated controls at high stimulation. Control experiments conducted in cells transfected with the inactive MPSD-Ala peptide showed normal low variance values. These results indicate that complete dilation of the fusion pore and transition to full collapse, seen with high stimulation, can be blocked by preventing the function of myosin II and MARCKS, or by inhibiting the function of PKC.

We then conducted control experiments to isolate the target of PMA-dependent PKC activation. Cells transfected with MPSD or pretreated with blebbistatin were exposed to PMA and stimulated at 0.5 Hz. PMA treatment alone resulted in low-capacitance variance, indicating fusion pore dilation to drive full collapse. Preventing the function of myosin II or MARCKS kept variance levels elevated compared with control cells, whereas the MPSD-Ala had no effect (Figure 6D). These data are consistent with the results obtained from the actin assay (Figure 3) as well as the amperometric technique (Figures 4 and 5), and indicate that PKC activation acts through both MARCKS and myosin II to play a role in regulating cortical actin and the exocytic mode.

amperometric spikes were analyzed and plotted as in the previous figure. The cumulative probability plot for spike amplitude of treated cells is shown (symbols). The solid lines represent control data from untreated cells stimulated at 0.5 Hz replotted from Figure 4B for comparison. The inset box-and-whisker plots show that disruption of normal MARCKS function decreased median spike amplitude compared with control cells. Statistical significance for median values was determined by a Mann-Whitney nonparametric median analysis. * $p < 0.001$ compared with untreated control. (C and D) Cells were pretreated with blebbistatin to directly inhibit myosin II function and stimulated at 15 Hz ($n = 14$ in C) or at 0.5 Hz after PMA treatment ($n = 10$ in D). Single amperometric spikes were analyzed and plotted. The cumulative probability plot for spike amplitude of treated cells is shown. The solid lines represent control data from untreated cells stimulated at 0.5 Hz replotted from Figure 4B for comparison. The inset box-and-whisker plots show that disruption of normal MARCKS function decreased median spike amplitude compared with control cells. Statistical significance for median values was determined by a Mann-Whitney nonparametric median analysis. * $p < 0.001$ compared with untreated control.

Figure 6. MARCKS or myosin II inhibition blocks fusion pore dilation under high stimulation or PKC-activated conditions. Capacitance variance was used to probe the roles of MARCKS and myosin II in maintenance of kiss-and-run Ω -form exocytosis. (A) A cell held in the perforated-patch configuration was stimulated at 15 Hz. Evoked membrane current (top trace) and measured cell capacitance (bottom trace) are shown for an example single AP stimulus. In response to Ca^{2+} influx, cell capacitance increases by ~ 14 fF, indicating the fusion of eight or nine granules with the cell surface. The solid lines indicate linear fits to pre- and postpulse capacitance. The dotted lines indicate the SD (δ) of these segments and the variance (δ^2) is calculated after each stimulation. (B) Cartoon illustrating the equivalent electrical circuit of a patch-clamped cell with a granule undergoing kiss-and-run exocytosis. Addition of circuit elements G_{fp} , R_v , and C_v during kiss-and-run exocytosis increase cell capacitance variance. (C) Variance was calculated throughout stimulus trains of APs delivered at 0.5 or 15 Hz in control and cells treated with agents that perturb MARCKS or myosin II function. Numbers of cells in each condition are as follows: Unstim. 9, 0.5 Hz. 12, 15 Hz. 12, Bleb 12, MPSD 15, MPSD-Ala 10, MPSD + Bleb 12, Gö 12. Pooled data for each condition are represented as mean $\pm$ SEM ($n \geq 10$ cells for each condition). * $p < 0.05$ compared with control (Student's t test). For comparison across conditions, dotted lines provide control variance values for 0.5-Hz (top) and 15-Hz stimulation (bottom). Icons indicate the mode of fusion at each control variance level. (D) Cells were treated with MARCKS or myosin II inhibiting agents as well as PMA, stimulated at 0.5 Hz, and variance was calculated. Numbers of cells in each condition are as follows: PMA. 9, Bleb. 15, MPSD 9, MPSD-Ala 9, MPSD + Bleb 12. ($n \geq 10$ cells for each condition). * $p < 0.05$ compared with control (Student's t test). Dotted lines indicate control variance for 0.5-Hz (top) and 15-Hz stimulation (bottom). Icons indicate the mode of fusion at each control variance level.

Myosin II Activity Is Necessary for Chromogranin Release

In chromaffin cells, catecholamines and peptide transmitters are copackaged in secretory granules; yet, they are differentially released as a function of stimulus intensity (Watkinson *et al.*, 1990; Takiyuddin *et al.*, 1994; Cavadas *et al.*, 2002; Fulop *et al.*, 2005). Data to this point indicate that MARCKS and myosin II regulate pore expansion. However, pore expansion and transition to full granule collapse may not necessarily result in peptide transmitter release (Angleson *et al.*, 1999). We wanted to determine the role of MARCKS and myosin II in peptide transmitter release. The most abundant peptide transmitters in chromaffin granules are CgA/B. The chromogranins are precursors to the catestatins family of neuroactive peptides (O'Connor and Frigon, 1984). Immunocytochemistry followed by a quantitative image cross-correlation analysis was used to determine whether preventing myosin II or MARCKS phosphorylation inhibited CgA/B release under elevated stimulation conditions. The approach is summarized in Figure 7A (for a description of the technique, see Zanella *et al.*, 2002; Fulop *et al.*, 2005) and is designed to estimate the probability of neuropeptide release. Cells were bathed and stimulated in the presence of AM1-43, which labels surface membrane. On endocytosis, the membrane stain is trapped. In this manner, stimulation

of cells in the presence of the dye, and subsequent washing of the dye from the bath will leave only recently internalized membrane stained; thus serving as a label for newly formed endosomes. Cross-correlation of CgA/B staining to AM1-43 staining identifies a recently fused granule that did not release peptide transmitters. Cells stimulated in the presence of AM1-43 were fixed, permeabilized, probed for CgA/B with a pan CgA/B antibody, and visualized with a rhodamine-labeled secondary antibody. Example images from this protocol are provided (Figure 7B). A Pearson's cross-correlation analysis was used to calculate the degree of spatial correlation between the CgA/B and endosomal membrane. The Pearson's algorithm calculates a magnitude-independent cross-correlation between two images and reports statistical scores between -1 and 1 , with a significance barrier for colocalization of 0.6 or greater. As shown in Figure 7C, the mean Pearson's score for low stimulation reached a highly significant value of 0.690 ± 0.020 . These data indicate reinternalization of newly formed endosomes before peptide transmitter release. This behavior is expected under the kiss-and-run mode of exocytosis (Fulop *et al.*, 2005). Pretreatment with PMA to activate PKC and low stimulation resulted in a Pearson's score of 0.350 ± 0.031 . Similarly, the mean Pearson's score for high stimulation did not reach significance at 0.351 ± 0.035 , each

Figure 7. Pearson's cross correlation analysis of peptide release. (A) Cartoon summarizing the experimental protocol is provided. Cells were bathed in 10 μ M animated styryl dye AM1-43 (green) and stimulated with either low or high potassium as described in *Materials and Methods*. Internalized fluorescence represents recently retrieved endosomal membrane. Cells were fixed 10 min after stimulation and permeabilized. CgA/B were detected with an antibody and visualized with an Alexa Fluor-594 IgG secondary antibody (red). (B) Sample images for unstimulated, low potassium-, and high potassium-stimulated cells are provided and show differential distribution of endosomal membrane depending on stimulus intensity. (C) Pearson's cross-correlation analysis was performed on data collected from low potassium- and high potassium-stimulated cells. A score greater than 0.6 (dotted line) represents a high degree of cross-correlation. Low-stimulated cells retain their protein core within their endosomes, whereas endosomes generated under high stimulation are emptied. Cells in which MARCKS activity was inhibited did not provide a significant Pearson's score. However, cells treated with blebbistatin to inhibit myosin II activity reached a highly significant value, indicating that CgA/B was strongly correlated to newly formed endosomes. $n \geq 20$ cells for each condition.

indicating release of CgA/B as expected under the full collapse mode of exocytosis. However, blocking PKC activation under

high stimulation conditions resulted in a Pearson's score of 0.606 ± 0.060 , indicating that PKC activity is necessary to facilitate peptide transmitter release.

We repeated this protocol in cells transfected with the MPSP peptide to determine whether the phosphorylation of MARCKS is required for CgA/B release. After MPSP treatment, the mean Pearson's score at high stimulation was 0.490 ± 0.048 , indicating that CgA/B did not colocalize with endosomes. Similarly, MPSP-Ala treatment had no statistically significant effect compared with untransfected cells; CgA/B did not colocalize with endosomes after high stimulation. We then determined whether activation of myosin II is required for CgA/B release. Cells were treated with blebbistatin, and the analysis was repeated. As expected, blebbistatin had no effect at low stimulation conditions (data not shown). However, the mean Pearson's score calculated from cells stimulated with high potassium and in which myosin II was inhibited reached a highly significant value of 0.630 ± 0.020 , indicating that CgA/B was strongly correlated to newly formed endosomes. Thus inhibition of myosin II under high stimulation conditions prevents CgA/B release.

DISCUSSION

Activity-dependent fusion pore dilation promotes the transition from an Ω -form kiss-and-run exocytic mode to full granule collapse. This transition has been shown to facilitate peptide transmitter release from adrenal chromaffin cells under electrical stimulation mimicking sympathetic stress input (Fulop *et al.*, 2005). Similar regulation of exocytosis has been shown to depend on fusion pore dynamics in several other cell types. For example, work in pancreatic β -cells showed that simple granule fusion is not sufficient to elicit insulin release. Rather, dilation of the fusion pore is required for exocytosis (Takahashi *et al.*, 2002). However, simple pore expansion may not necessarily result in peptide transmitter release. Work in pituitary lactotrophs showed that prolactin release is regulated at a step even beyond pore expansion (Angleson *et al.*, 1999). Here, we provide data demonstrating MARCKS and myosin II both play a role in activity-dependent rearrangement of the actin cytoskeleton and transition in mode of exocytosis. The data presented in this study indicate that both MARCKS- and myosin II-dependent control of fusion pore behavior takes place after the fusion event has already been initiated. MARCKS or myosin II inhibition did not affect the charge of amperometric spike feet, a parameter largely interpreted as dependent upon initial pore formation during fusion. However, our data suggest that myosin II plays an additional role at the sites of granule fusion to regulate peptide transmitter release.

In chromaffin cells, F-actin forms a dense cortical network near the cell periphery, which was thought to act as a physical barrier that must be dissolved before granule recruitment for sustained exocytosis (Aunis, 1998; Bader *et al.*, 2002). Previous work from our group (Doreian *et al.*, 2008) and work in the present study have shown that under low stimulus conditions, the F-actin cortex is not disrupted, rather it acts to stabilize an Ω -form fusion intermediate. We showed that recycling in this case takes place near the membrane to resupply functional secretory granules without cortical actin disassembly (Fulop *et al.*, 2005). Under high stimulation conditions, the F-actin cortex is disrupted, and granules fuse in the full collapse mode (Trifaró *et al.*, 2000; Bader *et al.*, 2002; Doreian *et al.*, 2008). In addition, myosin II activity, presumably also requiring actin as a substrate, is required to drive the granule into the collapse mode (Doreian *et al.*, 2008; Neco *et al.*, 2008) and directly acts to destabilize the

fusion pore (Berberian *et al.*, 2009) to facilitate release of peptide transmitters. Together, these data provide what seems to be a paradoxical dependence on F-actin for granule collapse. On one hand, disruption of the F-actin cell cortex is necessary to allow granule collapse; yet, control of the collapse step exhibits an additional myosin II-dependent component. It is possible that this complex dependence on F-actin identifies two pools of functional F-actin in the collapse process. Recent work in *Xenopus laevis* oocytes showed that brief perturbation of actin assembly prevented collapse of cortical granules, whereas long-term actin disruption led to rapid granule collapse into the plasma membrane (Sokac *et al.*, 2003; Yu and Bement, 2007). Interpretations from these data together suggest that pre-existing cortical F-actin provides a structural support for exocytosis and conditions in which transmitter release occurs through a narrow fusion pore. Under high stimulation, bulk cortical actin needs to be disrupted to destabilize the Ω -figure. The literature (Hartwig *et al.*, 1992; Trifaro *et al.*, 2000) and data from this study show that this process includes phosphorylation of MARCKS. In basal calcium conditions, MARCKS has been shown to act as a cross-linking protein to stabilize F-actin networks. Increased calcium and PKC activity can lead to MARCKS phosphorylation, resulting in a decreased affinity for F-actin and subsequent destabilization of the cortex (Rose *et al.*, 2001). This process was predicted to play a role in the actin-mediated granule collapse observed under elevated electrical stimulation observed in chromaffin cells. Data presented here (Figure 2) indicate that MARCKS phosphorylation does indeed occur only under high stimulus conditions and that this phosphorylation is dependent on PKC. Block of MARCKS phosphorylation inhibited activity-dependent disruption of the F-actin cortex and led to maintenance of kiss-and-run exocytosis even under elevated stimulation (Figures 3, 5, and 6).

We also show that phospho-activation of myosin RLC contributes to depolymerization of the F-actin cortex. Representative images shown in Figure 1B and quantified data in Figure 1C show that myosin II RLC phosphorylation occurs under high stimulus conditions and correlates to disruption of the actin cortex and fusion pore dilation (Figures 5 and 6). Blocking myosin II RLC phosphorylation or directly blocking myosin II motor activity prevented activity-mediated disruption of the actin cortex. Neco *et al.* (2008) showed that cells expressing a dominant-negative myosin II mutation displayed slowed fusion pore dilation. These findings were further supported and expanded upon in secretory epithelial cells where myosin II was found to regulate fusion pore size and stability (Bhat and Thorn, 2009). Data from this study show that PKC inhibitors blocked myosin light chain kinase-mediated myosin II RLC phosphorylation and subsequent disruption of the actin cortex under elevated stimulation. Thus, with low stimulation, both myosin II and MARCKS remain unphosphorylated, the actin cortex remains intact, and secretion occurs through an Ω -form kiss-and-run event. High stimulation results in PKC activation, phosphorylation of both myosin II and MARCKS, dissolution of the actin cortex, and granule collapse.

Yet, these data present an apparent paradox; granule collapse is observed only after disruption of the F-actin cortex, yet it depends on myosin II motor function. However, myosin II motor function depends on the presence of F-actin as a physical substrate. A potential second activity-dependent regulatory mechanism for focal F-actin polymerization may help explain these results. Work from several laboratories has cumulatively shown that peripheral puncta of F-actin are still observed in chromaffin cells under elevated stimu-

lation (Vitale *et al.*, 1995; Trifaro *et al.*, 2002). Data collected in bovine adrenal chromaffin cells show that actin polymerization takes place in an N-WASP and Arp2/3-dependent mechanism near or at the site of granule fusion (Gasman *et al.*, 2003). This focal F-actin assembly could provide a physical substrate for myosin II activity to generate force to effect granule collapse (Berberian *et al.*, 2009). Thus, two separate molecular signaling mechanisms may alter F-actin in opposite directions in an activity-dependent manner, with both contributing to granule collapse. This complex interplay between bulk actin depolymerization and focal actin polymerization indicates the need for careful interpretation and description of data when analyzing the effects of F-actin on exocytosis.

Full collapse exocytosis has been assumed to lead to peptide transmitter release, but this may not necessarily be the case. As has been shown in chromaffin cells (Perrais *et al.*, 2004) and other neuroendocrine cells (Angleton *et al.*, 1999), fusion pore dilation does not necessarily lead to peptide transmitter release. Although we show that both MARCKS and myosin II regulate disruption of the actin cortex, only myosin II regulates peptide transmitter release. Under physiological conditions where MARCKS and myosin II are not phospho-activated, CgA/B did not undergo exocytosis during granule fusion. Elevated stimulation and thus MARCKS and myosin II phosphorylation did result in release of the chromogranins. However, blocking MARCKS function, with intact myosin II function still led to CgA/B exocytosis. The reverse situation, block of myosin II and intact MARCKS activity, prevented CgA/B release. Thus, although MARCKS and myosin II both contribute to activity-dependent disruption of the F-actin cortex, myosin II activation plays a greater role at the level of the fusion pore and ultimately controls peptide transmitter release.

ACKNOWLEDGEMENTS

We thank Dr. Shyue-An Chan for expert help in design of the experiments as well as critical comments of the manuscript, and Katherine Trueblood-Doreian for critical comments of the manuscript. This work was supported by National Science Foundation grant IBN-0344768 and National Institutes of Health grants 1R01 NS-052123 (to C. S.) and T32 HL-07887 (for support of B. D.).

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