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Dynamin and Myosin Regulate Differential Exocytosis from Mouse Adrenal Chromaffin Cells

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Abstract

Neuroendocrine chromaffin cells of the adrenal medulla represent a primary output for the sympathetic nervous system. Chromaffin cells release catecholamine as well as vaso- and neuro-active peptide transmitters into the circulation through exocytic fusion of large dense-core secretory granules. Under basal sympathetic activity, chromaffin cells selectively release modest levels of catecholamines, helping to set the “rest and digest” status of energy storage. Under stress activation, elevated sympathetic firing leads to increased catecholamine as well as peptide transmitter release to set the “fight or flight” status of energy expenditure. While the mechanism for catecholamine release has been widely investigated, relatively little is known of how peptide transmitter release is regulated to occur selectively under elevated stimulation. Recent studies have shown selective catecholamine release under basal stimulation is accomplished through a transient, restricted exocytic fusion pore between granule and plasma membrane, releasing a soluble fraction of the small, diffusible molecules. Elevated cell firing leads to the active dilation of the fusion pore, leading to the release of both catecholamine and the less diffusible peptide transmitters. Here we propose a molecular mechanism regulating the activity-dependent dilation of the fusion pore. We review the immediate literature and provide new data to formulate a working mechanistic hypothesis whereby calcium-mediated dephosphorylation of dynamin I at Ser-774 leads to the recruitment of the molecular motor myosin II to actively dilate the fusion pore to facilitate release of peptide transmitters. Thus, activity-dependent dephosphorylation of dynamin is hypothesized to represent a key molecular step in the sympatho-adrenal stress response.

Keywords

Adrenal; Catecholamine; Fusion pore; Neuropeptide; Sympathetic stress

Introduction

Chromaffin cells of the adrenal medulla receive stimulatory input through the sympathetic splanchnic nerve (Aunis 1998). Under basal sympathetic tone, secreted catecholamines help regulate homeostatic functions including vascular tone, enteric activity, and insulin release. Acute stress causes sympathetic activation and increases catecholamine release many fold (Klevans and Gebber 1970), setting the organism into a “fight or flight” state of energy expenditure. Elevated serum catecholamine levels result in increased pulmonary ventilation, cardiac output, shunting of blood flow from the viscera to skeletal muscle and increased glucagon secretion (Habib et al. 2001). In addition to elevating serum catecholamines, stress

activation causes chromaffin cells to release vaso- and neuro-active peptide transmitters including the chromogranins (precursor peptides for the catestatins, pancreastatin, and secretolysin), neuropeptide Y, atrial natriuretic factor, tissue plasminogen activator, and enkephalin (Carmichael 1983; Crivellato et al. 2008). These peptide transmitters exist within a gel-like dense granule core (Rahamimoff and Fernandez 1997) and are co-packed with catecholamine in the same secretory granules (Winkler and Westhead 1980; Crivellato et al. 2008). Thus it was assumed that both types of transmitter are released by a single exocytic mechanism. However, this simplification is inconsistent with reports of differential release of catecholamine and peptide transmitters (Watkinson et al. 1990; Cavadas et al. 2002) and transmitter levels measured in the circulation (Takiyyuddin et al. 1994; Giampaolo et al. 2002). Consequently, the idea that chromaffin granule fusion with the plasma membrane represents the final step in the control of transmitter release is insufficient to describe the observed behavior. Regulation of peptide transmitter release must occur after fusion of the secretory granule membrane with the cell surface. Below, we discuss previous publications and we provide new data demonstrating the roles of dynamin I, syndapin, N-WASP, and myosin II in regulation of fusion pore dilation and transition from kiss and run to full collapse exocytic mode.

Activity Dependence of Catecholamine Quantal Size

Adrenal chromaffin cells exhibit a diverse variety of exo- and endo-cytic membrane trafficking behaviors depending on stimulus intensity (Smith and Neher 1997; Artalejo et al. 2002). In 2001, the Artalejo group determined that secreted catecholamine quantal size increases with elevated external calcium (Elhamdani et al. 2001). Subsequent studies provide mechanistic insight into this observation by demonstrating that chromaffin cells shift modes of exocytosis in an activity-dependent and calcium-dependent manner to control transmitter release. Electrical stimulation designed to mimic chromaffin cell action potential firing under basal sympathetic input leads to only modest elevations in basal cytosolic Ca^{2+} (Chan et al. 2003) and evokes Ω -form kiss and run fusion (Ryan 2003) of granules with the plasma membrane. Kiss and run exocytosis is characterized by transient fusion of the granule with the cell membrane, maintenance of basic granule morphology, and retention of the dense proteinaceous granule core. Granule membrane is then internalized by direct retrieval, undergoing a rapid and local recycling into functional secretory granules (Fulop et al. 2005). In this mode of exocytosis, chromaffin cells selectively release freely soluble catecholamines through a restricted fusion pore (Elhamdani et al. 2006; Fulop and Smith 2006) while a portion of catecholamine remains in the granule (Wightman et al. 1995), presumably co-sequestered in the core. In contrast, under electrical stimulation designed to mimic the sympathetic acute stress response, catecholamine quantal size increases with elevated Ca^{2+} (Fulop and Smith 2006). At the same time, fusion pore dilation and granule collapse facilitates the release of peptide transmitters (Doreian et al. 2009). Exocytosis-coupled endocytosis following fusion pore dilation internalizes surface membrane through a clathrin-mediated mechanism and is followed by prolonged processing prior to re-generation of secretory granules (Elhamdani et al. 2001; Chan and Smith 2003).

Regulation of Fusion Pore Dilation

Over the last few decades, highly sensitive electrophysiological as well as electrochemical assays for secretion have been developed. These advances led to a re-examination of adrenal physiology at greater resolution, with a growing focus on determining the mechanism for activity-dependent differential transmitter release. Work from our group (Fulop and Smith 2006; Doreian et al. 2009), as well as others (Elhamdani et al. 2006) has combined to provide a simple size-exclusion hypothesis for this process. As outlined above and schematized in Fig. 1, an activity-dependent regulation of the fusion pore, through a shift in

exocytic mode, provides a size-dependent selectivity filter for transmitter sub-classes. Such a regulatory role for fusion pore expansion was predicted over a decade ago (Rahamimoff and Fernandez 1997) but only recently has experimental evidence for this been obtained (Elhamdani et al. 2006; Fulop and Smith 2006).

The pore-dilation hypothesis was based on studies showing a Ca^{2+} -dependent control of fusion pore dynamics in diverse cell types including horse eosinophil (Hartmann and Lindau 1995), pancreatic β -cells (Takahashi et al. 2002), and peritoneal mast cells (Fernandez-Chacon and Alvarez de Toledo 1995). Initial experimental support for post-fusion regulation of peptide transmitter exocytosis came from rat pituitary lactotrophs. Angleson and Betz described a novel mechanism whereby dopamine-dependent cytosolic cAMP signaling regulated the release of the dense granule core at a step after granule collapse (Angleson et al. 1999). Moreover, work in bovine chromaffin cells showed that secretion differs even between peptide transmitters as a function of size and mobility. The Almers group (Perrais et al. 2004) showed that neuropeptide Y (MW = 1,080) readily underwent exocytosis after granule fusion while tissue plasminogen activator (MW = 70,000) was only released after a significant delay, if at all. Work from our lab (Fulop et al. 2005) showed that activity-dependent dilation of the fusion pore can screen molecules according to molecular weight and leads to chromogranins release only under full collapse exocytosis evoked by elevated stimulation.

Considerable efforts are currently defining specific roles for SNARE proteins (Fang et al. 2008; Dean et al. 2009) as well as phospholipid and related phospho-regulatory molecules (Gong et al. 2005; Zhang et al. 2009) in the initial stages of granule fusion and pore formation. Yet, the molecular basis for the physiologically important regulation of pore dilation remains poorly understood. In early observations, the activity-dependent transition in exo- and endocytosis from what is now recognized as kiss and run to a full collapse fusion mode showed a dependence on calcineurin activity (Engisch and Nowycky 1998; Chan and Smith 2001). Of the potential calcineurin substrates, dynamin is a likely target in this context (Cousin and Robinson 2001) and may represent a key molecule in the control of the fusion pore (Graham et al. 2002).

Activity Dependence of Dynamin I Function

Dynamin is a GTPase found to be necessary for cleavage of newly forming synaptic endosomes (Praefcke and McMahon 2004). The *D. melanogaster* temperature-dependent dynamin I mutant, *shibire*, results in an accumulation of coated invaginations at the neuromuscular junction (van der Blik and Meyerowitz 1991) and a depletion of synaptic vesicles under persistent stimulation (Ramaswami et al. 1994). Utilization of the non-hydrolyzable GTP analog, GTP- γ -S (guanosine, 5'-O-[3-thiotriphosphate]) to disrupt dynamin's GTPase activity directly inhibits fusion pore closure (Holroyd et al. 2002). These and other examples led to the interpretation that dynamin I is essential for rapid recycling of secretory vesicles (Cousin and Robinson 2001; Tsuboi et al. 2004). Blocking dynamin function disrupts efficient granule recycling in neuroendocrine cells (Artalejo et al. 1995) and inhibits synaptic vesicle endocytosis completely (Newton et al. 2006). Recent imaging data by total internal reflection fluorescence microscopy indicate that dynamin is constitutively present at the site of granule exo- and endocytosis (Felmy 2009).

Dynamin has been found associated with secretory granules in adrenal chromaffin cells (Galas et al. 2000). The initial step in dynamin activation is an increase in cytosolic calcium during stimulation. Calcium binds to calmodulin, which then allosterically activates the protein phosphatase calcineurin. Dynamin is a primary substrate for calcineurin dephosphorylation at multiple serine residues (Cousin and Robinson 2001; Anggono et al.

2006). Dephosphorylation of dynamin reveals binding sites for accessory proteins required for clathrin-mediated endocytosis including endophilin and amphiphysin (McMahon et al. 1997; Cousin and Robinson 2001). The dynamin pleckstrin-homology (PH) domain binds phosphatidylinositol 4,5-bisphosphate (PIP₂) (Salim et al. 1996), a lipid component of the plasmalemma. Upon association with the membrane, dynamin GTPase activity increases and membrane scission activity is enhanced (Lin and Gilman 1996) leading to pinching off of dynamin-collared clathrin-coated invaginations and formation of new endosomes. Disrupting this chain of molecular interactions at any step results in the block of clathrin-mediated endocytosis. However, another consequence of dynamin dephosphorylation has also been documented, indicating a second potential action for dynamin. Previous work in adrenal chromaffin cells indicates that the dynamin I isoform plays a key role in regulating catecholamine secretion through regulation of quantal size (Artalejo et al. 1997; Chen et al. 2005) and rapid granule recycling (Artalejo et al. 1995). Disruption of dynamin/PIP₂ association alters secretory fusion pore behavior (Gong et al. 2005). Transfection with a peptide inhibitor for dynamin SH3-domain binding (Shupliakov et al. 1997) affects both exocytosis and endocytosis of chromaffin secretory granules (Fulop et al. 2008). The later study showed that, in chromaffin cells, dynamin-dependent membrane trafficking events were differentially dependent on the intensity of cell stimulation. Acute transfection with the dynamin SH3 peptide fragment inhibited granule re-internalization specifically under low frequency stimulation as expected. However, catecholamine quantal size was significantly decreased under high frequency stimulation. These data demonstrated that dynamin I activity is required under high frequency stimulation for the full granule collapse mode of exocytosis, thus suggesting a dual function for dynamin I in both kiss and run as well as full collapse membrane cycling. Further studies need to be conducted in neuroendocrine chromaffin cells to test the effect of total dynamin I deletion on either kiss and run or full collapse exocytosis.

Work by the Robinson and Cousin groups provides a mechanistic framework for the activity-dependent regulation of dynamin phospho-status and its effect on membrane trafficking. Over several studies they showed an activity-mediated, calcineurin-dependent dephosphorylation of dynamin I that acts to facilitate syndapin binding that in-turn is essential for membrane internalization in neuronal synaptosomes (Cousin and Robinson 2001; Clayton et al. 2009). To test if a similar activity-mediated dephosphorylation of dynamin I may occur in neuroendocrine chromaffin cells, we performed single cell immunostaining. Previously unpublished data provided in Fig. 2 demonstrate immunostaining for phospho-dynamin (see legend for abbreviated Methods). We used a phospho-specific antibody raised against Ser-774, a residue within the proline-rich domain (PRD) syndapin-binding pocket and a calcineurin substrate. We found phospho-Ser-774 immunoreactivity decreased significantly under stimulation with 30 mM external potassium; a chemical stimulus calibrated to match intracellular Ca²⁺ as well as catecholamine secretion expressed under high frequency action potential stimulation (Fulop and Smith 2007). Stimulation with low concentrations of external potassium (10 mM) elicited catecholamine secretion matching kiss and run fusion under basal chromaffin cell stimulation (Fulop and Smith 2007) but failed to alter dynamin Ser-774 phosphorylation. Thus, as in neuronal preparations utilized by Cousin and Robinson's groups, adrenal chromaffin cells respond to elevated stimulation with a decreased dynamin phosphorylation at Ser-774. This dephosphorylation correlates to the shift in exocytic mode from kiss and run to full granule collapse.

Myosin Motor Proteins and Exocytosis in Chromaffin Cells

Myosin motor proteins have been established as regulatory molecules that control the availability of secretory granules. Chromaffin cells express myosin V and several members of the non-muscle myosin II gene family. A body of work has demonstrated that myosin V

helps to mobilize chromaffin granules from the interior space of the cell to the periphery (Trifaro et al. 2008). Upon stimulation, myosin V dissociates from the granules (Rose et al. 2002). Myosin II is activated by phosphorylation of the regulatory light chain subunits by a Ca^{2+} -dependent activation of myosin light chain kinase (MLCK). Thus, myosin II motor function is activated through elevated cytosolic Ca^{2+} as experienced under increased cell firing. At the cell periphery, myosin II plays a role in regulating final steps of granule recruitment to the plasma membrane (Neco et al. 2004). Myosin II phosphorylation increased the rate of fusion pore dilation (Neco et al. 2008) while inhibition of myosin II activity led to a stabilization of the secretory fusion pore in adrenal chromaffin cells (Berberian et al. 2009). Myosin II is selectively phospho-activated under elevated firing conditions where it drives collapse of the X-form kiss and run fusion event to full granule collapse (Doreian et al. 2008) and chromogranin release (Doreian et al. 2009). Together, these myosin II-dependent processes represent key molecular steps that control pore dilation and thus increase catecholamine quantal size and control the release of neuropeptide transmitters under elevated adrenal stimulation.

Linking Dynamin to Myosin: The Role for Syndapin in Pore Expansion

Syndapin, also called protein kinase C and casein kinase substrate in neurons (PACSIN), is part of a family of cytoplasmic phosphoproteins that interact with N-WASP, synaptojanin, and dynamin (Qualmann et al. 1999; Anggono et al. 2006). Recent evidence has demonstrated that N-WASP, the Arp2/3 complex, and F-actin accumulate at sites of exocytosis and endocytosis (Gasman et al. 2004). Further work demonstrated that dynamin-mediated endocytosis is dependent on syndapin and cytoskeletal rearrangement (Kessels and Qualmann 2002, 2006). Thus, dynamin and syndapin are involved in focal F-actin coordination during membrane trafficking events.

As outlined above, myosin II activity is required for full granule collapse and expulsion of peptide transmitters. The control of this exocytic process requires that myosin II is active at the site of granule fusion, either directly pulling upon the granule membrane or through dynamic re-arrangement of filamentous actin surrounding the granules. This action could compress the granule or exert a force driving full granule collapse. Furthermore, dynamin I is dephosphorylated at the PRD Ser-774 syndapin-binding pocket only under high stimulation conditions. This leads to a working hypothesis where syndapin is recruited to bind de-phosphorylated dynamin I under high stimulation conditions. This, in turn, recruits the syndapin-binding partners N-WASP and Arp2/3 to coordinate F-actin at the site of fusion. The hypothesized dynamin–syndapin–N-WASP–Arp2/3–F-actin and myosin II multi-molecular complex is what we refer to as the pore expansion complex (Fig. 1), and may represent myosin II dependence for pore expansion as described (Neco et al. 2008; Doreian et al. 2009). To test this initial hypothesis, we determined the effects of inhibiting syndapin/dynamin binding and N-WASP activation on activity-dependent fusion pore dilation and present the findings in Fig. 3. We measured granule collapse where N-WASP activity was compromised by pre-treatment with wiskostatin. We also utilized the DynI_{768–784}AA peptide fragment that serves as a competitive inhibitor to dynamin I/syndapin binding (Anggono et al. 2006; Clayton et al. 2009). The DynI_{768–784}AA peptide has been shown to compete with native dynamin I and blocks dynamin I/syndapin-dependent synaptic vesicle cycling. The phospho-mimetic DynI_{768–784}EE peptide does not bind syndapin and serves as a negative control for the active inhibitory peptide. We synthesized each peptide as previously described (Anggono et al. 2006) with the addition of a fluorescein tag to confirm transfection.

We utilized a perforated-patch electrophysiological voltage-clamp approach to determine the status of fusion pore dilation by measuring cell capacitance and calculating variance of the

signal. Cell capacitance is an index of cell surface area; increasing with exocytic fusion of secretory granules and decreasing with endocytic membrane internalization (Neher and Marty 1982). When measured in the frequency domain, variance of the cell capacitance signal can be adapted to provide a diagnostic assay for exocytic mode. Granule fusion through full collapse exocytosis, in which the fusion pore fully dilates, maintains a low capacitance variance. Kiss and run exocytosis increases the complexity of the cell electrical equivalent circuit by adding the electrical components of the fusion pore. Thus, accumulation of Ω figures under kiss and run exocytosis increases capacitance variance (Chen and Gillis 2000; Fulop and Smith 2006). Patch-clamped cells were either left unstimulated as control or stimulated with trains of action potentials at 0.5 or 15 Hz. Data were pooled for each condition and are presented (Fig. 3). As expected, we found no difference between control and the 0.5 Hz condition. However, the 15 Hz condition showed a specific increase in capacitance variance in the DynI₇₆₈₋₇₈₄AA-transfected cells. No increase in variance was observed in the DynI₇₆₈₋₇₈₄EE-transfected cells (data not shown). Likewise, inhibition of N-WASP, by pre-treating cells with wiskostatin (20 μ M), mimicked this effect. Variance was elevated under 15 Hz stimulation, but had no effect at 0.5 Hz stimulation. These data point to a role for dynamin I/syndapin binding and N-WASP activation in the dilation of fusion pore and transition from kiss and run to full collapse normally observed under elevated stimulation. Control data confirmed these DynI₇₆₈₋₇₈₄AA- and wiskostatin-dependent effects were not due to inhibition of Ca²⁺ influx or inhibition of granule fusion in general (data not shown), but were limited to regulation of pore dilation.

Fusion Pore Regulation as a Key Element of the Stress Response

The adrenal medulla accepts various frequency inputs from the innervating splanchnic nerve depending on the sympathetic state. Input is translated into specific hormonal profiles in an activity-dependent manner to help meet metabolic demand (Klevans and Gebber 1970; Crivellato et al. 2008). In essence, the adrenal medulla acts as a differentiator for sympathetic activity, increasing catecholamine quantal size and releasing neuro- and vaso-active peptide transmitters in an activity-dependent manner through the regulated dilation of the secretory fusion pore. In this report we review literature and provide previously unpublished data supporting an activity-dependent dephosphorylation of dynamin I at Ser-774 as a critical first step in the regulation of fusion pore dilation, facilitating the differential transmitter release consequent from the transition from kiss and run to full collapse exocytosis. Yet, the challenge in the future will be to incorporate contemporary mechanistic understanding of fusion pore dilation obtained in isolated chromaffin cells to the intact tissue of the sympatho-adrenal signaling complex. How do physiologically important modulating factors such as splanchnic synaptic input and autocrine/paracrine signaling contribute to fusion pore behavior? How do native cell–cell contacts and local intracellular signaling mechanisms between individual chromaffin cells and between chromaffin and glial cells affect this process? To this end, our current working models of the molecular and cellular mechanism for adrenal chromaffin cell function will need to be tested in a more systems-oriented preparation to preserve the physiological context of the adrenal medulla.

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Fig. 1.

Proposed mechanism for activity-dependent pore expansion. A proposed activity-dependent regulation of fusion pore expansion in adrenal chromaffin cells. The *upper* path outlines proposed kiss and run exocytosis as expressed under modest stimulation while the *lower* path exhibits proposed activity-dependent dephosphorylation of dynamin I that in turn leads to recruitment of the pore expansion complex (syndapin, N-WASP, Arp2/3, and F-actin). In this manner, the dense granule core, including sequestered peptide transmitter contents, are selectively secreted under full collapse exocytosis triggered by elevated stimulation

Fig. 2.

Activity-dependent dephosphorylation of dynamin I. Phosphospecific dynamin immuno-reactivity was not altered by modest potassium stimulation but decreased significantly under high-potassium stimulation. Methods: Isolated mouse chromaffin cells were treated with control HEPES-buffered Ringer or potassium containing Ringer (“Low Stim.” = 10 mM K⁺ and “High Stim.” = 30 mM K⁺) for 5 min, to mimic low and high native stimulation (Fulop and Smith 2007). Following stimulation, cells were immediately fixed with 4% paraformaldehyde in phosphate buffer for 30 min as previously described (Doreian et al. 2009). Phospho-dynamin I (Ser-774) was detected by incubation with a phospho-specific antibody (Invitrogen PS774). Phospho-dynamin immune-reactivity was determined by an Alexa488-conjugated secondary antibody, imaged and quantified as described (Doreian et al. 2009). Quantified data for fluorescence IR is supplied in the *top* plot and representative images for each condition are provided below each category (*scale bar* = 10 μ m). Sample size and *P* values are as follows; control = 17, low stim. = 15, high stim. = 14, * *P* < 0.001, one-way ANOVA)

Fig. 3.

Dynamin I and N-WASP inhibition blocks transition to full collapse. Perturbation of dynamin–syndapin-binding blocked the activity-dependent decrease in capacitance variance observed under 15 Hz stimulation seen in control cells. Likewise, inhibiting N-WASP auto-activation also blocked the activity-dependent decrease in capacitance variance. Methods: Cells were either transfected with DynI_{769–784}AA phospho-box competitive syndapin-binding inhibitor or DynI_{769–784}EE phospho-mimetic negative control peptide or pretreated the N-WASP inhibitor wiskostatin (20 μ M; Sigma-Aldrich). Cells were then held in the perforated-patch configuration (Fulop et al. 2005) and stimulated with 0.5 or 15 Hz trains of action potential equivalent voltage templates (Chan and Smith 2001). Cell capacitance variance was measured (Fulop and Smith 2006) to determine the mode of granule fusion. Capacitance variance data from untransfected control cells as well as cells transfected with DynI_{769–784}AA peptide or treated with wiskostatin are plotted. *Dotted lines* reflect control variance measured under either 0.5 Hz stimulation (*upper line*) or 15 Hz stimulation (*lower line*). Sample size and *P* values are as follows; unstimulated control = 9, 0.5 Hz control = 9, 15 Hz control = 12, 0.5 Hz DynIAA = 14, 15 Hz DynIAA = 14, 0.5 Hz Wisko. = 10, 15 Hz Wisko = 11. * *P* < 0.001, one-way ANOVA)