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Dynamin I plays dual roles in the activity-dependent shift in exocytic mode in mouse adrenal chromaffin cells

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Abstract

Under low stimulation, adrenal chromaffin cells release freely-soluble catecholamines through a restricted granule fusion pore while retaining the large neuropeptide-containing proteinaceous granule core. Elevated activity causes dilation of the pore and release of all granule contents. Thus, physiological differential transmitter release is achieved through regulation of fusion pore dilation. We examined the mechanism for pore dilation utilizing a combined approach of peptide transfection, electrophysiology, electrochemistry and quantitative imaging techniques. We report that disruption of dynamin I function alters both fusion modes. Under low stimulation, interference with dynamin I does not affect granule fusion but blocks its re-internalization. In full collapse mode, disruption of dynamin I limits fusion pore dilation, but does not block membrane re-internalization. These data suggest that dynamin I is involved in both modes of exocytosis by regulating contraction or dilation of the fusion pore and thus contributes to activity-dependent differential transmitter release from the adrenal medulla.

Keywords

Electrophysiology; Capacitance; Fusion Pore; Dynamin I; Clathrin; Chromaffin

Introduction

Chromaffin cells of the adrenal medulla package multiple classes of transmitters and neuroactive peptides into large dense-core secretory granules. These granules contain both small, freely-soluble transmitters that include ATP and catecholamines as well as larger peptide transmitters packaged in a proteinaceous dense granule core. Neuropeptides include chromogranins, neuropeptide Y, atrial natriuretic factor and enkephalin [1]. Chromaffin cells are innervated by the sympathetic splanchnic nerve [2]. Upon sympathetic stimulation, secretory granules fuse with the cell surface in a calcium dependent manner. Previous studies have established that chromaffin cells regulate the activity-dependent release of catecholamine versus the larger proteinaceous neuropeptide-containing core by limiting the size of the fusion pore between granule lumen and cell exterior [3,4]. Under low frequency stimulation set by the basal sympathetic tone, an Ω -form 'kiss and run' fusion transient results in selective release of low molecular weight, soluble catecholamines through a restricted fusion pore [5]. Elevated stimulation frequency, as experienced under the sympathetic stress response, results in a shift

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in exocytosis from an Ω -form fusion event to a mode where the fusion pore dilates until the granule collapses into the cell surface [6], and both catecholamine as well as the neuropeptide-containing core are expelled from the granule lumen.

Such activity-dependent regulation of fusion pore dynamics has several physiological implications. Rapid dilation of the fusion pore acts to complete exocytosis of the granule content, while a restricted fusion pore can limit the rate of release. Stabilization of the pore before dilation also acts to maintain segregation of the granule and cell plasma membrane [7]. Reversal of the fusion pore represents the endocytosis of the granule membrane, terminating the fusion event prior to release of the dense granule core [3,8,9]. The molecular mechanism of fusion pore regulation has received much attention over the years. Several studies have investigated the role of the small GTPase, dynamin I, in the regulation and reversal of the fusion pore in neuroendocrine cells [10,11]. However, description of the specific molecular mechanism that determines whether a fusion pore closes versus dilates, selecting between 'kiss and run' and 'full collapse' in chromaffin cells, remains sparse. The importance of this transition, and thus determination of the mode of exocytosis, is of particular physiological significance in the adrenal chromaffin cells where it seems to be responsible for the activity-dependence of release for different transmitter classes [3,5]. This transition contributes to the shift in metabolic status of the organism from a basal 'breed and feed' to the stress-induced 'fight or flight' response [12,13].

It is the overall goal of this study to delineate the molecular steps required for activity-mediated shift in the mode of granule fusion and subsequent membrane re-internalization in the chromaffin cells of the adrenal medulla. We utilize single cell electrophysiological, electrochemical, quantitative fluorescence imaging and acute peptide transfection techniques to investigate permissive conditions for maintenance of Ω -form 'kiss and run' exocytosis versus dilation of the pore leading to full granule collapse. Our data confirm the basic finding that low frequency stimulation, designed to mimic conditions experienced under basal sympathetic tone, elicits a transient Ω -form exocytosis and endocytosis. Granule fusion and subsequent fission is dependent upon the normal function of dynamin I but is not dependent on normal association of synaptotagmin to endophilin, a key step in clathrin-mediated endocytosis [14]. Block of membrane internalization under 'kiss and run' mode does not transition granules fusion to full collapse; rather granules are maintained in an Ω -figure with a restricted fusion pore to the cell surface. We further show that stimulation at elevated frequencies, designed to mimic sympathetic input under the acute stress response, reverses this dependence and elicits a full collapse mode of fusion that requires conventional synaptotagmin-endophilin-mediated uptake to retrieve membrane from the cell surface. Moreover, disruption of normal dynamin I-amphiphysin function has the effect of limiting the dilation of the fusion pore under elevated firing conditions. Thus, dynamin I represents a key molecule in the transition of adrenal chromaffin cell secretory behavior.

Materials and Methods

Cell preparation

Chromaffin cells were isolated from the adrenal medullae of adult C57/BL6 mice (Jackson Laboratories, Bar Harbor, ME) as previously described [3]. All anesthesia and euthanasia protocols were reviewed and approved by the institutional animal care and use committee (IACUC) of Case Western Reserve University, an accredited oversight body (federal animal welfare assurance # A3145-01). During recording, the cells were superfused in a Ringer solution of the following composition (in mM); 150 NaCl, 10 HEPES-H, 10 Glucose, 2.8 CaCl_2 , 2.8 KCl, 2 MgCl_2 . Final osmolarity was adjusted to 320 mOsm with mannitol. Experiments in which 10 or 30 mM K^+ was used as a secretagogue utilized an isotonic substitution of KCl for NaCl. Cells were stimulated by switching from perfusion in normal

Ringer to elevated potassium Ringer for 10 minutes in 10 mM K⁺-Ringer or 5 minutes in 30 mM K⁺-Ringer to match the durations of 0.5 and 15 Hz APe trains, respectively (see [15] for further details). Additionally, both control and experimental protocols were alternated such that experiments for several data sets were run on each cell preparation. Sample numbers for each data set were determined by an analysis of the variance versus mean values.

Electrophysiological recording

For cell electrophysiology measurements, patch pipettes were pulled from borosilicate glass. They were partially coated with molten dental wax and fire polished. All electrophysiological recordings in this study were performed in the perforated patch configuration. The perforated patch pipette solution contained (in mM): 135 CsGlutamate, 10 HEPES-H, 9.5 NaCl, 0.5 TEA-Cl and 0.53 amphotericin B; pH 7.2 and osmolarity 320 mOsm. Amphotericin B (Sigma, St. Luis, MO) was prepared as a 100x stock solution in DMSO (Sigma) daily and diluted into the standard internal pipette solution. Patched cells were allowed to perforate to less than 30 M Ω series resistance prior to recording. Liquid junction potential was measured to be approximately 13 mV and all potentials were corrected for this factor. Cells were held at -80 mV command potential. Cells with leak currents greater than -30 pA were discarded from analysis. Voltage clamp records were acquired through an EPC-10 amplifier (HEKA Elektronik, Lambrecht Germany) under control of 'Pulse' software (v 8.80; HEKA Elektronik). Cell capacitance (Cm) was estimated using a software "Sine + D.C." lock-in module with a sine wave amplitude of 25 mV at 635 Hz. Patched cells that exhibited sufficient access conductance and low leak were stimulated with trains of simulated action potentials at either 0.5 or 15 Hz. In order to compare data across frequencies, stimulation protocols were balanced to evoke the fusion and release of a similar amount of catecholamine in each condition [3].

Cell capacitance variance analysis was performed as previously described [5] with the following modification for circumstances specific to cells where endocytosis is blocked. Variance is a function of the resistance of the cell equivalent circuit. With an inhibited endocytic function, cell surface area increases unabated and acts to decrease cell membrane resistance. Thus, variance measured from all cells was corrected for increased surface area, and thus decreased cell input resistance, from pre-stimulus values (see [5] for a full derivation of the variance method) by updating Rm (membrane resistance) and Cm (membrane capacitance) with each stimulus. This allows for the detection of Ω -figures independent of the non-specific convolution introduced by large changes in cell size.

Electrochemical amperometric recordings

Amperometric currents were recorded through a dedicated amplifier (VA-10x with a 1 G Ω head stage, ALA Scientific). Commercially available carbon fiber electrodes (ALA Scientific, Longneck, NY) of 5 μ m tip diameter were used for amperometric catecholamine detection. Carbon fibers were cut fresh prior to each recording. A +650 mV potential was placed on the carbon fiber once it was in the bath, and the background current was allowed to relax to a steady value. If the resting current was larger than 10 pA or was unstable, the fiber was re-cut or replaced. Oxidative amperometric currents, indicating catecholamine release, were passed through an analog 1.3 kHz Bessel filter and sampled at 20 kHz through an ITC-1600 (Instrutech, Port Washington, NY) with an acquisition macro written in IGOR Pro (WaveMetrics Inc., Lake Oswego, OR). Amperometric records were analyzed with a modified peak detection routine based on the 'Spike' macro initially described by the Borges group [16].

Imaging

Fluorescence images were acquired on an Olympus IX81 inverted microscope with a 100x oil immersion objective (N.A. = 1.3). Cells were superfused with the above-described Ringers. The Ringer additionally contained 10 μ M AM1-43 (Biotium, Hayward, CA) or 10 μ M FM2-10

(Invitrogen, Carlsbad CA) as indicated in the text. Excitation light was provided by a monochromator (TILL Photonics, Eugene OR) that was shuttered to illuminate cells only during image acquisition. Images were captured with a cooled CCD camera (QImaging Corporation, Burnaby BC, Canada) under the control of SlideBook software (v. 4.10; 3I Inc. Denver, CO) at a set exposure time of 50 ms to allow for comparison of signal magnitude between cells and to minimize photobleach during the illumination, and stored at 12-bit resolution. Image acquisition and data analysis were performed using custom-written macros in IGOR Pro. Cell intensity was background-subtracted and internalized dye was calculated as the average intensity inside the cell cytosol.

Peptide transfection

The Dyn peptide (figure 1A) was synthesized by Elim Biopharmaceuticals (Hayward, CA) after a previously-published sequence [17] and the PP-19 peptide (figure 1A) was synthesised by Sigma Genosys (The Woodlands, TX, USA) after a previously published sequence [18]. Cells were transfected by using the Chariot (Active Motif, Carlsbad, CA) transfection method. The transfection reaction was modified from the manufacturer's suggested protocol to increase efficiency in the adrenal chromaffin cell preparation (determined empirically). The reactions were prepared as follows: 2 ng Chariot reagent was dissolved in 50 μ l distilled water, the peptide was prepared as a solution of 500 ng protein in 50 μ l normal Ringer [19]. These solutions were sonicated 30 minutes and kept at room temperature for 30 minutes to allow the Chariot-peptide complex to form. The Chariot-peptide solution was then brought to a final volume of 5 ml in DMEM growth medium. Isolated chromaffin cells were incubated in this mixture for at least 1 hour prior to recording.

Immunocytochemical staining

Cells were stimulated as described in the text in the presence of the aminated styryl dye AM1-43 (10 μ M). Following stimulation, cells were perfused with a phosphate-buffered saline (PBS) containing 10% paraformaldehyde for 20 minutes. Cells were then washed for 5 minutes in normal PBS. Cells were then permeabilized for 30 minutes PBS in 0.15% Triton X-100 (Sigma). Next cells were washed again in PBS for 5 minutes and then washed in 4% normal goat-serum for 1-hour. Cells were incubated for 1 hour in PBS containing primary antibody against either dynamin I (Oncogene Research Products, Boston, MA) or clathrin heavy chain (Sigma) Cells were then washed and incubated in a secondary antibody tagged with AlexaFluor 594 (Molecular Probes, Eugene, OR). Cells were imaged as described above.

RESULTS

Recent work has demonstrated a mechanistic shift in the mode of exocytosis under different stimulation conditions in chromaffin and other secretory cell types [3,6,20,21]. We initiated a series of experiments designed to elucidate the molecular players responsible for this shift in exocytic mechanism. Molecular perturbation was carried out by transfecting cells with peptide fragments designed to disrupt native protein-protein interactions. To quantify the effect, we assayed granule fusion, catecholamine release and subsequent membrane trafficking events under either 0.5 Hz or 15 Hz action potential equivalent (APe) stimulation in single chromaffin cells. In order to maintain macromolecular and protein components in the cytoplasm, all recordings were made in the perforated patch voltage clamp configuration.

Membrane trafficking exhibits differential activity-dependent molecular requirements

It has previously been shown that exo- and endocytosis under 0.5 Hz APe stimulation proceeds through a transient Ω -form granule fusion intermediate [3] that internalizes membrane independent of the action of clathrin [19,22]. Dynamin I activity has been implicated in membrane endocytosis under moderate stimulation in chromaffin cells independent of the

actions of clathrin [4,23]. We set out to test if dynamin I function is required to support ‘kiss and run’ Ω -form exocytosis elicited under conditions that mimic native basal sympathetic tone [24,25]. Dynamin I function represents a core component for neuronal and neurosecretory function. Chronic disruption of dynamin I may result in overall poor cell health. Genetic knock out of dynamin I results in compromised animal health and viability [26]. In order to guard against non-specific effects of chronic dynamin I disruption, we chose an alternate disruption approach. We acutely transfected cells with a small synthetic peptide (Dyn) that contains the SH3 binding site. The Dyn peptide has been well characterized by several groups in multiple preparations and has been shown to specifically disrupt dynamin I(aa) binding to amphiphysin, which blocks endosomal scission and internalization [17,27]. We also took advantage of this peptide transfection technique to disrupt a key component in clathrin-mediated endocytosis. The well-described 19 amino acid peptide ‘PP-19’ acts to disrupt normal association between synaptojanin and endophilin and blocks the formation of clathrin-coated pits [18]. Previous work in chromaffin cells has shown PP-19 to effectively block endocytosis evoked by 15 Hz APe under similar experimental conditions [19]. We utilized both these peptides to probe for the molecular components supporting endocytosis under conditions that mimic basal sympathetic input versus the acute stress response.

The Dyn and PP-19 peptides were synthesized with a fluorescein tag on the N-terminus to confirm transfection (indicated by the green asterisk in Fig. 1A). Fluorescencepositive cells (Fig. 1Aii) were patch clamped in the perforated patch configuration and stimulated at either 0.5 Hz or 15 Hz with APe trains (Fig. 1B and C). Total cell capacitance was monitored as an index of cell surface during stimulation. Sample recordings for the 0.5 Hz stimulation condition are provided in panel Bi and show that Dyn transfected cells exhibited a much greater capacitance increase than either control or PP-19 transfected cells. These data are consistent with either an increased amount of exocytosis, or they indicate similar amounts of exocytosis in the absence of compensating endocytosis in the Dyn transfected cells compared to the other conditions. Total catecholamine output was determined by amperometry and was equal in all conditions (Table 1). This ruled out a greater amount of exocytosis in the Dyntransfected cells, leaving the expected interpretation; the greater capacitance increase is due to a decreased endocytic function. Data pooled from multiple experiments are provided in panel Bii and reflect mean capacitance values at the end of the stimulation train. These data confirm the finding shown in Bi; Dyn transfection acts to limit endocytic membrane internalization under 0.5 Hz APe stimulation. Cells transfected with the PP-19 peptide showed no difference from control cells (Chariot with no peptide, or no Chariot).

Cells stimulated with 15 Hz APe showed a different effect. Under these conditions, Dyntransfected cells behaved just as Chariot-only or untransfected controls while PP-19 transfected cells exhibited the larger-than-control capacitance response (Fig. 1C). Again, this could either be due to a greater exocytic activity, or a lack of endocytic activity, both resulting in a greater capacitance response. Direct assay of catecholamine release by amperometry showed that the first scenario was not the case; all cells released catecholamine in statistically identical amounts (Table 1). Thus the greater capacitance increase is due to a deficient membrane internalization under PP-19 transfection [19]. Taken together, these data indicate a differential activity-mediate molecular dependence of membrane internalization. Membrane internalization was inhibited under low frequency stimulation (0.5 Hz) by Dyn, but not PP-19 transfection. These data are consistent with an endocytic mechanism depending on normal dynamin I-amphiphysin binding and argue against a role for clathrin-mediated endocytosis. Elevated stimulation (15 Hz) reverses this dependence. Endocytosis is insensitive to Dyn transfection, but is blocked by PP-19, pointing to an insensitivity to disruption of dynamin I/amphiphysin and a need for normal clathrin-mediated processes [4].

Next we performed immunocytochemical staining of both newly-formed endosomes as well as dynamin I and clathrin to determine if there is an activity-dependent shift in their relative association. Cells were stimulated by bath application of elevated potassium Ringer calibrated to quantitatively match stimulation intensity under either 0.5 Hz versus 15 Hz APe stimulation (see [15] and Methods). During stimulation, cells were bathed in the styryl dye AM1-43. As endosomes are pulled from the cell surface during endocytosis, they incorporate the dye. Thus, AM1-43 fluorescence tracks newly formed endosomes. AM1-43 is similar to the well-characterized FM1-43 except that it contains an additional amine group, causing it to cross-link under standard fixation conditions, preserving its staining pattern for combination with immunocytochemical staining. We paired AM1-43 staining with immuno-staining for either dynamin I or clathrin heavy chain (Fig. 2A). As expected, unstimulated cells did not stain with AM1-43. Consistent with previous reports, dynamin I antibody immuno-reactivity (dynamin I IR) exhibited a peripheral staining pattern [4]. Clathrin heavy chain antibody immuno-reactivity (Clathrin HC IR) showed a more uniform staining pattern in unstimulated cells. Light stimulation resulted in AM1-43 staining at the periphery as well as some staining in the perinuclear region of the cell. Under the same stimulation condition, dynamin I IR redistributed to a more uniform pattern while clathrin staining remained qualitatively unchanged from unstimulated control. Consistent with previous characterization, under elevated stimulation conditions AM1-43 staining concentrated around the perinuclear region of the cell [3]. Dynamin I IR and clathrin IR staining shifted under elevated stimulation as well; dynamin I IR remained in a peripheral staining pattern while clathrin IR redistributed to the perinuclear region of the cell, matching the AM1-43 staining pattern.

The relative spatial distribution of AM1-43 staining compared to dynamin I and clathrin HC IR was quantified to test for activity-dependent co-localization of dynamin I and clathrin to newly-formed endosomes (Fig. 2B). We utilized the Pearson's cross correlation analysis approach to quantify the degree of correlation between AM1-43-stained endosome and dynamin I or clathrin and assign a cross-correlation coefficient [3,28]. A Pearson score above 0.6 indicates a statistically relevant co-localization of the signals. As shown in figure 2B, under light stimulation dynamin I IR is co-localized to new endosomes while clathrin IR is not. Alternately, under elevated stimulation, dynamin I is not co-localized to newly-formed endosomes while clathrin is colocalized. Thus, there is a significant activity-dependent switch in the relative association of dynamin I versus clathrin IR with newly-internalized membrane. This relationship is consistent with the data presented in figure 1 showing that normal dynamin I function is necessary for internalization under basal stimulation while clathrin-mediated processes are required for endocytosis under elevated stimulation.

Ω -figures persist under 0.5 Hz APe stimulation

Blocking membrane internalization under conditions that normally lead to 'kiss and run' endocytosis may be expected to allow the granule Ω -figure to collapse into the cell surface. We tested for the persistence of Ω -figures in the cell surface under control as well as Dyn and PP-19 transfected cells. We utilized capacitance variance analysis to assay for accumulation of Ω -figures at the cell surface. Briefly, the variance analysis approach [5] takes advantage of the fact that the major source of variance in frequency-domain capacitance signals is introduced as a resistance-dependent thermal noise from the cell equivalent circuit [29]. Ω -figures introduce additional resistive elements in the form of fusion pores (Fig. 3Ai). Accumulation of Ω -form fusion pores increase the variance in the signal by a predictable and measureable quantity. Therefore, monitoring capacitance variance provides a relative read-out of the complexity of the electrical properties of the membrane, and thus of 'kiss and run' exocytosis [5]. An example variance recording is provided in figure 3Aii where a cell was patch clamped and stimulated with 0.5 Hz APe to elicit 'kiss and run' exocytosis [3]. As seen in the lower capacitance trace, excitation elicits both a jump in capacitance, indicating insertion of granular

membrane during exocytosis, as well as an increase in signal variance (δ^2), indicating that the granule is maintained in an Ω -figure. Mean variance of the capacitance signal was measured under 0.5 and 15 Hz APe stimulation of control as well as Dyn and PP-19-transfected cells and were pooled and are plotted in figure 3B. As expected, cells stimulated at 0.5 Hz exhibited a greater mean variance than those left unstimulated or stimulated at 15 Hz [5]. Moreover, Dyn transfection and stimulation at 0.5 Hz resulted in a further increased variance compared to untransfected controls, consistent with an accumulation of Ω -figures in the membrane.

A role for dynamin I in high frequency-evoked exocytosis

Data to this point show that disruption of dynamin I/amphiphysin blocks membrane internalization and that dynamin I is associated with endosomal membrane under low frequency stimulation. Additionally, previous work using single-cell carbon fiber amperometry [30,31] has indicated that quantal size positively depends on cell Ca^{2+} levels [32] by transitioning from Ω -form fusion transients to 'full collapse' events, thus expelling all granule contents [3,8,9]. We measured the quantal size of catecholamine secretion in Dyn transfected cells compared to untransfected controls to determine the relative occurrence of Ω -form 'kiss an run' to 'full collapse'. We quantified spike amplitude as well as total spike integral (Fig. 4A), parameters that are both increased in the full collapse mode of exocytosis [5]. Data were collected and pooled for each stimulation frequency and transfection condition and are provided in panel B (i–ii). We did not measure a significant change in spike amplitude or charge under 0.5 Hz stimulation by transfection with either Dyn or PP-19 peptides. PP-19 transfection had no significant effect on either parameter under elevated stimulation. An unexpected outcome was that Dyn transfection had the effect of decreasing both spike amplitude and integral under 15 Hz stimulation compared to control. These data indicate that Dyn transfection has no effect on Ω -form exocytosis under 0.5 Hz, but limits fusion pore dilation collapse of the Ω -figure and full granule emptying under 15 Hz stimulation. This effect is not reflected in the more sensitive variance analysis provided in figure 3, indicating that dilation must occur to some degree, but does not proceed to completion. Indeed, recent work has suggested that mechanisms other than dynamin function contribute to fusion pore dilation [33,34].

Dyn and PP-19 transfection selectively alter endosomal uptake in an activity-dependent manner

All previous data have shown that the Dyn peptide alters fusion pore behavior in an activity-dependent manner. We next asked if Dyn or PP-19 transfection alters the formation of endosomes as assayed by bulk fluid-phase dye uptake. We utilized the fast styryl dye FM2-10 to reversibly stain surface membrane during stimulation with 0.5 versus 15 Hz APe. We monitored dye uptake and sequestration in newly-formed endosomes as a function of endocytosis. Uptake of the small molecular weight dye (M.W. 555.44) is not limited by a restricted fusion pore [35,36]. As in figure 1 and table 1, stimulus trains were balanced to elicit the release of identical amounts of catecholamine under both low and high frequency stimulation conditions. Similar to the more commonly used FM1-43, FM2-10 exhibits a low quantum efficiency in an aqueous environment, but a dramatic increase in fluorescence in a lipid environment [37]. A schematic of the cell staining protocol is provided in figure 5A. Cells were washed in Ringer containing 10 μM FM2-10. An immediate increase in total cell fluorescence was measured due to the staining of the cell plasmalemma. Cells that were not stimulated ($\square$) showed a stable fluorescence. Stimulated cells showed a continuous increase in fluorescence ($\blacksquare$). After stimulation, cells were washed in dye-free Ringer. Fluorescence measured in control cells rapidly decreased back to initial nominal levels due to the rapid wash-out of the FM2-10 from the membrane. Stimulated cells also decreased in fluorescence as the dye left the exposed surface membrane. However, some fluorescence remained due to dye retained in newly-formed endosomes that was no longer accessible to wash-out. We tested endosomal formation in cells stimulated at either 0.5 or 15 Hz stimulation. In control cells,

APe trains delivered at each frequency resulted in the same amount of stained endosomal membrane. As expected and consistent with data presented in figure 1, transfection with the Dyn peptide blocked endosomal uptake at 0.5 Hz while PP-19 transfection had no effect. Likewise, PP-19 transfection blocked endosome formation under 15 Hz stimulation while Dyn transfection had no effect. These data are consistent with the data obtained with the capacitance technique (Fig. 1) and show that exocytosis-coupled endosome formation is differentially blocked by disruption of normal dynamin I function or clathrin function in an activity-dependent manner.

DISCUSSION

An accumulating body of evidence is beginning to demonstrate that neuroendocrine adrenal chromaffin cells secrete transmitters by two mechanistically separable mechanisms depending on stimulation intensity. Electrical stimulation designed to match that experienced under basal sympathetic tone results in a transient fusion of granules with the cell surface and selectively releases a portion of granule catecholamines through a restricted fusion pore. Under these circumstances the granule retains its basic morphology as an intact Ω -figure in the cell surface. Granule membrane components remain segregated from the general cell surface and the granule pinches back basically intact. Endosomal recycling is rapid and the granules are available for re-release within a few minutes. Elevated electrical stimulation, matching the sympathetic acute stress response, specifically inhibits the first exo-endocytic mechanism and activates a second mode of exocytosis and endocytosis in a calcium dependent manner [19, 38]. Granule fusion under elevated firing results both in total release of catecholamine as well as the neuropeptide-containing proteinaceous granule core [3]. The fusion pore dilates until the Ω -figure ceases to exist and the granule collapses. Components of the granule membrane mix with the cell surface and coupled endocytosis retrieves bulk surface membrane through a clathrin-mediated process [3] (Figure 1, Figure 2 and Figure 5 of this study). Thus, a fundamental activity-mediated shift in the mode of exo- and endocytosis is responsible for the divergent secretory behavior exhibited by chromaffin cells under either sympathetic tone or the acute stress response [5]. System-wide consequences of this acute stress-mediated shift in exocytic mode underlie the transition from sympathetic 'breed and feed' to 'fight or flight' metabolic status. Biochemical and molecular studies have provided several candidate molecular processes that may correlate to either of these secretion modes [4,23]. We report that Dynamin I is involved in transmitter secretion under both modes of exocytosis, but that it controls two different aspects of granule fusion as a function of stimulation intensity.

Under low frequency APe stimulation matching splanchnic firing under basal sympathetic tone (0.5 Hz) [39–41], we show that transfection with a peptide fragment of the Dynamin I proline-rich domain (PRD) that competitively competes for binding to amphiphysin [17] has the result of blocking granule endocytosis. Consistent with previous work [4,19,25], disrupting clathrin-mediated endocytosis by blocking synaptojanin/endophilin binding has no effect on membrane retrieval in these conditions. Data presented here as well as provided in the literature, point to the major mode of exocytosis under light physiological electrical stimulation as a transient 'kiss and run' occurring through a stabilized Ω -figure. A somewhat surprising result is that block of Ω -figure internalization by Dyn transfection does not cause an immediate collapse of the granule into the membrane. Rather, based on amperometric analysis, the fused granule remains stable as an Ω -figure for at least the duration of catecholamine release. This behavior may simply be a result of the dense proteinaceous core within the granule lumen that blocks granule collapse, or it may also include other extrinsic factors such as stabilization by the cytoskeleton [33,34]. Further experimentation will be required to reveal the factors responsible for Ω -figure stabilization under light electrical stimulation.

Other evidence demonstrates a molecular as well as morphological shift in the mode of endocytosis under high frequency electrical stimulation or as a function of internal calcium concentration. Earlier work from our group [38] demonstrated an activity-mediated kinetic shift in membrane retrieval that also exhibited a shift in endocytic mechanism. Other groups showed a molecular shift in dynamin I isoform-dependence under sustained activity from a dynamin I, clathrin-independent retrieval process to a dynamin II, clathrin-dependent endocytosis [4,22]. Work presented here provides further evidence for a shift from a dynamin I/amphiphysin-dependent membrane internalization process at low frequency stimulation to a clathrin-dependent retrieval process under elevated activity. Thus, in the neuroendocrine adrenal chromaffin cell, it is becoming apparent that dynamin I plays a critical role in membrane internalization under conditions that match the basal sympathetic 'breed and feed' activity level. However, a second somewhat surprising role for dynamin I in the regulation of transmitter release was found. Under high frequency stimulation, where endocytosis was not blocked by the Dyn peptide, amperometric and spike morphology closely resembled that measured under low frequency stimulation (Fig. 4B). Thus, while the Dyn peptide did not block endocytosis under elevated firing conditions, disruption of normal dynamin I function did limit dilation of the fusion pore, causing less release of the catecholamine from the granule lumen.

A previous study [32] reported a Ca^{2+} -dependent quantal size and tested the role of dynamin I in the regulation of the quantal size. In this study dynamin I function was blocked by diffusion of anti-dynamin IgG antibodies through a whole-cell patch pipette. This had the initial effect of increasing catecholamine quantal size in elevated Ca^{2+} stimulation conditions. However, they also found that after the initial increase, quantal size in the dynamin I-block cells rapidly fell to levels below control, and continued to fall to values identical to quantal size measured under low Ca^{2+} conditions. The nature of the perforated-patch recording condition and acute transfection approach we chose in order to maintain integrity of the cytoplasm, and thus normal exo- and endocytic function [42–44], did not allow us to resolve quantal size immediately after dynamin block. Thus we cannot address the initial time-point resolved in the previous study. However, we can consider the time-dependent decrease in quantal size under dynamin block and elevated Ca^{2+} conditions previously reported. The original interpretation was that granules were blocked from efficiently recycling under dynamin I block, leading to an enhanced demand for fusion-competent granules and re-release of recycling granules prior to complete refilling. However, re-examination of these data in the context of our current data set provides an alternative: Quantal size fell with time as a function of anti-dynamin IgG antibody diffusion into the cell. Indeed, the predicted time constant for an IgG molecule of 150 kD diffusing into a cell through a patch pipette [45] is roughly the same as the time-dependent decrease in quantal size reported [32]. Further targeted experiments will be required to resolve the initial dynamin-block effect, but subsequent behavior is quite consistent with the current study.

An outstanding question that remains to be addressed is the apparent divergence in the function of dynamin I between cell systems. As discussed above, dynamin I seems to be associated with a membrane retrieval mechanism predominating under light stimulation. Block of dynamin I function has no effect on the final endosomal scission step under elevated stimulation conditions where a dynamin II and clathrin mediated process predominates in adrenal chromaffin cells [4,22]. This seems not to extend to neuronal preparations where dynamin I seems to be directly associated with clathrin-mediated internalization of small, clear vesicle membrane [46–48] where it is activated under elevated stimulus conditions [26,49–51]. This point is of note for several reasons. First, adrenal chromaffin cells often serve as a biological model system for the study of molecules involved in synaptic physiology [52]. The differential, and even contradictory, dependence of membrane recycling on the normal function of dynamin I may serve as a point of divergence between neuroendocrine and neuronal systems. Secondly, neuroendocrine cells utilize regulation of the fusion pore to control differential transmitter release [3,8,53], forming a critical point of control between 'breed and feed' and 'fight or flight'

metabolic states. While neuronal synapses do exhibit an activity-mediated shift between ‘kiss and run’ and ‘full collapse’ vesicle cycling [21,35,54], it is not clear if a parallel physiological basis would exist in synaptic systems where vesicles do not contain a proteinaceous core. Thus it may be that the divergent dependence of endocytosis on dynamin I in neuronal synapses reflects a different biological function for the transition between ‘kiss and run’ and ‘full collapse’ exo- and endocytosis [55].

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Figure 1.

Dynamin I blocking peptide selectively inhibits endocytosis under low frequency stimulation. A) Chromaffin cells were transfected with fluorescently-tagged Dyn or PP-19 peptides. ii) Cells were chosen for experimentation based on internalized fluorescence, indicating efficient peptide transfection. B) Cells were then held in the perforated patch configuration voltage clamp configuration and stimulated with trains of action potential equivalent (APe) waveforms at 0.5 Hz. Cell capacitance was recorded for control as well as Dyn and PP-19 transfected cells with representative traces shown (Bi). The change in cell capacitance (ΔC_m) was determined as the difference in capacitance during the stimulation train and data were pooled from multiple experiments from control and transfected cells and are plotted (Bii; n = 10 for each condition,

* = $P < 0.01$). C) The same protocol, as pictured in panel B, was repeated except the cells were stimulated with APe at 15 Hz. Representative traces for control, Dyn and PP-19 transfected cells are provided (Ci). Mean capacitance increases were measured as in panel B and pooled data for each condition are plotted (Cii; $n = 10$ cells for each condition; “*” = $P < 0.01$ by paired Student’s t-test compared to control).

Figure 2.

Dynamin I and clathrin associate with newly formed endosomes in an activity-dependent manner. A) Representative images of dynamin I immunoreactivity (Dynamin I IR), clathrin immunoreactivity (Clathrin IR) as well as AM1-43 staining of newly internalized membrane are provided. Cells were either unstimulated, or stimulated with iso-osmotic Ringer solutions containing 10 mM or 30 mM KCl to provide stimulation intensities quantitatively equivalent to electrical stimulation with action potential trains delivered at 0.5 and 15 Hz, respectively (see text for details). Scale bar = 10 μ m. B) Cross correlation between either dynamin I IR and AM1-43 staining or between clathrin IR and AM1-43 staining after basal and elevated stimulation was quantified by a Pearson's cross correlation analysis. Mean Pearson's scores

from 12 cells in each condition are shown. Statistical significance is reached at a Pearson's score of 0.6, this statistical threshold is indicated by a dotted line.

Figure 3.

Dynamin I blocking peptide does not affect cell capacitance variance under light stimulation. A) The equivalent electrical circuit of a patch-clamped cell is shown. R_a represents the access resistance of the pipette/cell junction. R_m and C_m represent cell membrane resistance and capacitance respectively. Variance of the capacitance signal is mainly a function of R_m (see text for details). 'Kiss and run' exocytosis results in accumulation of Ω -figures and additional electrical elements: granule capacitance (C_v), resistance (R_v) and fusion pore conductance (G_{fp}). Thus 'kiss and run' exocytosis increases cell variance by introducing additional conductances into the equivalent circuit. ii) Evoked current (top plot) and measured cell capacitance (bottom plot) are shown for a cell stimulated with a single APe. In response to the stimulus cell capacitance increases by approximately 6 fF. Solid lines indicate linear fits to pre- and post-pulse capacitance. Dotted lines indicate the standard deviation (δ) of these segments and variance (δ^2) is calculated after each stimulation. B) Mean variance was calculated during 0.5 Hz or 15 Hz stimulation in control and transfected cells. Mean data are provided ($n = 10$ cells for each condition; "*" = $P < 0.05$ by paired Student's t-test compared to control). Dotted lines indicate variance measured under 15 Hz stimulation and elevated variance under 0.5 Hz stimulation, respectively. Relative high variance values indicate the accumulation of Ω -form fusion intermediates while lower variance is consistent with full granule collapse.

Figure 4.

Amperometric analysis of activity-dependent catecholamine release. A) A schematic of the amperometric properties quantified. Spike amplitude and total event charge were measured from individual spikes elicited by stimulation at 0.5 and 15 Hz APe stimulation under control and transfected cells. B) For all conditions, mean values for all events under control, Dyn-transfected and PP-19-transfected cells are reported. Dotted lines indicate control values for 0.5 and 15 Hz APe stimulation. Pooled data were obtained from greater than 7 cells in each condition, and an asterisk indicates statistical significance by paired Student's t-test $P < 0.05$.

A

B

Figure 5.

FM2-10 dye internalization as an assay for membrane internalization. Cells were bathed in 10 μ M FM2-10 to stain surface membranes. A) Single fluorescence records are provided. Dye-containing Ringer stained cell membrane. Electrical stimulation with APe at 15 Hz caused a further increase in cell fluorescence compared to an unstimulated control cell (filled versus open symbols). After the stimulation period the dye-containing Ringer was washed out with dye-free Ringer. Fluorescence values fell as dye was washed from the external membrane, revealing a persistent fluorescence signal in the stimulated cell, representing stained internalized endosomal membrane. B) The same dye-staining protocol was repeated on multiple cells stimulated with 0.5 and 15 Hz APe trains in both control and transfected cells. Image acquisition parameters were kept constant across all recordings. Wash-resistant fluorescence from each cell were quantified as in panel A and reported. Pooled data were collected from 4 or greater cells in all conditions, an asterisk indicates statistical significance by paired Student's t-test $P < 0.01$.

Table 1

Mean analysis of amperometric parameters for all experimental conditions. Total catecholamine output was measured under 0.5 Hz stimulation and 15 Hz stimulation in control and transfected cells to ensure that transfection did not alter secretion properties of the cell. Amperometric records were base-line subtracted and integrated for each cell. Total catecholamine output was determined at the end of the stimulus train and pooled. Values for each of these parameters are represented as mean value $\pm$ SEM.

Condition		Total amperometric charge (pC)
0.5 Hz	Control	83 $\pm$ 10 (n=10 cells)
	Dyn	86 $\pm$ 17 (n=10 cells)
	PP-19	85 $\pm$ 10 (n=10 cells)
15 Hz	Control	123 $\pm$ 19 (n=10 cells)
	Dyn	95 $\pm$ 14 (n=10 cells)
	PP-19	108 $\pm$ 35 (n=10 cells)