The Role of Dynamin, Actin/Myosin II and Clathrin in Controlling Catecholamine Release from Mouse Adrenal Chromaffin Cells

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Contents

Abstract

Exocytosis is a vital cellular process that regulates hormone or neurotransmitter release. This release occurs mainly via intracellular vesicles that fuse with the cell membrane. This fusion creates a pore that enables the vesicles to release their contents into the extra-cellular space. The fusion pore is a channel created between the vesicle and the cell membrane and it can undergo a secondary expansion in response to multiple factors. The mechanism of controlling the fusion pore expansion (FPE) is poorly understood; however, the dynamin protein has been recently discovered to be a key player in FPE.

Dynamin is a GTP-ase that plays a well-known role in endocytosis and which can self-assemble and oligomerize into helices or rings. Through this GTP-ase activity, dynamin directly acts on the fission reaction at the neck of clathrin-coated pits to generate a free endocytic vesicle. Dynamin function *in vivo* depends on its ability for efficient GTP hydrolysis and its oligomerization, and this GTP-ase activity may be responsible for the regulation of fusion pore expansion. However, there is not enough research that shows dynamin as a significant part in the exocytosis process.

Furthermore, there are other proteins that may have a critical role in assisting dynamin function in FPE. One of these important binding proteins are the actin/myosin II cytoskeleton; however, the interaction mechanism between dynamin and actin to control FPE is unclear. Additionally, clathrin, a coat-protein that recruits dynamin to retrieve

vesicles during endocytosis, is also thought to play a role in exocytosis; however, there are no current studies to show its participation in the FPE.

In this project we had access to novel small molecules that stimulate dynamin through their GTPase activity. Dynamin's interaction with the actin/myosin II motor proteins and the involvement of clathrin in FPE was explored by using different chemical effectors. Mouse chromaffin cells were exposed to these chemical effectors, and the kinetics of vesicle release was measured using carbon fiber amperometry before and after treatment with these modulators.

This study found that quantal transmitter release increased significantly upon dynamin activation, and less during dynamin inhibition. This may indicate that dynamin alteration directly regulates the amount of catecholamine released per fusion event by either making the fusion pore open wider or for longer periods of time. Dynamin's ability to control FPE is assisted by actin/myosin II cytoskeleton, possibly by providing enough force on the vesicle to accelerate and evacuate vesicle content. For the first time, even though it is an endocytosis protein, clathrin has been found to play a role in exocytosis, either by controlling the recycling process of the vesicles or by interacting with actin/myosin II proteins.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that for the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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Chapter 1 - Introduction

1.1 Cell communication

Exocytosis is a biological process that involves the fusion of a vesicle with the cell membrane to enable the vesicle contents to be released to the extra cellular matrix. This fusion between the intracellular vesicle membrane and the plasma membrane creates a fusion pore that allows and controls the amount of molecules out of the cell (Edwardson & Marciniak 1995; Sugita 2008). Exocytosis is a central process of the nervous system whereby neurotransmitters signals or hormones are released during cell communications and signalling. After being secreted, these neural signals are then received by the post-synaptic membrane at the synaptic cleft, enabling the transmission process (Burgoyne & Morgan 2003). The vesicle is therefore fundamental for trafficking and for exocytosis to occur; whereas, the fusion pore is essential for controlling the quantity of the content released. However, this communication between neurons via vesicles is far from simple and there is still a large gap in the current knowledge regarding the molecular mechanism of fusion and how it occurs, or what controls fusion pore expansion. Determining what might control these processes is therefore the aim of this research.

1.2 The significance of exocytosis

Exocytosis is used in various biological processes. Examples of these include the production of proteins such as enzymes, and of peptide hormones, as well as the secretion of antibodies found on the cell surface (Sugita 2008). Other biological processes that require exocytosis include the acrosome reaction that occurs during fertilization, and the presentation of antigen, a process that occurs during an immune response (Wassarman 1999). This process is also important when recycling receptors that are bounded by membrane (Patzak & Winkler 1986). Moreover, the exocytosis process assists the presynaptic neurons to release neurotransmitters (Wu et al. 2014).

There are two main categories of exocytosis: constitutive and regulated (non-constitutive). Constitutive exocytosis occurs constantly and is not regulated by calcium, and is useful in transporting proteins such as receptors that are used in the cell membrane. Neuronal synaptic vesicles and the large dense core vesicles (LDCV) in endocrine cells are good examples of regulated exocytosis triggered by the increase of calcium (Ca^{2+}) levels in cytosol (Pang & Südhof 2010; Ramos & Teissié 2000). However, fusion does not occur spontaneously due to the negative charges found in the phospholipids that exist in the lipid layer of the organelles and vesicles. The fusion of the membranes requires energy as well as the interaction of adaptor molecules found in both plasma and vesicle membranes (Chan et al. 2010; Jahn et al. 2003). These molecules are very selective in that they only allow the fusion of vesicles with specific organelles' membranes (Skehel & Wiley 2000; Söllner et al. 1993; Théryet et al. 2009).

When the adapter molecules fuse with each other, stored energy is released in the form of ATP to form binding pores between the plasma and vesicle membranes (Whiteheart et al. 1994). In general, exocytosis is regulated when the cell receives signals from their exterior.

1.3 Synaptic system in exocytosis and endocytosis

Exocytosis is a fundamental phenomenon enabling neural communication that typically occurs at the synaptic terminal of axons. During this process, after the integration between the vesicle and cell membrane, the content of secretory vesicles is released. As a result of this merge, the fusion pore is formed, the released molecules then affect the post-synaptic receptors, and the vesicles are then retrieved by endocytosis via clathrin-mediated endocytosis (CME) to be recycled **(Figure 1.1)** (Sugita 2008). Prior to exocytosis, vesicles are filled with neurotransmitters and translocated to the active zone where docking occurs, and ATP assists the priming event for the purpose of activating fusion. The initial fusion is triggered by Ca^{2+} entry and SNARE proteins, which are well-known for their essential role in merging the two membranes together (Burgoyne & Morgan 2003). Calcium entry depends upon depolarizing the presynaptic membrane, which then enables the voltage-gated calcium channels to open, allowing a Ca^{2+} influx to enter into the intercellular cytoplasm causing docking and rapid fusion (Barclay et al. 2005). After release, endocytosis retrieves the fused synaptic vesicles to produce new ones that can be reused in exocytosis. The endo-exocytosis processes are closely related to each other as they are both involved in the synaptic vesicle cycle (Regazzi 2007).

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Figure 2.1 Summary of synaptic vesicle cycle

Vesicles are generated from budding early endosomes or clathrin coated vesicles in endocytosis, then refilled with neurotransmitters and translocated to the active zone in the presynaptic membrane via cellular traffic. The vesicle will then undergo exocytosis which involves docking, priming and fusion; then, content release which is triggered by the Ca2+ influx and the SNARE complex. Synaptic vesicles are then recycled by the endocytosis process (Image from Richmond, JE 2007).

1.4 Steps involved in Exocytosis

Initially, the vesicle forms around the cell materials to be secreted and is then transported near the membrane of the cell where it fuses with the vesicle membrane and the contents are then released.

1.4. 1. Vesicle trafficking

These steps require the movement of a vesicle over a large distance. The vesicles that transport proteins from the organelles such as the Golgi apparatus are required to move near the target before the tethering process begins. Microtubule and actin-based cytoskeletons are involved in these steps together with other motor proteins. When the vesicles reach their targets they interact with factors that can confine them (Bonifacino & Glick 2004; Südhof 2004).

1.4. 2. Vesicle Tethering

Vesicle tethering occurs when vesicles are linked over a distance, which is almost equal to the diameter of the vesicle. This step is used for increasing the concentration of the synaptic vesicles in the synapse. The Rab proteins present on the vesicle membrane interact in its active form with the complementary Rab effector located at the target membrane. This will produce enough force to join the two membranes together. The Rab protein will then interact with the SNARE (Soluble NSF-Attachment proteins Receptor

whereas NSF stand for N-ethylmaleimide sensitive factor) proteins in order to facilitate the docking and priming process (Bonifacino & Glick 2004; Südhof 2004).

1.4. 3. Vesicle docking

The docking process refers to the binding of membranes separated by a distance of not less than 5-10nm. Both the molecules and proteins that are involved in vesicle docking and tethering have coiled structures (Bonifacino & Glick 2004; Südhof 2004). Munc-18 is an example of a protein that promotes the docking process (Voets et al. 2001).

1.4. 4. Vesicle priming

Priming refers to the molecular rearrangement and modifications of ATP-dependent lipids and proteins, which are required after docking and before exocytosis. When the vesicles are at the docking stage, they are not competent to undergo the fusion process. Therefore, they need to be primed to enable them to rapidly fuse upon the influx of calcium. The latency that is normally experienced in between the entry of calcium into the synaptic terminal and the release of neurotransmitters, shows that there are some vesicles that need to connect with the plasma membrane when the calcium arrives (Bonifacino & Glick 2004; Südhof 2004).

In cells that have continuous secretion, priming is not required (Sugita 2008). This stage is thought to involve the partially assembled SNARE complexes. There are also other proteins that are involved in the process, including UNC-13 and RIM. The function of

UNC-13 is to facilitate the transformation of the target or t-SNARE (located in the membranes of target compartments) syntaxin. The t-SNARE is changed from a closed to an open formation and in turn, works to assist the assembling of the vesicle or v-SNARE (located in the membranes of vesicles) or the t-SNARE complexes. Past structural studies have been able to indicate that the SNARE formation (complex) provide sufficient energy that brings the synaptic vesicle membrane closer to the plasma membrane and this may prove to be essential for the fusion process to take place efficiently (Kasai et al. 2012; Richmond 2007; Shin 2014; Südhof & Rizo 2011).

The role of the SNARE proteins in the process of priming in exocytosis is displayed by the detection of SNARE interactions prior to fusion of the membrane, especially in the priming step (Sugita 2008) **(Figure 1.2)**. This role is also evidenced by the increase in the number of fusion-competent or primed synaptic vesicles. The complete formation of the SNARE complex or the complete zippering of the SNARE domain does not normally occur until the calcium signal is detected (Bharat et al. 2014; Hu et al. 2002; Südhof & Rizo 2011). Syntaxin contains the SNARE and carboxy-terminal domains and a globular amino terminal. When placed in a solution state, a default-closed configuration is adopted by syntaxin. Under this closed configuration, the amino terminus is folded over the SNARE within syntaxin. The amino terminus thus inhibits the formation of the SNARE complex. This indicates that both the priming and SNARE formation do not occur when syntaxin is in a closed state. The transformation from a closed state to an open one is therefore observed with the aid of other proteins. These

other proteins are said to bind onto the amino terminus. These proteins (Rim and UNC-13) work together to oversee the priming (Han et al. 2011; Koushika et al. 2001).

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https://www.ncbi.nlm.nih.gov/core/lw/2.0/html/tileshop_pmc/tileshop_pmc_inline.html?title=Click%20on%20image%20to%20zoom&p=PMC3&id=3225952_cshperspect-SYP-005637_F2.jpg

\ Südhof, 2011**]**

Figure 1.2 The SNARE protein cycle

Step one involves the initial assembly of the SNARE complex and vesicle priming. At the presynaptic terminal, syntaxin opens its closed conformation by turning off the SM protein. Membranes are then primed for fusion whilst the partial trans-SNARE complex is assembled, being controlled by chaperones. Step two involves the fusion pore opening with the trans-SNARE complex. In step three, the fusion pore expands until the two membranes are joined, and the trans-SNARE complex are converted into the cis-SNARE complex. At the final step, the structure of the SNARE complex is disassembled by NSF and SNAPs, and the vesicle is formed for further usage in the cellular process (Image from Südhof, 2011).

1.4.5. Vesicle fusion

This step is triggered by SNARE proteins and involves the union of the target protein with the vesicle membrane, the results of which are the production of large biomolecules such as neurotransmitters in the synapse, being released into the extracellular space.

Neuronal SNARE components mediate the release of neurotransmitters in the synaptic vesicle by enhancing fusion of vesicles to the plasma membrane. Cognate t-SNAREs and v-SNAREs, produced by vesicles and the cell membrane, bind, causing apposition

of the membranes at the c-terminal ends **(Figure 1.3)**. The calcium sensing and SNARE zippering processes are modulated by synaptobrevin, syntaxin-1, SNAP-25 and synaptotagamin (Südhof, 2011). In addition, Munc18 accelerates the fusion reaction by directly connecting both t- and v-SNAREs (Shen et al. 2007). Whilst, the synaptic vesicle "priming" protein, Munc 13 catalyses the transition from syntaxin-Munc18 complexes to the *trans*-SNARE complexes (Ma et al. 2011).

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https://science.sciencemag.org/content/339/6118/406 \ Hughson 2013**]**

Figure 1.3: Synaptic vesicle fusion in neural communication.

When the synaptic vesicle reaches the presynaptic membrane, specific proteins located in both membranes start to facilitate the fusion process in order to take its place. Synaptotagamin and synaptobrevin are both vesicle trans-membrane proteins (V-SNAREs). While the proteins located on the target membrane are SNAP-25 (peripheral membrane protein) and syntaxin (trans-membrane protein). These proteins are called the t-SNAREs. Both V-SNAREs and t-SNAREs react with each other forming the SNARE complex which helps with vesicle release and its stabilization in a process called ‘zippering. Munc18 accelerates fusion, while, Munc13, catalyses the transition from syntaxin-Munc18 complexes to the trans-SNARE complexes (Image from Hughson 2013).

1.4.6. Fusion pore expansion FPE:

SNARE protein complexes produce energy through their ‘zippering’ process that forces the two membranes to form the initial fusion pore. However, they do not show any further function in controlling the fusion pore expansion and the zippering process appears to be only crucial for the initial formation of fusion pores. Previously, it was thought that SNARE proteins controlled the fusion process. However, recent studies suggest that the endocytosis protein dynamin not only plays a significant role in endocytosis in pinching-off newly formed vesicles; but, also controls fusion pore expansion and the fusion mode. After releasing their content, vesicles are retrieved either through a slow process (clathrin-mediated endocytosis) when full fusion occurs (**Figure 1.4),** or through a fast process (reused locally ‘kiss and run’ and ‘kiss or stay’), when partially released (Südhof 2004).

Kiss and run fusion is a form of synaptic vesicle release which involves the transient opening and closing of the vesicle pore that results in a partial release of their content. Whereas, the full fusion form represents the full collapse of a vesicle membrane into the cell membrane which is then retrieved by using clathrin mediated endocytosis (CME).

CME recruits different scaffold proteins for vesicle retrieval. One of these important proteins is dynamin which is known for its primary role in the fission process. However, it has recently been suggested that dynamin is a bi-directionally regulator of fusion pore expansion (FPE). Therefore dynamin plays a more significant role than previously thought.

1.5 DYNAMIN

Dynamin is a large GTPase with mechanochemical characteristics that enable it to tubulate and constrict membranes (Obar et al. 1990; Shpetner & Vallee 1989). Dynamin forms a helix that surrounds the embryonic vesicle. The GTP hydrolysis assists with the length of the helix and as a result, the neck of the budding vesicle breaks producing a new vesicle **(Figure 1.4)**. Dynamin is not a single class of protein; but, rather a super family of proteins that is categorized into various groups such as classical dynamins, Mx proteins, mitofusins, OPA and GBS (Praefcke & McMahon 2004).

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Figure 1.4: The role of dynamin in endocytosis. In vesicle formation clathrin coats start to react with adaptin receptors forming a semi-spherical shape around the newly formed vesicle. Dynamin facilitates the separation between the membrane and the vesicle by its helical structure that wraps around the tubules forcing the two membranes to separate. The coated vesicle then will discard the CCP, which makes it available to be used (image from Alberts et al. 2008).

Dynamin is found in different isoforms that may be directly or indirectly responsible for organelle division and endocytosis. Some bacteria possess GTPases, which have similar multidomain architecture to those found in mammalian and plant cells (Praefcke & McMahon 2004). The architecture is made up of DLPS (Dynamin-like proteins). Bacterial DLPs have qualities such as a helical-self-assembly. DPL1 is a type of mammalian dynamin that is useful for cellular distribution of organelles such as endoplasmic reticulum and mitochondria which are found in cultured cells. Some dynamin are important for vesicle formation especially in endocytosis processes, caveolae internalization and recycling of synaptic vesicles (Grohm 2011). Besides dynamin having an N-terminal GTPase domain, it also comprises: a middle domain that forms a “stalk” with the GTPase effector domains (GED); a pleckstrin homology (PH)

domain that is used in binding membranes, and a proline arginine rich-domain (PRD) that allows SH3-domain-containing-proteins to interact.

1.5.1 ISOFORMS OF DYNAMIN

Dynamin consists of different isoforms (*DYN1*, *DYN2*, and *DYN3*) that have been found in mammalian cells (Liu et al. 2008; Urrutia et al. 1997). Dynamin-I was found to be involved in the formation of synaptic vesicles and therefore thought to be neuron specific (Ferguson et al. 2007). Dynamin-II is the most abundant and it is responsible for regulating clathrin-mediated endocytosis (Kasai et al. 1999). Dynamin-III has a limited expression, and only regulates the postsynaptic transmissions in the brain and testes (Ferguson et al. 2007).

1.5.2 Clathrin mediated endocytosis

In general, endocytosis is the opposite process to exocytosis; however, they are intimately linked by a number of proteins. Endocytosis is a process through which cells absorb and engulf molecules such as proteins. This process is mostly utilized because the majority of the materials that are crucial to the cells are larger and polar in shape and therefore, cannot pass through the cell membrane or the hydrophobic plasma. Endocytosis takes places through certain pathways, and these pathways are classified into four groups (Gratton et al. 2008). They include caveolae, phagocytosis, clathrin-mediated pathway and the macropinocytosis. Each pathway is composed of distinct

compartments that handle internalizing molecules that recycle them to the surface. Some of the principle components of these pathways are early endosomes, late endosomes and lysosomes (Huotari & Helenius 2011). The early endosomes are found in the cell periphery and receive vesicles from the surface of the cell. The late endosomes receive materials from the early endosome and then transport them to the lysosomes. However, prior to this, these components conduct a final sorting of the materials before they are released to the lysosomes.

Caveolae are non-clathrin coated plasma buds that exist on cell surfaces (Hansen & Nichols 2010). These structures are made up of caveolin, a cholesterol-binder on protein. The caveolae are flask-shaped pits with a diameter of approximately 50 nm and resemble a cave; hence, the name caveolae. The phagocytosis refers to the process in which the cells bind and then internalize particulate substances that are more than 0.70 µm diameter. These substances may include dust, cell debris, apoptotic cells or micro-organisms. These are the processes of invagination of the cell membrane that form a pocket. This pocket is later pinched off by dynamin to form a vesicle that is filled with extracellular fluid and molecules (Oh, MacIntosh & Schnitzer 1998). The vesicle then fuses with other vesicles like the lysosomes in the cytosol. The final pathway is one which is clathrin-mediated (Liu et al. 2001). Small vesicles characterised by morphological coats and made up of complex proteins mediate this process. These proteins are associated with clathrin, a cytoplasmic protein.

1.5.3 Dynamin and clathrin-mediated endocytosis

Clathrin acts as a mediator for endocytosis. Clathrin is a protein involved in the formation of coated pits on the surface of the plasma membrane (Liu et al. 2001; Ungewickell & Branton 1981). These coated pits serve to distort the donor membranes into producing vesicles, and are known to exclude particular proteins in their formation. These coated vesicles have a diameter of approximately 35 nm and only survive for less than a minute. There are two proteins that assist the formation of vesicles by clathrin; namely, dynamin and adaptin (Robinson 1994). The formation of the clathrin-coated vesicles undergo a series of five stages. These stages include; nucleation, selection of the cargo, clathrin coat assembly, the scission of the vesicles and finally, uncoating and recycling of the clathrin component.

Dynamin plays a role in the clathrin-mediated endocytosis, and its function is seen in the pre-stages (Miwako et al. 2003). At these early stages, the dynamin regulates the maturation of the clathrin-coated pits, whilst the final stages involve catalysing the fission of the membrane and the formation of the clathrin-coated vesicles. Dynamin-1 and dynamin 2 are the two members of the dynamin family that are known to assist in clathrin-mediated endocytosis (Hinshaw 2000). Dynamin's role in endocytosis has in the past been evaluated using biochemical, genetic, ultra-structural and biophysical studies. These studies have tried to glean functional data from imaging of live cells of dynamin at endocytosis sites. Rappoport et al. (2008), undertook a study to assess the role of dynamin, specifically, dynamin1 and dynamin 2, in the clathrin-mediated endocytosis of PC12 cells. During the study, it was discovered that both dynamin isoforms portrayed

co-localization with clathrin (Rappoport et al. 2008). Most studies on the effect of both dynamin 1 and dynamin 2 elicited increases in fluorescence prior to the disappearance of clathrin spots on the surface of the cells. After the rise of the fluorescence, the intensity of dynamin drops and this is attributed to the outward movement of the vesicle from the evanescent field (Mattheyses et al. 2011). Dynamin is recruited at the endocytosis sites by particular binding partners such as amphiphysin, intersectin and endophilin (Hill et al. 2001). These partners also assemble dynamin at the neck of the clathrin-coated pits ready for the fission process.

Once dynamin assembles in the necks of the coated pits, it regulates the release of the vesicles (Hill et al. 2001; Perrais & Merrifield 2005). The release can only take place when there is a proper assembly of dynamin at the neck. This assembly is typically initiated by the other binding proteins of dynamin. Inadequate provision of these proteins leads to insufficient recruitment of dynamin. The arrangement of dynamin at the neck of the clathrin-coated pits is followed by various conformational changes, constrictions, coiling and twisting as well as a re-arrangement of domains such as the PH domain, and all of these processes are said to be regulated by the cycling GTP (Hinshaw 2000).

1.6 Dynamin binding proteins

1.6.1 Bar domains proteins

The Bar domain proteins are divided into three families (Frost et al. 2009). These families are the F-bar (Fes/CIP4 homology-BAR), the I-bar (Inverse-BAR) and the classical Bar. There exist some common elements within these three families, including dimerization modules. These modules are composed of curved and charged surfaces (positively charged). There are two basic residues within the domains that interact with the phospholipid found in the membrane (Peter et al. 2004). These phospholipids are negatively charged and allow for the interaction to take place. The classical proteins are made up of the N-Bar as well as other lipid-binding domains (Ren et al. 2006). An amphipathic helix portrays the difference between the N-bar and the other domains. The helix lies at the front of the domain and it functions to enhance the ability of the N-bar to bind liposomes. The I-bar protein is a cigar-shaped structure that exists as a dimer with six helices. I bar domains contain amino acids that are located at one of its ends. These amino acids act as binding sites for actin and their membranes (Itoh et al. 2005). I-bar domains are bonded to the lipid layers via a convex surface. They are responsible for the formation of membrane protrusions (Peter et al. 2004).

The third family, the F-bar domain, is referred to as the extended domain (Ahmed et al. 2010). This domain plays a role in binding the membrane's phospholipid and also in dimerization. Unlike the N-bar domain, three basic residues are located in the F-bar protein. These residues bind the lipids and are also responsible for mediating the

tabulation activity of the membranes. The domain induces a curved helical dimer having a length of approximately ~250-600 Å membrane (tubulated). The F-bar domain couples with the dynamics of actin to remodel the membrane in the formation of filopodium and endocytic pathways.

1.6.2 Amphiphysin proteins

Amphiphysin is a type of protein that is encoded by an AMPH gene in humans and is associated with dynamin proteins (Takei et al. 1999). The AMPH gene in turn encodes an individual protein that is associated with synaptic vesicles. Amphiphysin is made up of a lipid interaction (N-terminal); clathrin; membrane-bending-bar domain; dimerization and an SH3 domain (c-terminal). The function of these proteins in the brain are to recruit dynamin to the endocytosis sites.

1.6.3 Endophilin

These are one of the Bar domain proteins that have been widely studied. They function in apoptosis and receptor trafficking (Kjaerulff et al. 2011). They also play a role in vesicle endocytosis and other processes that involve membrane structure remodelling. In mammalian cells, endophilin controls the tubulovesicular endocytic pathways that are usually independent of clathrin (Gallop et al. 2006). Endophilin is activated by the ligand that binds to the cargo receptors and are also impeded by dynamin inhibitors (Kjaerulff et al. 2011). Endophilin contains the SH3 domain and its removal may result

in the prevention of micro-vesicle formation in the plasma membrane. The lack of the SH3 domain also inhibits endophilin from binding with dynamin (Sundborger et al. 2011).

1.6.4 Cortactin

Cortactin is another protein that is found in the cell cytoplasm (Kaksonen et al. 2000). This protein is usually activated by an external stimulus so as to promote the rearrangement and polymerization of the cytoskeleton (Lanzetti 2007). These proteins recruit Arp2 or 3 proteins to actin microfilaments and in the process, facilitate and stabilize the nucleation regions enabling the branching of actin. Apart from being involved in endocytosis, cortactin promotes the formation of lamellipodia and invadopodia, migration cells (Kaksonen et al. 2000; Sung et al. 2011). The structure of cortactin is made up of a thin and elongated monomer that consists of the amino-terminal acidic region (Weed & Parsons 2001). It also contains the SH3 domain; a region that is rich in Proline and 37-residue segments. Tyrosine kinases are tasked with the responsibility of activating cortactin through a process called phosphorylation. Once the cortactin is activated, it can bind to the filamentous actin or the F-actin. The SH3 domain of cortactin is known to interact with dynamin

1.6.6 Grb2

The Grb2 is an adaptor protein that is associated with signal cell communication (Giubellino et al. 2008). This protein is crucial to cellular functions and its inhibition results in the impairment of the development processes in almost all organisms. It may also block any proliferation and transformation of the various types of cells. Grb2 consists of both SH2 and SH3 domains. The SH2 domain binds the phosphorylated tyrosine, whilst the SH3 domain binds to the peptides that are rich in Proline (Hinshaw 2000). Grb2 associates with dynamin in a variety of cell types and also under conditions that are able to stimulate signal transduction. Also, Grb2-dynamin association increases after the stimulation of insulin in hepatocytes (Ando et al. 1994).

1.6.7 Intersectin

This particular type of protein consists of two N-terminal domains. These domains bind epsin, the SH3 domains as well as other Proline-rich proteins (Okamoto et al.1999). These proteins assist in anchoring the endocytic proteins by binding them together at various endocytic zones (Evergren et al. 2007). The possibility of a direct interaction between dynamin and intersectin was displayed in vitro through the domains of intersectin. Intersectin, unlike amphiphysin, interacts with dynamin in *Drosophila* (Hinshaw 2000).

Another significant protein binder are the phosphatidylinositides. These proteins assist endocytosis that is mediated by clathrin (Buczynski et al. 1997). The PH domain comes from the pleckstrin protein in the platelets, and dynamin binds directly through its PH domain into the phospholipids (Achiriloaie et al. 1999). These domains are in many proteins such as those found in the signalling events of kinases. They may also be found in the protein-to-protein interactions and membrane binding processes (Wang et al. 1998). The PH domains typically prefer binding to the phosphoinositides, and every protein confers a different preference to each Isoelectric Point (PI) group. The dynamin PH domain that favours its binding to the PI is crucial to the localization of the dynamin membrane and the receptor-mediated process of endocytosis (Hinshaw 2000). The oligomerization of the domain significantly enhances the binding of the lipids. The tetramers of the dynamin confer a ridged lipid-binding feature, with the subsequent oligomerization of the domain being mediated further through the dynamin self-assembly characteristic (Chappie & Dyda 2013).

Shank and Pacsin are other proteins that bind with dynamin. Shank specifically interacts with dynamin-2 and has also been associated with cortactin (Modregger et al. 2000). Whereas, pacsin is a cytoplasmic protein consisting of an N-terminal, a coil together with a C-terminal enclosed by an SH3 domain. .Pacsin has three isoforms, and include pacsin 1, and pacsin 2 and pacsin 3. These three isoforms perform almost the same function in the various tissues that they are expressed in.

1.7 Dynamin in exocytosis

Under normal conditions, during exocytosis, the signalling molecules are released from the excitable cells. The process itself requires the vesicles to connect with the plasma membranes and the fusion pores to be formed so as to enable the vesicle contents to be released to the extracellular space. Dynamin typically regulates membrane fission in endocytosis; however, another role of dynamin has become evident during exocytosis (Di et al. 2003; Graham et al. 2002). Dynamin has been found to exist in various stages of the fusion process, such as the vesicle fusion in neuroendocrine cells, virus fusion with host cells, cell-cell fusion and acrosomal reactions. The protein works through its GTPase activity (oligomerisation-stimulated) and in the process, it can assemble itself into helical complexes or rings.

Experiments performed in early 2000 discovered that dynamin played a role in kiss and run (Graham et al. 2002). Kiss and run is one of the modes of exocytosis found in neuroendocrine cells. During this particular type of exocytosis, only a partial amount of the vesicle contents are released. Dynamin enables the vesicle mouth to reclose, which is usually performed by a mechanism that resembles the formation of vesicles in the endocytosis process. Myosin II controls kiss and run (Graham et al. 2002), as it causes the contraction of the muscle in the cells. It consists of two chains with each chain having a length equal to 2000 amino acids. These chains contain the N-terminal domain in the head and the C- terminal at the tail end.

Dynamin-1 is involved in speeding up the expansion of fusion pores in chromaffin cells. In an attempt to reveal whether or not dynamin is a bidirectional regulator in chromaffin cells, a test was performed using small molecules that modulate dynamin function (Jackson et al. 2015). Through the use of a single cell, it was discovered that dynamin activators or inhibitors increased or decreased respectively the catecholamine amounts that were released during exocytosis (Jackson et al. 2015). Dynamin 2, on the other hand, is found more in cells secreting insulin (Min et al. 2007). This therefore means that dynamin-1 and dynamin-2 exist in exocytosis.

Dynamin also controls the fusion events through the actin cytoskeleton dynamics (González-Jamett et al. 2013). In this case, actin is rearranged in neuroendocrine cells. The arrangements act as a barrier and carrier for the secretory granules to access the plasma membrane. Actin filaments are involved in the fusion process and pore expansion. Since the actin organization is by dynamin, the actions of dynamin rely on their ability to modulate the actin dynamics. It has also been proposed that dynamin supports the expansion of the pores that connect the HIV-1 envelopes and endosomal membranes (Miyauchi et al. 2009). Recently, the role of dynamin-2 further extends to the myoblast fusion that results in the formation of multinucleated myotubes (Shin et al. 2014).

1.7. 1 Dynamin and the cytoskeleton dynamics

The polymerization of the cytoskeleton is regulated by dynamin-actin interactions (Kaksonen et al. 2006; Orth et al. 2002; Schafer 2004). Dynamin is typically recruited by various actin structures, such as the phagocytic cups, podosomes lamellipodia, listeria actin and filopodia. Dynamin-2 and actin have a regulatory function in the activation of the T cells, endocytosis (clathrin-dependent), and phagocytosis (Gomez et al. 2005; Otsuka et al. 2009).Both actin and dynamin display a synergistic action in which one of them modulates the other's recruitment, enabling both of them to be involved in the remodelling and scission of the membrane. Dynamin promotes the assembly of actin in such a way that it relies on the GTPase activity. The actin filaments promoted oligomerisation of the dynamin and, the dynamin induced the elongation of the F-actin. Due to these interactions, a model was developed whereby dynamin favours the polymerization of the actin protein through the removal of its capping **(Figure 5)** (Roux & Plastino 2010).

This Image has been removed due to copyright restriction. Available online from [https://www.ncbi.nlm.nih.gov/core/lw/2.0/html/tileshop_pmc/tileshop_pmc_inline.html?title=Click%20on%20image%20to%20zoom&p=PMC3&id=2982767_emboj2010263f1.jpg \ Roux & Plastino 2010**]**

Figure 1.5 Dynamin oligomerizes via uncapping the short actin filaments that promotes actin elongation (image from Roux & Plastino 2010).

Actin is the most abundant type of protein in eukaryotic cells (Dominguez & Holmes 2011). This protein participates in protein-to-protein associations and interactions. Actin is made up of several proteins, with two major types being G-actin and F-actin. The G-actin binding protein performs many functions and emanates from a different family. The majority of the G-actin binds in the actin cleft and are said to insert amphiphilic helices to the same clefts. Other helices involved are the gelsolin, cofilin and the vitamin-D protein or DBP (Dominguez 2004). All these helices face the opposite direction to that of the G-actin. Actin is a crucial player in various cellular functions due to its ability to transmit through the filamentous (or the F-actin) and the monomer (or the G-actin). Its duties range from maintenance of the shape of the cells and cell motility to

transcription. The filamentous also interacts with myosin, resulting in a muscle contraction.

Gelsolin originates from the family of actin capping proteins such as villin, adseverin, supervillin and advillin (Nag et al. 2013). It is composed of approximately six domains that are regulated by phosphoinositides and tyrosine phosphorylation. Gelsolin usually adopts a configuration that is very compact and is stabilized by intramolecular associations at actin-binding locations. Gelsolin is cytoplasmic in nature though it is a long isoform that circulates in the plasma. This isoform induces a mechanism called the actin scavenger system whereby actin is released into the bloodstream when the cells are dead, or the tissue has been injured. The actin scavenger system causes depolymerization and the subsequent removal of the parent actin. The cofilins are in all eukaryotes and are involved in recycling the actin monomers (Pollard & Borisy 2003). The recycling is done by the depolymerization of the filament. The F-actin is made up of two major chains that gradually turn around forming two chained helices. The actual symmetry has about thirteen molecules that repeat themselves after each six turns and have axial distances of nearly 35nm.

1.7 Chemical effectors

1.7.1 Dynamin modulator

Ryngos

Ryngo 3-32 and Ryngo 3-37 are novel cell permeable small molecules that activate dynamin1 isoforms (Prof. Phil Robinson, unpublished data, and personal communication). Ryngo 3-32 and Ryngo 3-37 are the next generation of Ryngo-1. Ryngo 1 was used previously by our laboratory, with the results showing increased cell secretion (Jackson et al. 2015). However, this new generation of Ryngo does not have an inhibitory effect on dynamin even in very high concentrations unlike Ryngo- 1. These small molecules do however induce the GTP-ase activity and stabilize dynamin ring formation by reducing the disassembly rate. Furthermore, Ryngo's activate dynamin-actin interactions which results in promoting both the dynamin oligomerisation and actin polymerization process.

Ryngo drugs are novel small molecules that are valuable research tool; however, further investigation is needed to characterize their mode of action in both *in vitro* and *in vivo* studies.

Dynole 34-2

Dynole 34-2 is a potent, cell permeable dynamin I and II inhibitor that targets dynamin GTPase domain activity and receptor-mediated endocytosis (RME) (Hill et al. 2009). This dynamin inhibitor binds to an allosteric site in the dynamin GTPase domain which

is thought to provide it with less off-target effects than any other dynamin inhibitors such as Dynasore dynamin inhibitor. In addition, Dynole 34-2 has been used extensively to study different cellular systems and endocytosis processes (Chircop et al. 2011; Harper et al. 2013; Merezhko et al. 2014; Smith & Chircop 2012). Recent work by the Robertson lab (2014), demonstrated that Dynole 34-2 anti-proliferative effects could be used as a new approach to cancer therapy as it has less cytotoxic effects on non-tumerigenic proliferating cells and a high sensitivity to dividing cancer cells (Robertson et al. 2014).

1.7.2 Actin/myosinII inhibitors

Latrunculin B is an inhibitor of actin polymerization that is commonly used to disrupt the actin cytoskeleton cells (Spector et al. 1989; Wakatsuki et al. 2001). Unlike latrunculin A, LatB has less unwanted/undesirable effects and is more ideal for short-term studies.

Cytochalasin B is myotoxin that is cell-permeable that binds to the actin terminal (MacLean-Fletcher & Pollard 1980; Urbanik & Ware 1989). Cytochalasin B has metabolites that have the capability of adhering to actin filaments and inhibiting the process of polymerization and the elongation of actin. Therefore, Cytochalasin B has the ability to alter the cellular morphology and inhibit biological processes such as cell division. This inhibitor can also permeate the plasma membrane and impede cellular translocation, and it can also prevent protein synthesis.

Blebbistatin is a small molecule that shows high affinity for myosin II (Dou et al. 2007; Kovács et al. 2004). Blebbistatin rarely competes with nucleotide binding for skeletal myosin fragment, and preferentially fixes to ATPase intermediate. It therefore does not inhibit the production of phosphate, and does not interfere with the binding of actin and myosin. Moreover, it does not interfere with actomyosin dissociations that are induced by ATP. The inhibitor blocks myosin terminals with the aqueous cavity localized between the actin–binding interface and nucleotide pocket. Blebbstatin also blocks the muscle protein myosin II.

1.1.3 Clathrin inhibitors

Pitstop 2 prevents clathrin terminal domains from specifically interacting with its endogenous ligands and thus inhibits clathrin mediated endocytosis (CME) and synaptic vesicle recycling (von et al. 2011). As such, this drug can be used to investigate clathrin function and can be used in pharmacological applications.

Pitstop 2 can be used as a tool to assess the function of the clathrin mediated endocytosis and is a potential therapeutic drug. CME is a specific process that utilizes clathrin-coated vesicles to internalize cargo which occurs in almost all cell types (Brodsky 2012; Perrais & Merrifield 2005). A recent study showed that the nuclear pore complex (NPC) permeability barrier collapses after treatment with Pitstop 2, causing its ultrastructure to be modified and Importin β binding to be reduced (Liashkovich et al. 2015). This highlights for the first time, a functional indication between NPC and clathrin scaffold

proteins, and could be used to improve the second generation of NPC permeability barrier-disrupting drugs. In another study, Pitstop 2 was found to inhibit the clathrin globular N-terminal β-propeller domain (TD) which connects the three legs of clathrin heavy chains (CHCs) in a triskelion structure by their C termini at a central vertex (Robertson et al. 2014). This inhibition of TD by Pitstop 2 increased the lifetimes of clathrin coat components which suggests that coated pit dynamics are regulated by clathrin TD (von et al. 2011). Clathrin also plays another critical non-endocytic role in mitosis. When Pitstop 2 is incubated with dividing cancer cells, it exclusively induces apoptosis and inhibits proliferation of those cells (Smith et al. 2013). However, Pitstop 2 did not affect non-tumourigenic fibroblasts suggesting the specificity of this drug towards the terminal domains of clathrin.

Aim and significance

Dynamin is a mechanochemical GTP-ase enzyme that has a known role in endocytosis by directly acting on the fission reaction at the neck of budding vesicles that generates a free endocytic vesicle. However, recent studies in our lab have shown that dynamin is a key player in regulating the exocytosis process specifically by controlling the dilation of the fusion pore, and possibly discriminating between full fusion and kiss and run fusion. However, the mechanism underlying this process is still unclear and needs further investigation. Therefore, in this project we are aiming to reveal the molecular mechanism of dynamin-mediated fusion pore expansion by applying chemical effectors that target dynamin, actin/myosin II and clathrin proteins. Chromaffin cells were used in this study, as they are a long-used model for investigating exocytosis process.

Aims of this project

1- To determine how dynamin modulators affect the quantal release of catecholamine during exocytosis.

2- To investigate whether the inhibition of actin and myosin can block the effect of dynamin inhibition in vesicle release.

3- To examine whether clathrin affects vesicle release when actin/myosin are inhibited

Hypothesis

Dynamin, through a mechanism involving actin/myosin II and clathrin is able to control transmitter release bi-directionally from vesicles by affecting the fusion pore expansion (FPE).

Chapter 2 - Methods

2.1 Primary culture and isolation of mouse chromaffin cells (MCCs)

Isofluorane was used to humanely euthanize ten-week-old male mice C57Bl/6, as approved by the Flinders University Animal Welfare Committee. Cervical dislocation and the removal of two adrenal glands were performed on each mouse and immediately stored in a 15 ml Falcon tube that contained ice-cold Locke's solution (154mM NaCl, 5.6mM KCl, 3.6mM NaHCO3, 5.6mM D- Glucose, 5.0mM HEPES, pH 7.4). The adrenal medulla was separated from the adipose tissue and then the adrenal cortex, which was then digested in 5ml of 1.5mg/ml collagenase type A in the shaking water bath for three intervals of 15 minutes, 10 minutes and 5 minutes at 37^{o}C. The suspension was slowly and carefully triturated with 1 ml pipette between each interval. Cold Locke's solution was then used to dilute the collagenase, and the cells were then pelleted in a refrigerated centrifuge at 168 (× g) for ten minutes at 4^{o}C. Prior to the tissue pellet being re-suspended in 5 ml of DMEM (Dulbecco's modified Eagle's medium), Locke's solution was then removed. The DMEM was supplemented with 10% (v/v) heat inactivated foetal calf serum, 100 units/mL penicillin and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). The solution was then pipetted through a 40μm nylon mesh filter (Sigma-Aldrich, USA), to remove connective tissue. This was then centrifuged again at 168 (× g) for ten minutes at 4^{o}C. The pellet of chromaffin cells was then re-suspended in 1ml of fresh DMEM after the removal of the supernatant. 200 μl of cell-containing media were plated into the centre of the pre-treated 35mm x 10 mm plastic tissue culture dishes (Corning Incorporated, NY, USA). These were subsequently

incubated at 37 ^{o}C with 5% CO_2 for 1 hour. 2mLs of fresh DMEM was then added to each plate prior to the cells being left to incubate in media for a maximum of three days before the DMEM needed to be removed and replaced with fresh DMEM.

2.2 Amperometric measurements of catecholamine released from mouse chromaffin cells

Carbon fibre amperometry is an electrophysiological technique that measures the amount of catecholamine release. In all of the experiments, Krebs solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes; pH 7.4) was used as the standard bath solution. The temperature was controlled using an automatic temperature controller at 35-37^{0}C (TC-344B; Warner Instrument Corporation, Hamden, CT, USA). A gravity-facilitated perfusion system was used to apply all solutions to the cells. A carbon fibre electrode CFE (5 μm diameter, ProCFE, Dagan Corporation, Minneapolis, MN, USA) was lowered a few microns above a single chromaffin cell, whereby the peak oxidation current (+800 mV) for catecholamine under voltage clamp conditions was applied to the bath. For 30 seconds, the cells were perfused with Krebs solution, then with high K^+ Krebs solution 70 mM (75mM NaCl, 70mM KCl, 2 mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 10mM D-Glucose, pH 7.4) for a further 60 seconds that was then followed by a wash with standard Krebs solution for 60 seconds. Once the CFE oxidises the catecholamine released from a single vesicle, it is represented as a current 'spike' on a trace using an EPC-7 amplifier (List Medical, Darmstadt, Germany), which is controlled with Pulse software (V8.78; HEKA

Electronik GmbH, Lambrecht/ Pfalz, Germany), sampled at 10 kHz and low pass filtered at 1 kHz.

In order to measure the effect of dynamin on fusion pore kinetics, chromaffin cells were treated with different drugs. The first drugs to be tested were those that were dynamin activators; namely, Ryngo 3-32 (10μM) and Ryngo3-37 (5μM), cells were incubated for 30 minutes. The second phase of testing was to analyse the influence of actin/myosin II inhibition on spike parameters, and to ascertain whether they block the effect of the dynamin inhibitor Dynole 34-2 on fusion pore kinetics. The cells were treated with cytochalasin B (0.5μM), latrunculin B (0.5μM) or blebbistatin (0.5μM) for 30 minutes then with dynole 34-2 for another 30 minutes. Single chromaffin cells were then stimulated for 60 seconds and amperometric traces recorded. To show the effect of the clathrin inhibitor Pitstop 2 and to test whether clathrin affects vesicle release when actin/myosin are inhibited, the cells were treated with either cytochalasin B (0.5μM), latrunculin B (0.5μM) or blebbistatin (0.5μM) for 30 minutes. These cells were then treated with Pitstop 2 for another 30 minutes. Amperometric traces were then recorded for 60 seconds from single cells that had been stimulated.

2.3 Amperometric data analysis

All amperometric records were sampled at 10 kHz low pass band filter, and converted to Axon Binary Files (ABF Utility, version 2.1, synaptosoft, USA). Spikes were analysed for a period of 60 seconds of stimulation, and only cells with spikes between 10 and 200

where selected. To avoid producing any misleading data, only spikes with an amplitude ≥10 pA were selected. However overlapping secretory spikes were excluded. The spike frequency for each chromaffin cell was calculated in Hz. The rise time for each spike was calculated from 50%-90% of the rising phase, in order to exclude any misleading data that may have originated from the Pre Spike Foot signals (PSF).

In the spike kinetics analysis, only spikes that met the threshold criteria were included. For each amperometric record, spike values were placed into relevant groups and the average was calculated for each spike parameter. Spike parameters included; amplitude, rise time, area, half-width and decay time. Selected PSF signals needed to be equal to or greater than 1 ms in duration and equal to or higher than 4 times the root-mean-squared (RMS) noise of the baseline. Similar to the spike parameters, the PSF were also analysed from pooled results and averaged for foot duration, area and amplitude. The interval time between the onset (when the signal exceeds the peak-to-peak noise of a 5 ms time segment) and the end of the PSF (the inflection point of between the PSF signal and the spike) was used to calculate the PSF duration. Between the onset and the time when the spike current fell to 2.5 times the RMS (Zanin et al., 2011). Noise of the baseline was used to calculate the area of the PSF signal. All spike values were evaluated using the Kruskal- Wallis ANOVA test with a Dunn's post hoc test to gauge their statistical significance. Only when p was less than 0.05, were data sets considered to be significantly different.

Chapter 3 - Results

3.1. Dynamin regulates fusion pore expansion

Dynamin, through its a GTPase activity, controls vesicle fission during clathrin-mediated endocytosis (Trouillon & Ewing 2013). Dynamin has been recently investigated as a bi-directional regulator in fusion pore expansion (FPE) and vesicle release (Jackson et al. 2015). Previous work by our lab showed that dynamin, through mechanisms involving actin/myosin II proteins and clathrin (Jackson 2013), regulates the quantal release of vesicles through the early fusion pore. Therefore, to understand the mechanism underlying this process we took this research further where we aimed to demonstrate the role of dynamin in FPE by investigating the effect of chemical effectors targeting dynamin and its binding proteins in mouse chromaffin cells. Exocytosis was measured by using amperometry, which determines the kinetics of vesicle release. In general, the results showed that the activation of dynamin causes an increase in the release from a single vesicle, whereas, the inhibition causes a reduction in the secretion. Also, the blocking of motor proteins actin/myosin reduced the clathrin inhibition effect on spike kinetics.

3.1.1. Carbon Fiber Amperometry

Carbon Fiber Amperometry is an electrophysiological technique that has been used to demonstrate the amount of oxidized molecules released from a single vesicle. This project was divided into three sections: the first part involved investigating dynamin activators Ryngo 3-32 (10μM) and Ryngo 3-37 (5μM) by applying those drugs to chromaffin cells for a period of 30 minutes. The second part examined the effect of actin/myosin II inhibitors and dynamin inhibitor on chromaffin cells. This was done by treating the cells with either Latrunculin B (0.1μM), Cytochalasin B (0.5μM) or Blebbistatin (0.5μM) for 30 minutes followed by adding Dynole 34-2 (0.5μM) for another 30 minutes. The last section studied the same actin/myosin II inhibitors combined with the clathrin inhibitor, Pitstop 2 (0.5μM), following the same procedure. In all experiments, single chromaffin cells were then stimulated for 60 seconds by passing a +800mV voltage through a Carbon Fiber Electrode (CFE) and the quantal release of catecholamine recorded as an amperometric trace. These traces represent groups of spikes that reflect the current produced by the transfer of electrons onto the CFE during catecholamine oxidation. Every single spike that was analyzed provided us with different information and parameters such as the spike amplitude, rise time, area, half-width, decay time and the pre-spike foot (PSF) signal. The PSF signal represents the release of catecholamine through the early fusion pore **(Figure 3.1)**.

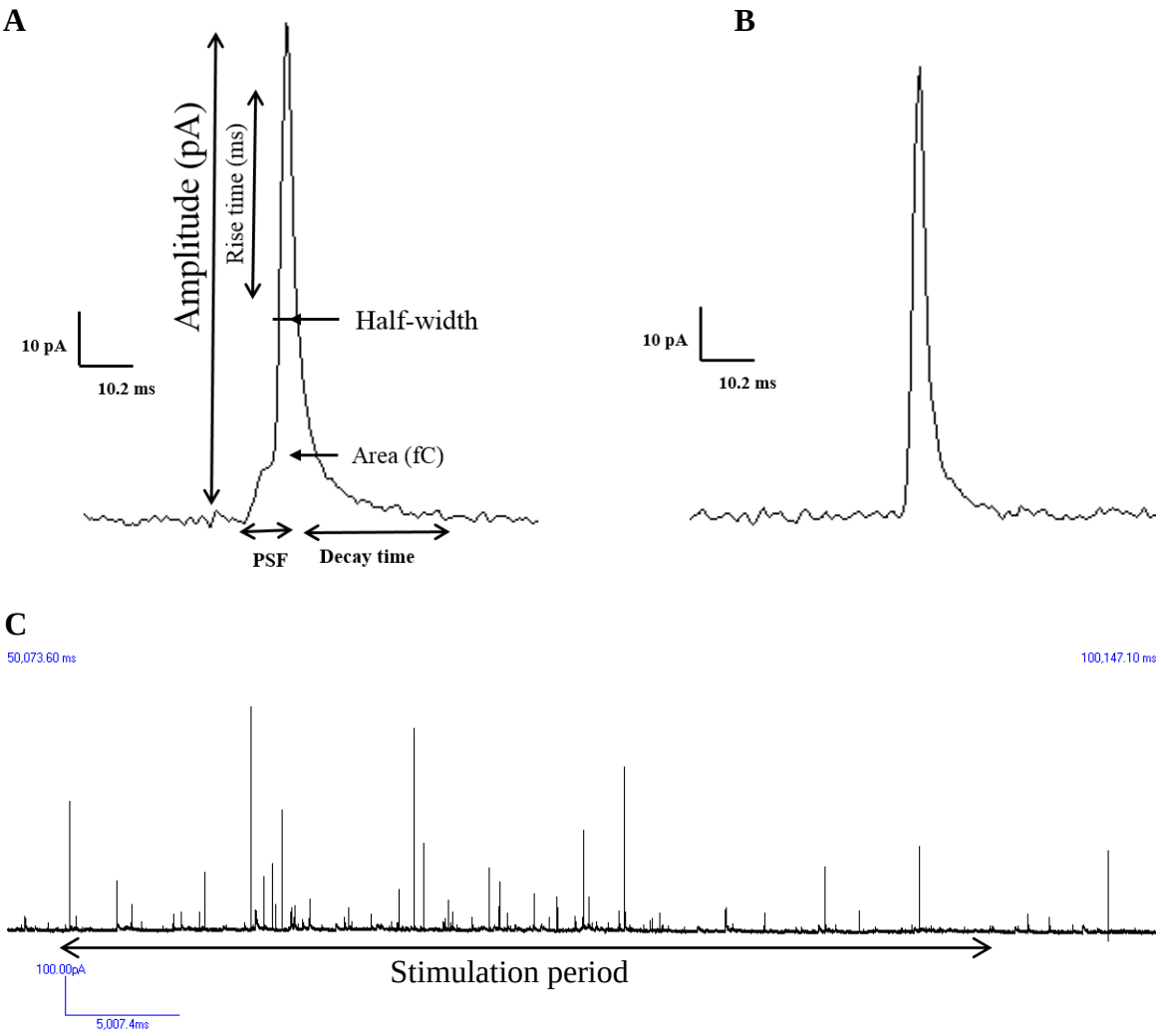

Figure 3. 1 Amperometric recording from a single mouse chromaffin cell

(**A**) Single amperometric spike parameter includes; amplitude, rise time (90%-50%), area, half-width, decay time (90%-37%), and a pre-spike foot (PSF) signal, which reflect the release through the early fusion pore. (**B**) Another single spike example without the PSF signal. (**C**) Amperometric recording from a control chromaffin cell stimulated for 60 seconds with 70mM K^+ Krebs solution.

3.2. Ryngo 3-32 and Ryngo 3-37 increase the total amount of catecholamine released per fusion event

Previous findings published by our lab demonstrate that dynamin activity directly affects the quantal release of neurotransmitters (Faelber et al. 2012). The Ryngo family are novel chemical effectors that activate dynamin GTPase activity and actin polymerization through dynamin-actin interactions, in which it stabilizes dynamin in a ring form allowing more catecholamine to be released (Prof. Phil Robinson, unpublished data, personal communication). Therefore, Ryngo 3-32 and Ryngo 3-37 were used to determine if the activation of dynamin will increases a single vesicle secretion in the same manner that Ryngo 1-23 did in our previous study (Jackson et al. 2015). As both drugs increased cell secretion and were causing the same changes to spike kinetics, we provide an example of a single amperometric record for only one of them, Ryngo 3-32 **(Figure 3.2)**.

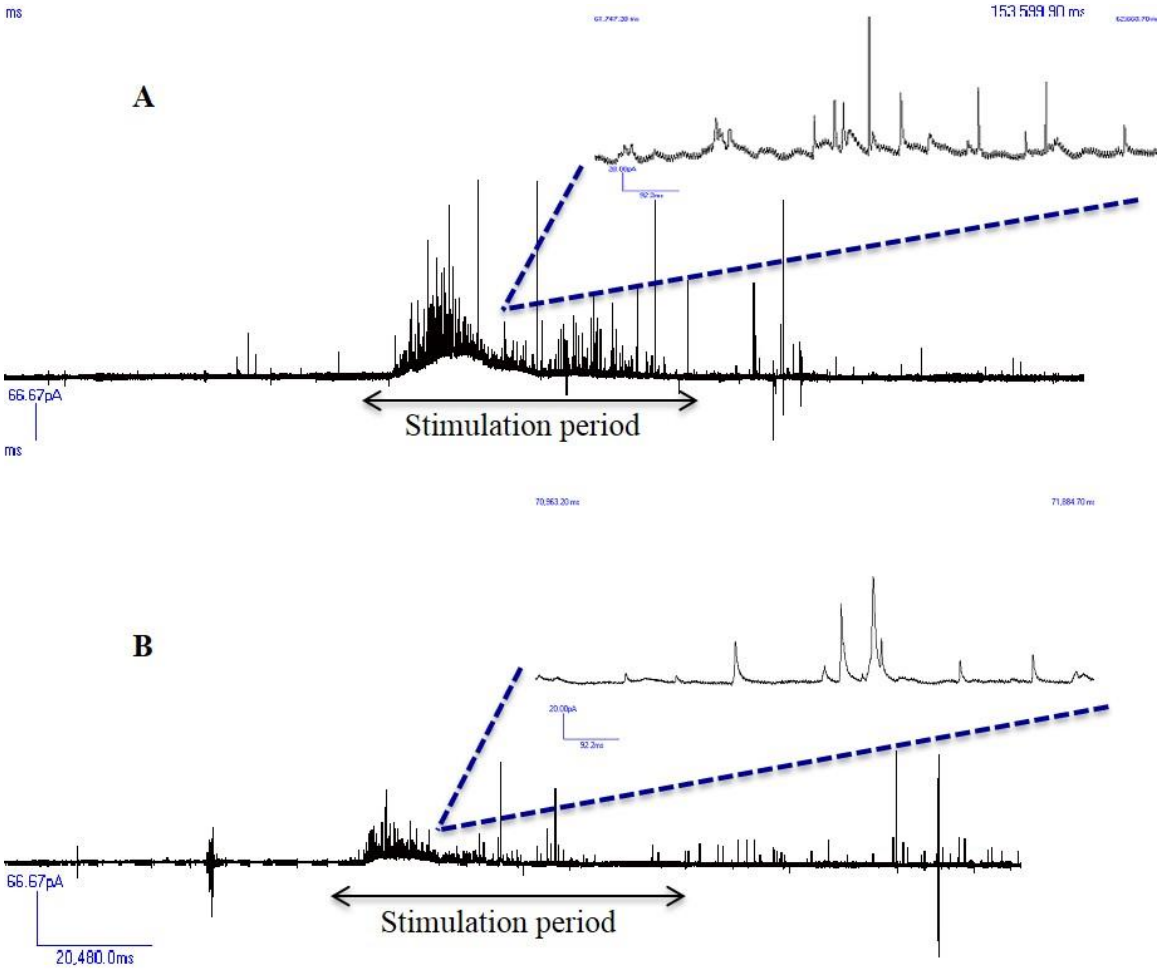

Figure 3.2 Amperometric recording from a single chromaffin cell before and after being treated with 10µM Ryngo 3-32.

(**A**) Amperometric recording from a control chromaffin cell stimulated for 60 seconds with high Krebs solution K^+ 70mM. (**B**) Amperometric recording from the same plate treated with 10µM Ryngo 3-32.

3.2.1. Ryngo 3-32

The average spike area increased significantly when chromaffin cells were treated with 10μM Ryngo 3-32 (409.8 ± 16.25 fC) compared to the control cells (290.8 ± 9.14 fC) **(Figure 3.3 A)**. Also, the average amplitude in cells incubated with 10μM Ryngo 3-32 decreased significantly (30.41 ± 1.6 pA) to more than half of the amplitude for the control cells (65.74 ± 3.02 pA) **(Figure 3.3 B)**. In addition, the average spike rise time for 10μM Ryngo 3-32 incubated cells (1.75 ± 0.12 ms) was greater than the control values (0.67 ± 0.03 ms) **(Figure 3.3 C)**. Moreover, the average spike half-width and the decay time for cells incubated with 10μM Ryngo 3-32 increased dramatically (34.2 ± 0.17) and (11.16 ± 0.58 ms) compared to control cells (10.46 ± 0.54) and (4.51 ± 0.19 ms), respectively **(Table 3.1)**. On the other hand, the average PSF amplitude for 10μM Ryngo 3-32 treatment displayed a significant decrease (9.76 ± 1.36) compared to the controls (13.89 ± 1.07) **(Figure 3.4 A)**. Interestingly, the average of the pre-spike foot (PSF) signal duration increased with 10μM Ryngo 3-32 treatment (21.84 ± 1.44 ms) compared to the control PSF (5.55 ± 0.4 ms) **(Figure 3.4 B)**.

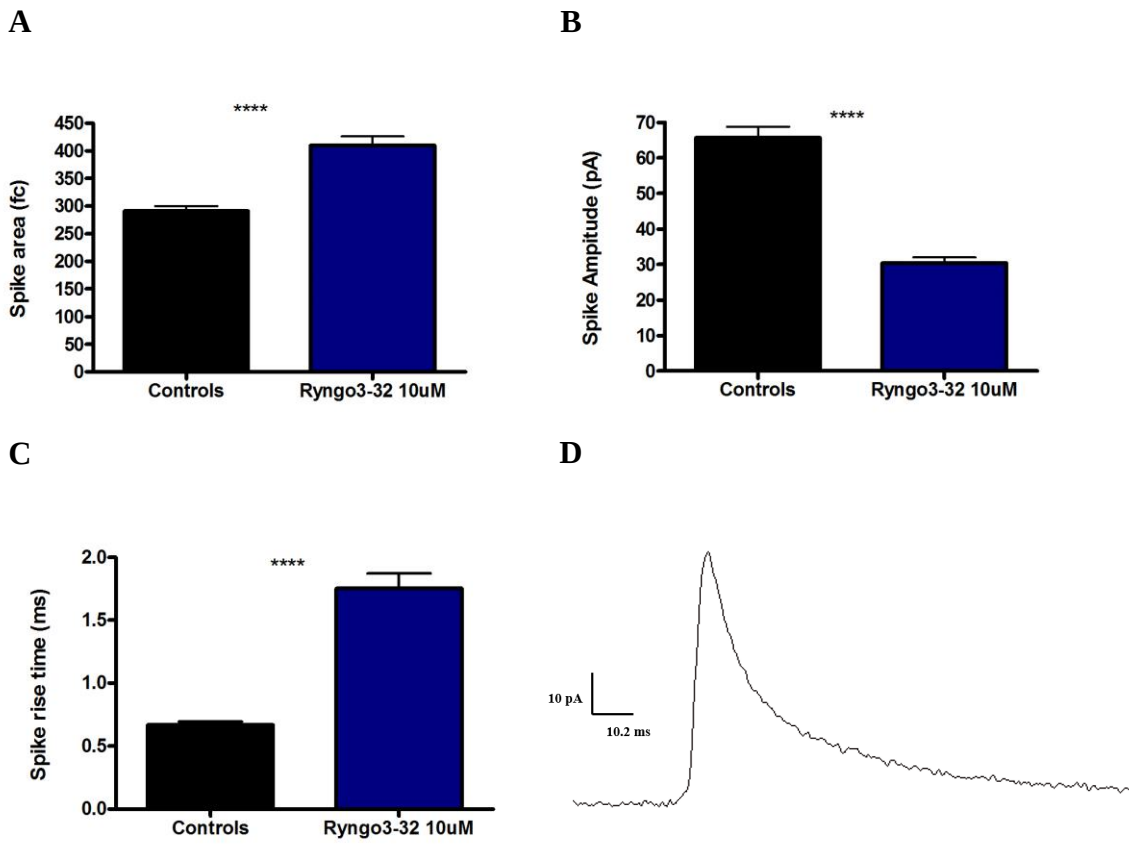

Figure 3.3 The effect of 10µM Ryngo 3-32 on chromaffin cell secretion

The average spike **(A)** area **(C)** rise time increased significantly when the cell was treated with 10µM Ryngo 3-32 compared to the control chromaffin cells **(B)** Average amplitude decreased significantly in cell treated with 10µM Ryngo 3-32 compared to the control. **(D)** Example of a single spike recorded from a cell treated with 10µM Ryngo 3-32. Control n= 8 cells, 585 spikes; 10µM Ryngo 3-32 n = 7 cells, 236 spikes. **** $p<0.0001$, Kruskal-Wallis ANOVA test, Dunn's post-hoc test.

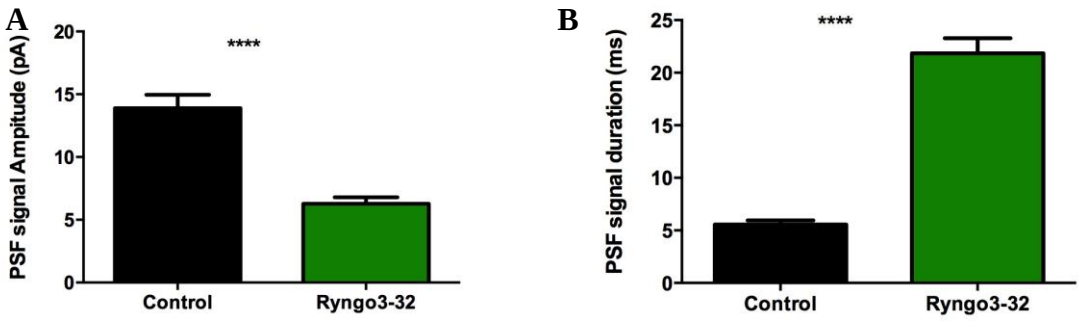

Figure 3.4 Ryngo 3-32 increases the amplitude and duration of the pre-spike foot signal (PSF)

Average PSF signal **(A)** amplitude decreased considerably when the cell was treated with 10μM Ryngo 3-32 compared to the control. However, the average PSF signal duration **(B)** increased significantly when the cell was treated with 10μM Ryngo 3-32. Control n= 8 cells, 270 PSF signals; 10μM Ryngo 3-32 n = 7 cells, 50 PSF signals. **** $p<0.0001$, Kruskal-Wallis ANOVA test, Dunn's post-hoc test.

3.2.2. Ryngo 3-37

When cells were treated with 5μM Ryngo 3-37, the average spike area was significantly greater (553.1 ± 27.89 fC) than control values (258.7 ± 11.14 fC) **(Figure 3.5 A)**. Also, during the 60 seconds stimulation with 70mM K^+, the average spike amplitude for the 5μM Ryngo 3-37 treated cells showed a notable decrease (65.01 ± 6.99 pA) compared to the chromaffin cell control (87.71 ± 5.32 pA) **(Figure 3.5 B)**. In comparison to the average rise time for control cells (0.69 ± 0.048 ms), the 5μM Ryngo 3-37 displayed a significant increase in average rise time (1.77 ± 0.13 ms) **(Figure 3.5 C)**. Other spike parameters such as spike frequency, half-width and decay time showed significant changes as well when 5μM Ryngo 3-37 was applied to the cells **(Table 3.1)**. The average PSF amplitude for 5μM Ryngo 3-37 treatment demonstrated the same significant decrease (13.22 ± 1.75 pA) compared to the controls (19.21 ± 1.57 pA) **(Figure 3.6 A)**. Moreover, the average for the pre-spike foot signal (PSF) duration increased remarkably for cells incubated with 5μM Ryngo 3-37 (15.89 ± 1.18 ms) compared to the control PSF (6.75 ± 0.49 ms) **(Figure 3.6 B)**.

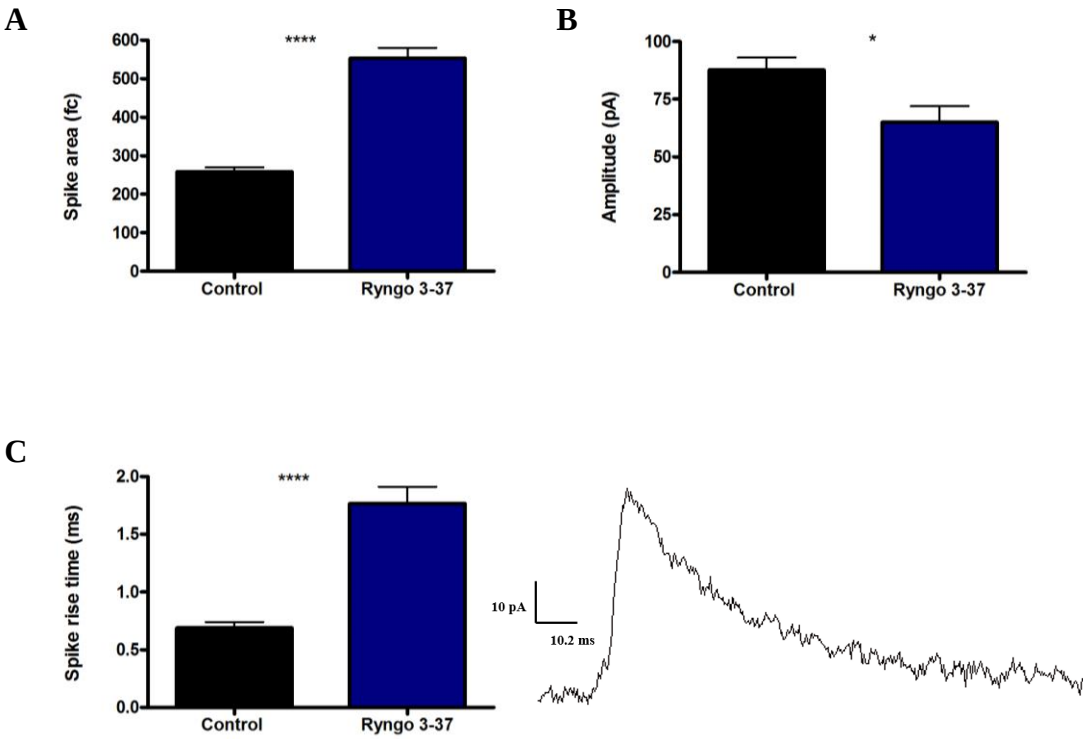

Figure 3. 5 The effect of 5µM Ryngo 3-37 on chromaffin cell secretion

The average spike **(A)** area **(C)** rise time increased significantly when the cell was treated with 5µM Ryngo 3-37 compared to the control chromaffin cells. Average amplitude decreased significantly **(B)** in cells treated with 5µM Ryngo 3-37 compared to the control cells. **(D)** An example of a single spike recorded from a cell treated with 5µM Ryngo 3-37. Control n= 7 cells, 320 spikes; 5µM Ryngo 3-32 n = 6 cells, 177 spikes. **** $p<0.0001$, *$p<0.05$, Kruskal-Wallis ANOVA test, Dunn's post-hoc test.

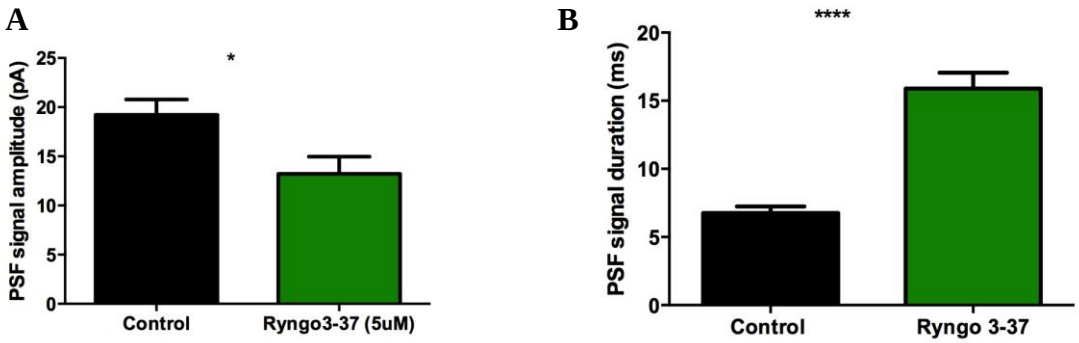

Figure 3.6 Ryngo 3-37 increases the amplitude and duration of the pre-spike foot signal (PSF)

Average PSF signal amplitude **(A)** decreased significantly in a cell treated with 5μM Ryngo 3-37 compared to the control. Average PSF signal duration **(B)** increased significantly when the cell was treated with 5μM Ryngo 3-37. Control n= 7 cells, 180 PSF signals; 5μM Ryngo 3-37 n = 6 cells, 41 PSF signals. **** $p<0.0001$, *$p<0.05$, Kruskal-Wallis ANOVA test, Dunn's post-hoc test.

3.3. The effects of Dynole 34-2 and actin/myosin II inhibitors on vesicle release

Dynamin GTPase activity has been known for its critical role in vesicle formation and recycling in endocytosis. Additionally, recent studies showed dynamin as a regulator for the early fusion pore expansion (Anantharam et al. 2011; González-Jamett et al. 2013; Jackson et al. 2015; Samasilp et al. 2012; Zanin et al., 2012). Dynamin-actin interaction is required for membrane remodelling and dynamin GTP-ase activity is essential for proper oligomerization and actin polymerization (Itoh et al. 2005; Koseoglu et al. 2013). According to previous data from our group, Dynole 34-2, a potent dynamin antagonist that inhibits dynamin GTP-ase activity, causes a significant reduction in chromaffin cell vesicle release (Gu et al. 2014). Therefore, we wanted to explore the molecular mechanism underlying this process by testing whether actin/myosin II inhibitors that target actin polymerization process, would block Dynole 34-2 inhibitory effects on chromaffin cells. The cells were treated with either one of two different actin/myosin II inhibitors; Latrunculin B (LatB), Cytochalasin B (CytoB) or the myosin II inhibitor Blebbistatin (Blebbi), for 30 minutes and then treated with Dynole 34-2 for another 30 minutes. Following the incubation, single chromaffin cells were stimulated for 60 seconds and amperometric traces were recorded to measure the effect of these drugs on vesicle release. In general, the quantal release of catecholamine was reduced significantly compared to the control cells upon treatment with these drugs. The data was then normalized to compare our data to the single drug treatment (Jackson et al. 2015).

3.3.1 Latrunculin B does not inhibit the action of Dynole 34-2 on vesicle release

The average spike area significantly decreases in cell incubated with 0.1μM LatB + 0.5μM Dynole 34-2 (1.71 ± 0.08 fC) compared to the control cell (300.5 ± 13.79 fC) **(Figure 3.7 A)**. The percentage of spike area for 0.1μM LatB + 0.5μM Dynole 34-2 treatment were reduced (0.62 ± 0.03%) more than the 0.5μM Dynole 34-2 single drug treatment (18.84 ± 3.52%) **(Figure 3.7 D)**. Likewise, there was a significant reduction in the average spike amplitude for cells treated with 0.1μM LatB + 0.5μM Dynole 34-2 (12.12 ± 0.28 pA) compared to controls (76.71 ± 3.85 pA) **(Figure 3.7 B)**. Similarly, the normalized amplitude was lower in 0.1μM LatB + 0.5μM Dynole 34-2 (17.43 ± 0.41%) than the 0.5μM Dynole 34-2 alone (29.74 ± 3.14%) **(Figure 3.7 E)**. The average spike rise time decreased significantly for 0.1μM LatB + 0.5μM Dynole 34-2 (0.051 ± 0.003 ms) compared to the control treated cells (0.55 ± 0.037 ms) **(Figure 3.7 C)**. We did observe significant decrease in the spike rise time between 0.1μM LatB + 0.5μM Dynole 34-2 (8.762 ± 0.46%) treated cell and 0.5μM Dynole 34-2 (16.90 ± 0.81%) **(Figure 3.7 F)**. Additionally the PSF signal duration was significantly reduced for the 0.1μM LatB + 0.5μM Dynole 34-2 groups (0.30 ± 0.06 ms) compared to controls (2.89 ± 0.30 ms) **(Table 3.2)**. Furthermore, similar reductions were observed with all other amperometric traces parameters for example spikes frequency, half-width, decay time and PSF amplitude **(Table3.2)**.

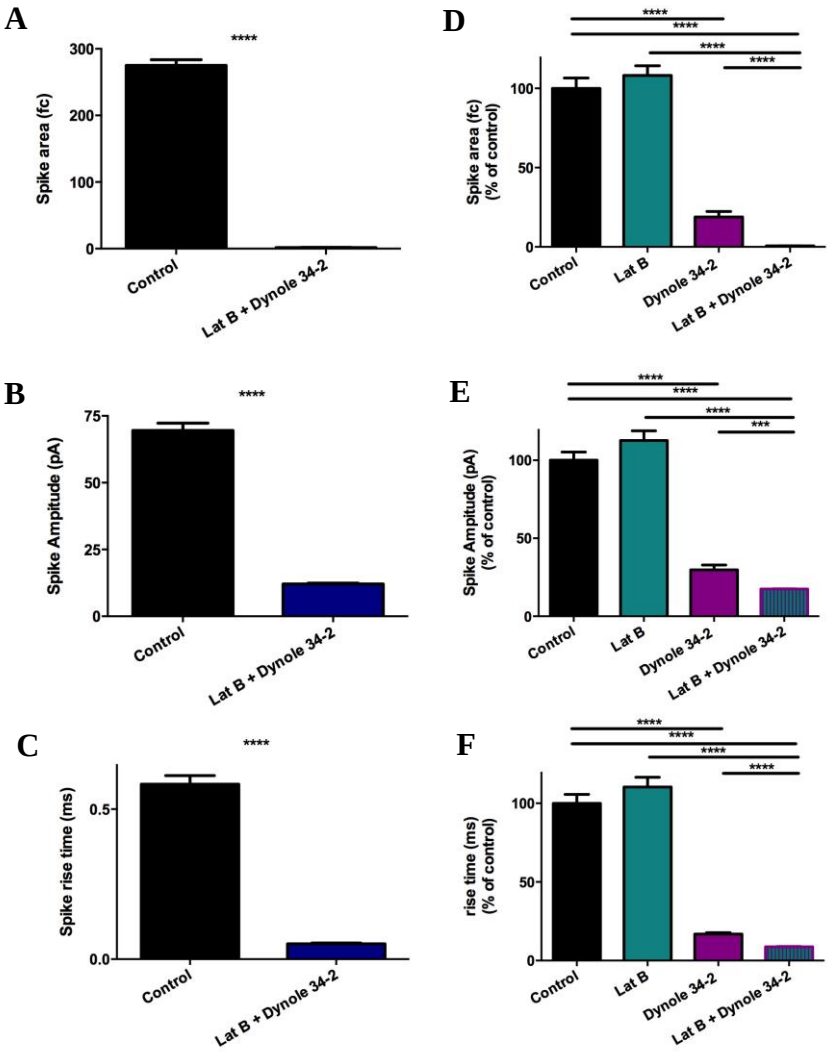

Figure 3. 7 The effect of Latrunculin B and Dynole 34-2 on chromaffin cell secretion

The average spike **(A)** area **(B)** amplitude and **(C**) rise time decreased significantly when the cell was treated with 0.1µM LatB + 0.5µM Dynole 34-2 compared to control chromaffin cells. **(D) (E)** and **(F)** show the comparison between the single drug treatment and the 0.1µM LatB + 0.5µM Dynole 34-2 treated cell calculated in percentage. Control n= 6 cells, 450 spikes; 0.1µM LatB + 0.5µM Dynole 34-2 n = 4 cells, 46 spikes. **** $p<0.0001$, *** $p<0.001$, Kruskal-Wallis ANOVA test, Dunn's post-hoc test.

3.3.2 Cytochalasin B does not inhibit Dynole 34-2 action on vesicle release

The data from cells treated with 0.5μM CytoB + 0.5μM Dynole 34-2 demonstrated a significant decrease in the average spike area (52.75 ± 4.91 fC) compared to the control cells (311.3 ± 7.67 fC) **(Figure 3.8 A)**. In the normalized data, area reduction in cells treated with 0.5μM CytoB + 0.5μM Dynole 34-2 was (16.95 ± 1.58%) the same as cell treated with 0.5μM Dynole 34-2 (18.84 ± 3.52%) **(Figure 3.8 D)**. The average spike amplitude diminished considerably in 0.5μM CytoB + 0.5μM Dynole 34-2 groups (22.69 ± 2.08 pA) compared to controls (89.97 ± 3.07 pA) **(Figure 3.8 B)**. Similarly, the amplitude reduction in normalized data for the 0.5μM CytoB + 0.5μM Dynole 34-2 treated cell (25.22 ± 2.32%) was the same as 0.5μM Dynole 34-2 single drug treatment (29.74 ± 3.14%) **(Figure 3.8 E)**. There was a slight decrease in the average spike rise time for cells incubated with 0.5μM CytoB + 0.5μM Dynole 34-2 (0.41 ± 0.05 ms) compared to controls (0.44 ± 0.07 ms) **(Figure 3.8 C)**. Unexpectedly, 0.5μM CytoB + 0.5μM Dynole 34-2 normalized data increased significantly (93.44 ± 10.56%) compared to 0.5μM Dynole 34-2 group (16.9 ± 0.81%) **(Figure 3.8 F)**. The PSF signal duration significantly decreased for the 0.5μM CytoB + 0.5μM Dynole 34-2 treated cells (1.58 ± 0.15 ms) compared to controls (4.39 ± 0.36 ms) **(Table 3.2)**. Moreover, similar variations were observed with other spike parameters for example spikes frequency, half-width, decay time and PSF amplitude **(Table 3.2)**.

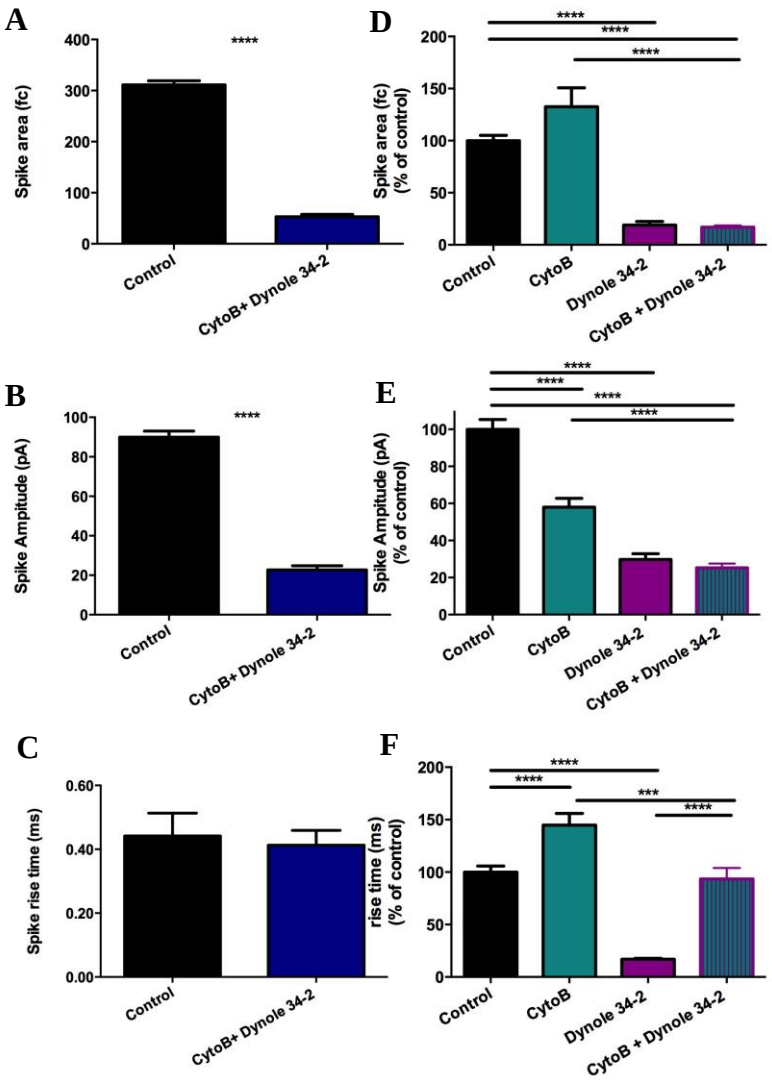

Figure 3. 8 The effect of Cytochalasin B and Dynole 34-2 on chromaffin cell secretion

The average spike **(A)** area **(B)** amplitude decreased significantly when the cell was treated with 0.5μM CytoB + 0.5μM Dynole 34-2 compared to the control cells. **(C)** There was no difference in the average spike rise time treated with 0.5μM CytoB + 0.5μM Dynole 34-2 compared to the control. **(D) (E) (F)** Display the comparison between results obtained from single drug treatment and 0.5μM CytoB + 0.5μM Dynole 34-2, calculated in percentage. Control n= 6 cells, 722 spikes; 0.5μM CytoB + 0.5μM Dynole 34-2 n = 6 cells, 73 spikes. **** $p<0.0001$, *** $p<0.001$, Kruskal-Wallis ANOVA test, Dunn's post-hoc test.

3.3.3 Blebbistatin partially inhibits Dynole 34-2 action on vesicle release

The average spike area decreased significantly in cells treated with 0.5μM Blebbi + 0.5μM Dynole 34-2 (124.7 ± 8.5 fC) compared to the control (367.1 ± 10.65 fC) **(Figure 3.9 A)**. In the normalized data, the 0.5μM Blebbi + 0.5μM Dynole 34-2 treated cells increased substantially (38.30 ± 2.62%) compared to the 0.5μM Dynole 34-2 treated cell (18.84 ± 3.52%) **(Figure 3.9 D)**. The average spike amplitude significantly reduced in the 0.5μM Blebbi + 0.5μM Dynole 34-2 group (31.09 ± 3.75 pA) compared to controls (54.61 ± 1.86 pA) **(Figure 3.9 B)**. The spike amplitude in the 0.5μM Blebbi + 0.5μM Dynole 34-2 treatment was higher (53.27 ± 6.42%) than cells treated with 0.5μM Dynole 34-2 (25.22 ± 2.32%) **(Figure 3.9 E)**. The average spike rise time showed was no different for cells incubated with 0.5μM Blibbi + 0.5μM Dynole 34-2 (0.80 ± 0.056 ms) compared to controls (0.86 ± 0.05 ms) **(Figure 3.9 C)**. While, normalized data showed the rise time at (93.90 ± 6.48%) which is greater than the 0.5μM Dynole 34-2 drug alone (16.90 ± 0.81%) **(Figure 3.9 F)**. There was a considerable reduction in the PSF signal duration for the 0.1μM Blebbi + 0.5μMDynole 34-2 cells (2.07 ± 0.25 ms) compared to controls (7.87 ± 0.55 ms) **(Table 3.2)**. Moreover, similar changes were seen with all other spike parameters for example spikes frequency, half-width, decay time and PSF amplitude **(Table 3.2)**.

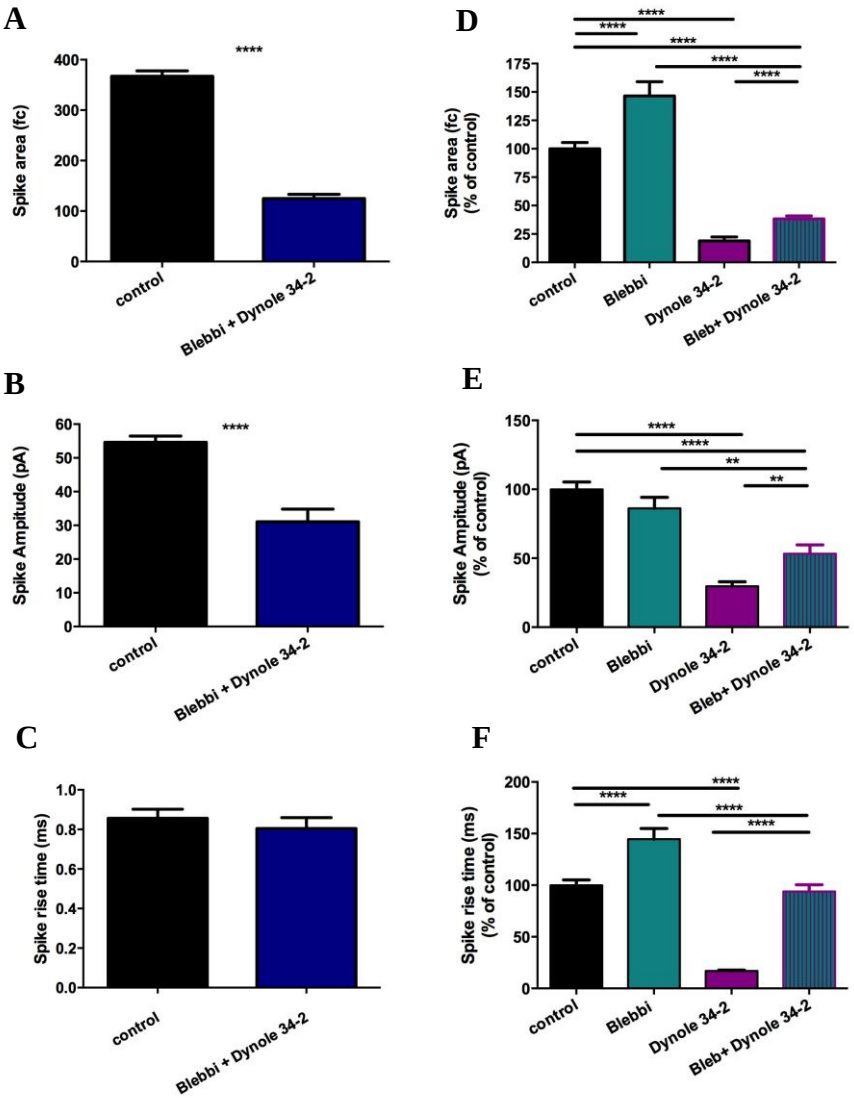

Figure 3. 9 The effect of Blebbistatin and Dynole 34-2 on chromaffin cell secretion

The average spike (**A**) area (**B**) amplitude reduced notably when the cells were treated with 0.5µM Blebbi + 0.5µM Dynole 34-2 compared to the control cells. (**C**) There was a slight decrease in the average spike rise time treated with 0.5µM Blebbi + 0.5µM Dynole 34-2 compared to the control. (**D**) (**E**) (**F**) Display the comparison between results obtained from single drug treatment and 0.5µM Blebbi + 0.5µM Dynole 34-2, calculated in percentage. Control n= 6 cells, 552 spikes; 0.5µM Blebbi + 0.5µM Dynole 34-2 n = 7 cells, 192 spikes. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, Kruskal-Wallis ANOVA test, Dunn's post-hoc test.

3.4. The effects of actin inhibitors and a clathrin inhibitor on vesicle release

During endocytosis, actin filaments interact with clathrin-mediated endocytosis to produce enough force to remodel the cell membrane for endocytosis (Collins et al. 2011; Jackson et al. 2015). This membrane invagination requires a direct interaction between dynamin, F-actin and clathrin. Interestingly, it was recently shown that the inhibition of clathrin reduces the release of catecholamine in chromaffin cells (Jackson, 2013). Therefore, we explored this further by investigating the effect of clathrin inhibition on FPE to see if this effect of clathrin on FPE involves the actin/myosin II network. For this, chromaffin cells were treated for 30 minutes with either one of Latrunculin B (LatB), Cytochalasin B (CytoB) and blebbistatin (Blebbi). Then cells were incubated additionally for another 30 minutes with the clathrin inhibitor, Pitstop 2 (0.5μM). Amperometry was used to measure the effect of these drugs on vesicle release. Then the data where normalized in percentage to be compared to the single drug treatment from previous work (Jackson, 2013).

3.4.1 Latrunculin B inhibits Pitstop 2 action on vesicle release

The average spike area considerably decreases in cells incubated with 0.1μM LatB + 0.5μM Pitstop 2 (156.3 ± 6.89 fC) compared to the control cell (243.2 ± 8.18 fC) **(Figure 3.10 A)**. However, the 0.1μM LatB + 0.5μM Pitstop 2 increased considerably (67.67 ± 2.98%) compared to 0.5μM Pitstop 2 treatment alone (7.91 ± 1.45%) **(Figure 3.10 D)**. The average spike amplitude reduced significantly in 0.1μM LatB + 0.5μM Pitstop 2 (29.27 ± 1.43pA) compared to controls (66.51 ± 2.92 pA) **(Figure 3.10 B)**. Interestingly, there was no change in the amplitude values in normalized data between the 0.1μM LatB + 0.5μM Pitstop 2 group for (49.47 ± 2.42%) and the 0.5μM Pitstop 2 (47.80 ± 5.31%) **(Figure 3.10 E)**. The average spike rise time increased significantly for 0.1μM LatB + 0.5μM Pitstop 2 (0.93 ± 0.06 ms) compared to controls treated cell (0.57 ± 0.04 ms) **(Figure 3.10 C)**. Normalized data showed that 0.1μM LatB + 0.5μM Pitstop 2 group (148.5 ± 9.46%) was higher than both 0.1μM LatB (110.4 ± 6.17%) and 0.5μM Pitstop 2 (24.57 ± 4.48%) treated cell **(Figure 3.10 F)**. Additionally, the PSF signal duration decreased significantly for the 0.1μM LatB + 0.5μM Pitstop 2 groups (2.73 ± 0.21 ms) compared to controls (7.60 ± 0.45 ms) **(Table 3.3)**. Furthermore, similar variations were observed with other amperometry traces parameters such as spikes frequency, half-width, decay time and PSF amplitude **(Table 3.3)**.

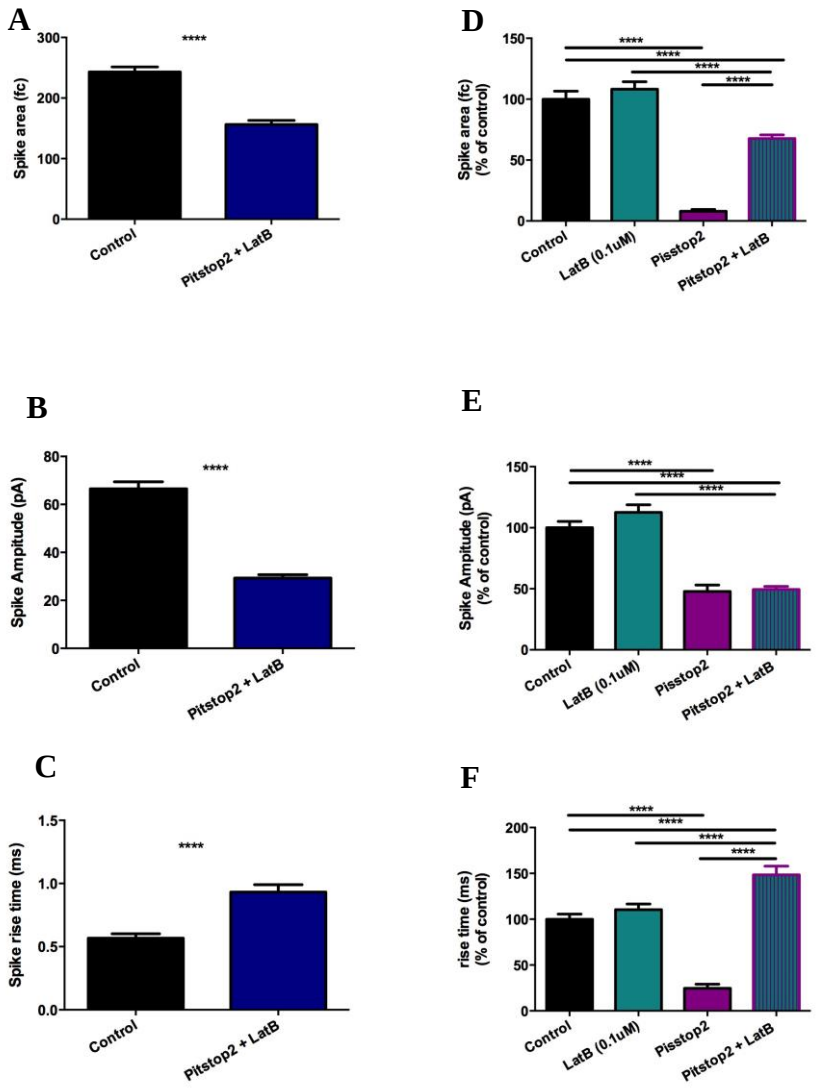

Figure 3. 10 The effect of Latrunculin B and Pitstop 2 on chromaffin cell secretion

The average spike (**A)** area **(B)** amplitude, decreased significantly when the cell was treated with 0.1μM LatB + 0.5μM Pitstop 2 compared to the control chromaffin cells. Average spike rise time **(C)** increased significantly upon treated with 0.1μM LatB + 0.5μM Pitstop 2 compared to the control. **(D) (E) (F)** A comparison between single drug treatment and 0.1μM LatB + 0.5μM Pitstop 2 calculated in percentage. Control n= 7 cells, 436 spikes; 0.1 μM LatB + 0.5μM Pitstop 2 n = 8 cells, 311 spikes. **** $p<0.0001$, Kruskal-Wallis ANOVA test, Dunn's post-hoc test.

3.4.2 Cytochalasin B inhibits Pitstop 2 action on vesicle release

The amperometry recording from cells treated with 0.5μM CytoB + 0.5μM Pitstop 2 resulted in a significant decrease in the average spike area (155.5 ± 8.95 fC) compared to the control cells (323.8 ± 9.51 fC) **(Figure 3.11 A)**. In the normalized data, spike area in cells treated with 0.5μM CytoB + 0.5μM Pitstop 2 increased considerably (43.57 ± 2.51%) compared to the effect of 0.5μM Pitstop 2 alone (7.93 ± 1.45%) **(Figure 3.11 D)**. The average spike amplitude decreased significantly in 0.5μM CytoB + 0.5μM Pitstop 2 treated cells (23.41 ± 1.49 pA) compared to controls (75.27 ± 3.29 pA) **(Figure 3.11 B)**. On the other hand, the amplitude reduction in normalized data was the same in all drugs treatment, (55.85 ± 3.58%) in 0.5μM CytoB + 0.5μM Pitstop 2, (48.19 ± 5.37%) in 0.5μM Pitstop 2, and (58.01 ± 4.69%) in 0.5μM CytoB treated cells **(Figure 3.11 E)**. The average spike rise time showed an increase for 0.5μM CytoB + 0.5μM Pitstop 2 (0.86 ± 0.06 ms) compared to controls (0.49 ± 0.10 ms) **(Figure 3.11 C)**. Conversely, normalized data showed a significant increase in 0.5μM CytoB + 0.5μM Pitstop 2 group (118.2 ± 8.40%) compared to cell treated with 0.5μM Pitstop2 (24.57 ± 4.88%) **(Figure 3.11 F)**. Moreover, the PSF signal duration increased significantly for the 0.5μM CytoB + 0.5μM Pitstop2 groups (5.28 ± 0.63 ms) compared to controls (11.84 ± 1.22 ms) **(Table 3.3)**. Additionally, similar variations were observed with other amperometry parameters for example spikes frequency, half-width, decay time and PSF amplitude **(Table 3.3)**.

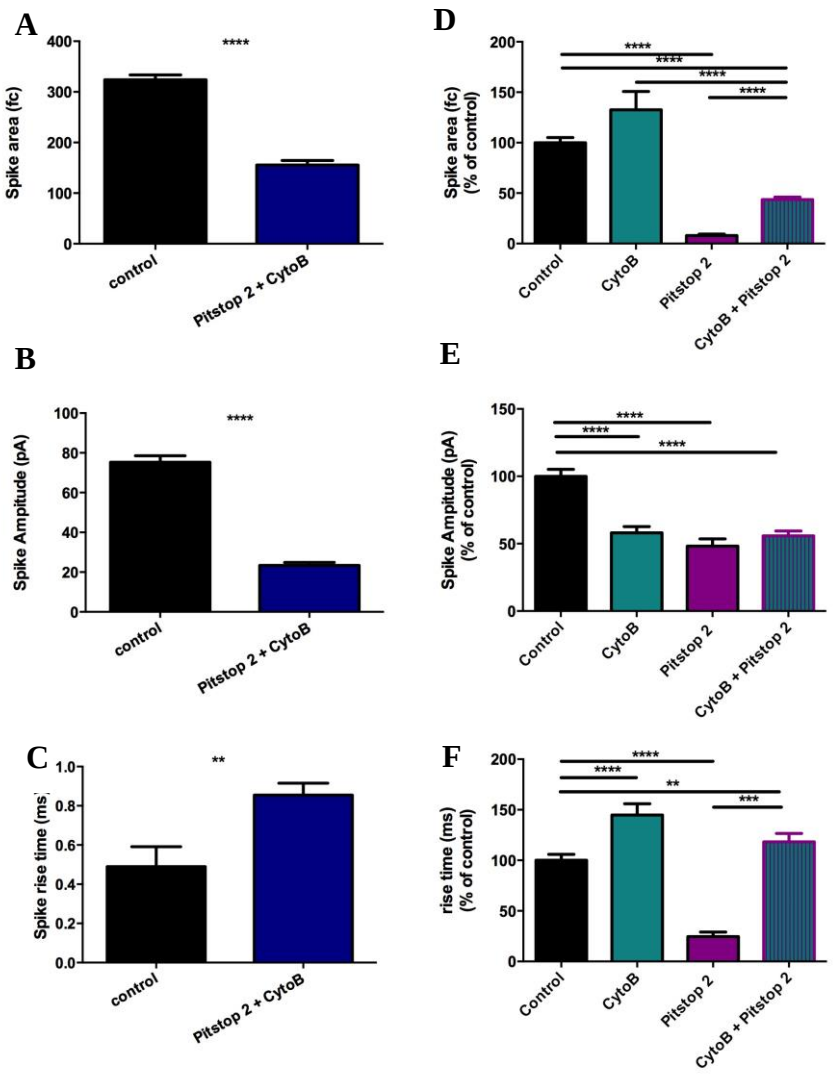

Figure 3. 11 The effect of Cytochalasin B and Pitstop 2 on chromaffin cell secretion

The average spike (**A**) area **(B)** amplitude decreased significantly when the cell was treated with 0.5µM CytoB + 0.5µM Pitstop 2 compared to the control chromaffin cells. **(C)** There was a significant increase in the average spike rise time treated with 0.5µM CytoB + 0.5µM Pitstop 2 compared to the control. **(D) (E) (F)** A comparison between single drug treatment and 0.5µM CytoB + 0.5µM Pitstop 2 calculated in percentage. Control n= 6 cells, 511 spikes; 0.5µM CytoB + 0.5µM Pitstop 2 n = 6 cells, 167 spikes. **** $p<0.0001$, *** $p<0.001$, ** $p<0.01$, Kruskal-Wallis ANOVA test, Dunn's post-hoc test.

3.4.3 Blebbistatin inhibits Pitstop 2 action on vesicle release

The average spike area decreased significantly in cells treated with 0.5μM Blebbi + 0.5μM Pitstop2 (204.2 ± 9.66 fC) compared to the control chromaffin cells (243.2 ± 8.18 fC) **(Figure 3.12 A)**. In the normalized data, the spike area for the 0.5μM Blebbi + 0.5μM Pitstop 2 treated cells increased considerably (83.97 ± 3.97%) compared to the spike area for the 0.5μM Pitstop 2 treated cell (7.93 ± 1.45%) **(Figure 3.12 D)**. The average spike amplitude decreased slightly but not significantly in 0.5μM Blebbi + 0.5μM Pitstop2 treated cells (47.75 ± 3.49 pA) compared to controls (66.51 ± 2.94 pA) **(Figure 3.12 B)**. The amplitude percentage data displayed a major increase in 0.5μM Blebbi + 0.5μM Pitstop 2 (71.80 ± 5.25%). Whereas the 0.5μM Pitstop 2 treated cell was (47.80 ± 5.31%**) (Figure 3.12 E)**. The average spike rise time showed an increase in cells incubated with 0.5μM Blebbi + 0.5μM Pitstop2 (0.92 ± 0.09 ms) compared to controls (0.57 ± 0.035 ms) **(Figure 3.12 C)**. Although, in normalized data the rise time increased massively with 0.5μM Blebbi + 0.5μM Pitstop2 (162.4 ± 16.26%) compared to the 0.5μM Pitstop 2 drug alone (24.57 ± 4.48%) **(Figure 3.12 F)**. Furthermore, the PSF signal duration increased significantly for the 0.5μM Blebbi + 0.5μM Pitstop2 groups (5.98 ± 0.62 ms) compared to controls (7.60 ± 0.45 ms) **(Table 3.3)**. Additionally, similar variations were observed with other amperometry traces parameters for example spikes frequency, half-width, decay time and PSF amplitude **(Table 3.3)**.

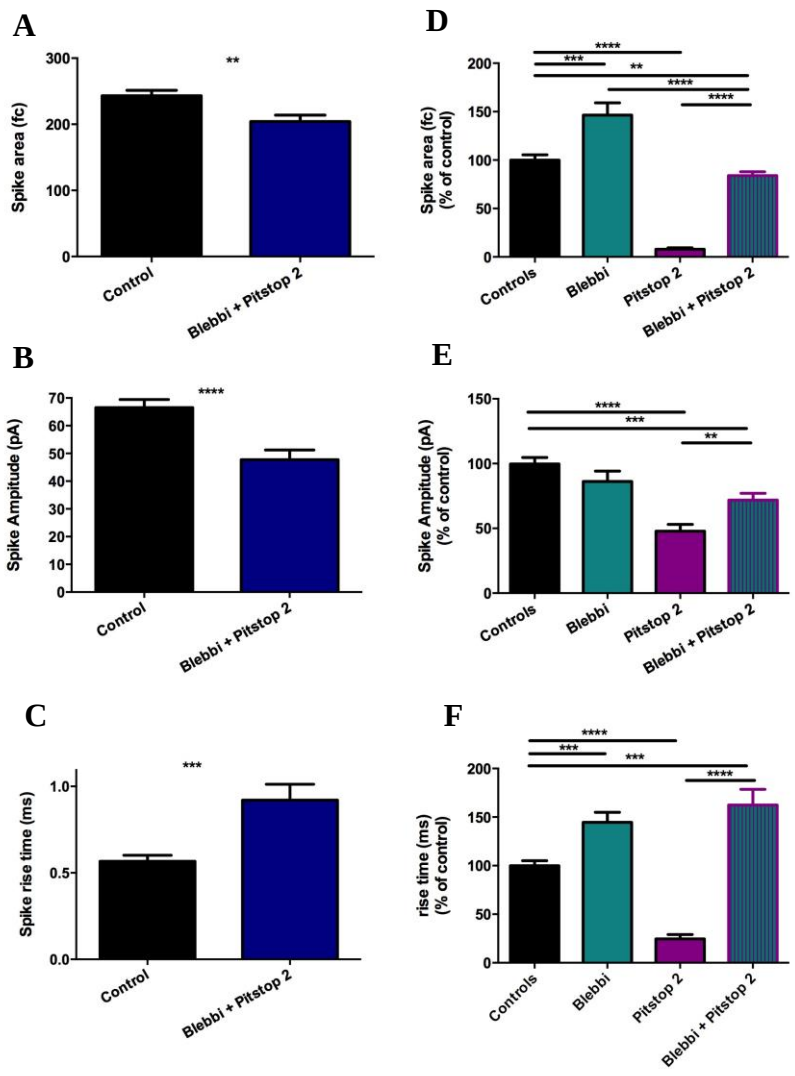

Figure 3. 12 The effect of Blebbistatin and Pitstop 2 on chromaffin cell secretion

The average spike **(A)** area **(B)** amplitude decreased significantly when the cell was treated with 0.5µM Blebbi + 0.5µM Pitstop 2 compared to the control chromaffin cells. Average spike rise time**(C)** increased significantly upon treated with 0.5µM Blebbi + 0.5µM Pitstop 2 compared to the control. **(D) (E) (F)** A comparison between single drug treatment and 0.5µM Blebbi + 0.5µM Pitstop 2 calculated in percentage. Control n= 7 cells, 435 spikes; 0.5µM Blebbi + 0.5µM Pitstop 2 n = 8 cells, 311 spikes. **** $p<0.0001$, ** $p<0.01$, Kruskal-Wallis ANOVA test, Dunn's post-hoc test.

Table 3.1 Ryngo3-32 and Ryngo3-37 increase the total amount of catecholamine release per fusion event

Chemical effectors	Frequency (Hz)	Amplitude (pA)	Area (fC)	Decay time (ms)	Rise time (ms)	Half-width	Pre spike foot signal data			
							Frequency (Hz)	Amplitude (pA)	Duration (ms)	Area (fC)
Ryngo3-32 control	1.45 ± 0.26	65.74 ± 3.02	290.8 ± 9.14	4.51 ± 0.20	0.67 ± 0.028	4.20 ± 0.17	0.56 ± 0.20	13.89 ± 1.07	5.55 ± 0.40	27.81 ± 2.34
Ryngo3-32 (10µM)	0.66 ± 0.18	30.41 ± 1.60	409.8 ± 16.25	11.16 ± 0.58	1.753 ± 0.12	10.46 ± 0.54	0.12 ± 0.05	9.76 ± 1.36	21.84 ± 1.44	34.03 ± 4.85
Ryngo3-37 control	0.71 ± 0.13	87.71 ± 5.32	258.7 ± 11.14	3.29 ± 0.2143	0.69 ± 0.05	3.25 ± 0.19	0.43 ± 0.16	19.21 ± 1.57	6.753 ± 0.49	36.05 ± 2.70
Ryngo3-37 (5µM)	0.39 ± 0.08	65.01 ± 6.99	553.1 ± 27.89	9.73 ± 0.60	1.77 ± 0.15	8.93 ± 0.54	0.14 ± 0.07	13.22 ± 1.75	15.89 ± 1.18	109.8 ± 12.36

Table 3.2 The effects of actin/myosinII and Dynole 34-2 inhibitors on spike parameters										
Chemical effectors	**Frequency (Hz)**	**Amplitude (pA)**	**Area (fC)**	**Decay time (ms)**	**Rise time (ms)**	**Half-width**	**Pre spike foot signal data**			
							Frequency (Hz)	**Amplitude (pA)**	**Duration (ms)**	**Area (fC)**
0.5µM Dynole 34-2 + 0.1µM LatB Control	1.88 ± 0.50	69.54 ± 2.72	275.2 ± 8.36	3.48 ± 0.16	0.58 ± 0.03	3.65 ± 0.20	0.62 ± 0.28	15.71 ± 0.94	4.8 ± 0.42	26 ± 2.35
0.5µM Dynole 34-2 + 0.1µM LatB	0.20 ± 0.11	12.12 ± 0.28	1.71 ± 0.08	0.10 ± 0.01	0.05 ± 0.003	0.12 ± 0.006	0.03 ± 0.01	0.65 ± 0.16	0.30 ± 0.06	0.20 ± 0.05
0.5µM Dynole 34-2 + 0.5µM CytoB Control	1.10 ± 0.21	89.97 ± 3.07	311.3 ± 7.67	2.70 ± 0.10	0.44 ± 0.07	2.68 ± 0.10	0.63 ± 0.31	13.31 ± 0.70	5.44 ± 0.33	13.31 ± 0.70
0.5µM Dynole 34-2 + 0.5µM CytoB	0.15 ± 0.06	22.69 ± 2.08	52.75 ± 4.91	2.24 ± 0.23	0.41 ± 0.05	2.13 ± 0.23	0.13 ± 0.07	5.356 ± 0.77	1.66 ± 0.15	4.05 ± 0.66
0.5µM Dynole 34-2 + 0.5µM Blibbi Control	1.95 ± 0.52	54.61 ± 1.86	367.1 ± 10.65	4.879 ± 0.2	0.86 ± 0.05	4.88 ± 0.20	0.63 ± 0.14	10.05 ± 0.79	7.74 ± 0.53	38.45 ± 3.08
0.5µM Dynole 34-2 + 0.5µM Blibbi	0.52 ± 0.17	31.09 ± 3.75	124.7 ± 8.52	3.75 ± 0.2	0.80 ± 0.06	3.94 ± 0.27	0.21 ± 0.06	5.99 ± 0.58	2.575 ± 0.30	7.49 ± 1.05

Table 3.3 The The effects of actin/myosin inhibitors and pitstop 2 on spike parameters

Chemical effectors	Frequency (Hz)	Amplitude (pA)	Area (fC)	Decay time (ms)	Rise time (ms)	Half-width	Pre spike foot signal data			
							Frequency (Hz)	Amplitude (pA)	Duration (ms)	Area (fC)
0.5µM Pitstop2 + 0.1µM LatB Control	1.88 ± 0.23	66.51 ± 2.94	243.2 ± 8.18	2.83 ± 0.14	0.57 ± 0.04	2.8 ± 0.15	0.9 ± 0.27	14.03 ± 1.33	7.6 ± 0.45	46.61 ± 4.28
0.5µM Pitstop2 + 0.1µM LatB	0.72 ± 0.09	29.27 ± 1.43	156.3 ± 6.89	4.36 ± 0.20	0.93 ± 0.06	4.47 ± 0.22	0.31 ± 0.05	7.13 ± 0.56	2.73 ± 0.21	11.80 ± 1.29
0.5µM Pitstop2 + 0.5µM CytoB Control	1.86 ± 0.67	75.27 ± 3.29	323.8 ± 9.53	3.38 ± 0.14	0.49 ± 0.10	3.58 ± 0.17	0.36 ± 0.08	7.95 ± 0.83	11.84 ± 1.22	100.9 ± 14
0.5µM Pitstop2 + 0.5µM CytoB	0.54 ± 0.13	23.41 ± 1.49	155.5 ± 8.95	4.44 ± 0.23	0.86 ± 0.06	4.55 ± 0.24	0.22 ± 0.06	4.27 ± 0.42	5.28 ± 0.63	19.10 ± 3.1
0.5µM Pitstop2 + 0.5µM Blibbi Control	2.57 ± 0.44	66.51 ± 2.94	243.2 ± 8.18	2.83 ± 0.14	0.57 ± 0.04	2.8 ± 0.15	0.9 ± 0.27	14.03 ± 1.33	7.6 ± 0.45	46.61 ± 4.28
0.5µM Pitstop2 + 0.5µM Blibbi	0.47 ± 0.26	47.75 ± 3.49	204.2 ± 9.66	3.87 ± 0.29	0.92 ± 0.09	3.50 ± 0.27	0.17 ± 0.08	10.04 ± 1.36	5.98 ± 0.62	36.61 ± 3.28

Chapter 4 - Discussion

Previous work in our laboratory showed that incubating chromaffin cells with dynamin modulators, actin/myosin II inhibitors or clathrin inhibitors affect transmitter release from vesicles. Based on this data, this project aimed to expand our knowledge about dynamin's ability to direct the dilation of the fusion pore by investigating whether actin/myosin II inhibition blocks the effect of dynamin modulators or clathrin inhibition on vesicle release. This study provides new insight into the cell's molecular mechanism by using drugs that afforded us the opportunity to identify the functions of dynamin and clathrin in exocytosis; separate from their functions in endocytosis. Our results showed that chromaffin cells treated with dynamin activators, Ryngo 3-32 and Ryngo 3-37, increased the amount of catecholamine released per fusion event. Furthermore, actin/myosin II inhibition did not block the effect of Dynole 34-2 as it did with Ryngo-1. Moreover, the acute approach of inhibiting actin/myosin II, partially blocked the clathrin inhibitor, Pitstop 2, from reducing transmitter release.

4.1 Fusion pore dilation is directly controlled by Dynamin GTP-ase activity

Dynamin GTPase activity enables the scission of endocytic vesicles from the plasma membrane to sustain the balance of fusion and fission process (Kaksonen et al. 2006). Dynamin also has a new role in regulating exocytosis through the fusion pore, as this process is critical as it controls the quantal release of vesicle content to the extracellular space (Anantharam et al. 2011; Ferguson & De Camilli 2012; Jahn et al.

2003). My experimental results indicated that dynamin GTP-ase activity plays a key role in the regulation of the expansion of the fusion pore as well as the release of transmitters. When chromaffin cells were treated with the dynamin activators Ryngo 3-32 or Ryngo 3-37, a significant increase in most spike parameters were seen such as spike area, rise time and PSF signal.

Different factors can affect amperometric spike parameters such as dissociation and diffusion of catecholamine (Anantharam et al. 2011). The dissociation rate for catecholamines from the cell to the extracellular matrix is an important factor as well as the diffusion rate from the cell that affect spike parameters along with vesicle size, fusion pore size and fusion pore open time. As the small molecules used in this study are not expected to affect vesicles size (Galas et al. 2000), we assume that the diffusion or dissociation rates of catecholamine released from these vesicles are not affected as well. Our assumption therefore, is that the changes that we see in spike area which represent the amount of catecholamine released from a single vesicle are due to changes in fusion pore expansion, opening and/or closing. For this reason most of the following discussion focuses more on these parameters.

It is presumed that dynamin regulates the size of the diameter of the fusion pore and requires oligomerisation–stimulated activity of GTPase to do so (Jackson et al. 2015). This is assumed to be the mechanism targeted by Ryngo-3 compounds to increase the release of neurotransmitters. Dynamin is a large GTPase that is used in the scission of clathrin-coated pits (CCPs) and is thought to regulate the early stages of clathrin mediated endocytosis (CME) in conjunction with the actin cytoskeleton. It

does so by developing higher order oligomers such as helices and rings, depending on its GTPase hydrolysis, at the neck of deeply invaginated CCPs where it mediates membrane fission and the release of clathrin coated vesicles (González-Jamett et al. 2013; Liu et al. 2013; McMahon & Boucrot 2011). Based on how dynamin functions during the fission process, it can be assumed that Ryngo-3 stimulates the GTPase domain which induces the formation and maintenance of dynamin rings around the neck of the initial fusion pore that is created between the vesicles and cell membrane, augmenting the quantal release of transmitters. This stimulation by Ryngo-3 compounds will most likely effect the 'kiss and run' fusion (Stand Alone Foot signal SAF) which represents only half of the fusion frequency in chromaffin cells as demonstrated using total internal reflection fluorescence microscopy (Kozlov et al. 2010), more than effecting the full fusion events. The changes that we observed in spike kinetics supports our theory that Ryngo-3 promotes fusion pore opening. These changes include an increase in the duration time, rise/ decay time, and spike area which could indicate that the fusion pores are taking longer to open and close, giving us a wider spike with smaller amplitude. This increase in some spike parameters suggests that there is a direct impact on dynamin GTPase activity and actin polymerization by Ryngo-3, which consequently acts on fusion pore expansion and vesicle release.

4.1.1 Dynamin phosphorylation is required for fusion pore expansion

This type of fusion pore expansion that we see with Ryngo -1 and 3 is also seen under acute stress response. This whole process of fusion pore expansion acutely occurs in nature and serves a function in the adrenal medulla in chromaffin cells. Catecholamine levels increases in response to acute stress (fight or flight response) which thrusts blood flow to the muscles and increases cardiac output (Jackson et al. 2015). Thus, high stimulation in chromaffin cells increases the amount released from individual vesicles, and this process involves dynamin-syndapin interaction and calcineurin-dependent dephosphorylation of dynamin (Liashkovich et al. 2015; Samasilp et al. 2012). In addition, this study showed that the fusion pore expansion is mediated by calcineurin activity, dynamin I–syndapin interaction and N-WASP activation and that any inhibition of these routes is followed by a decrease in cells secretion and restricts the expansion of the fusion pore. Syndapin, through N-WASP, regulates actin polymerisation and has also been found to limit dynamin's ability to expand the fusion pore when the interaction between syndapin and dynamin are disrupted (Samasilp et al. 2012; Shieh 2010). One proposed model suggests that dynamin working in association with N-WASP is able to organise actin localisation at the exocytosis site that may propel the initial expansion of the fusion pore and control its expansion rate (Quan & Robinson 2014). Since dynamin phosphorylation is critical in this pathway, it is logical to ask if Ryngo's are affecting this step. We do not think that we are affecting the phosphorylation of dynamin by these drugs, because we know that they effect ring formation. However given that there is a similar outcome in terms of more release per vesicle, perhaps this phosphorylation of

dynamin with syndapin also stabilizes dynamin into a ring structure, providing a potential link between what we are doing pharmacologically and the physiological reaction. The potential for future work to look at whether this phosphorylation of dynamin involving syndapin also results in stabilizing the ring formation of dynamin may be a worthwhile area of examination.

4.2 Actin/myosin II cytoskeleton participation in the fusion pore

Dynamin's ability to initiate membrane fission and produce membrane curvature is well known; however, recent findings suggest that dynamin plays another role in regulating the actin cytoskeleton and fusion pore (Berberian et al. 2009; González-Jamett et al. 2013; Robertson et al. 2014; Shieh 2010). Dynamin was found to interact with short actin filaments to promote actin polymerization or elongation by displacing the actin capping protein gelsolin from their barbed ends (Lanzetti 2007). However, how significant this interaction is for vesicle release and what the molecular mechanism underlying this process, are yet to be discovered and need further investigation. Previous data from our lab demonstrated that by inhibiting either actin or myosin II, the activation of dynamin by Ryngo-1, which target dynamin GTPase domain, was prevented (Jackson et al. 2015). This took us to the next step of testing whether actin/myosin II inhibition would block dynamin's inhibitory effect on cell secretion.

The results of our experiments support the notion that the dynamin-actin interaction is critical for cell secretion and fusion pore formation. When both actin and dynamin

were inhibited, the spike frequency was reduced which could be most likely due to blocking endocytosis and vesicles recycling (Gu et al. 2010). Also, we noticed a significant decrease in the average spike area similar to cells treated with Dynole 34-2 alone (Jackson et al. 2015). This decrease in cell secretion in those cells treated with Dynole 34-2 and actin/myosin II inhibitors may be due to the inability of the fusion pore to open and expand. In chromaffin cells, dynamin's GTPase activity was require for regulating the early fusion pore expansion and F-actin comets (Anantharam et al. 2011; Orth et al. 2002; Pang & Südhof 2010) and that F-actin and myosin II were found to accelerate and facilitate the fusion pore expansion by providing enough force for the vesicles to release their content (Doreian et al. 2008; Gu et al. 2014) Thus these studies, along with our findings, support our theory that fusion pore opening and expansion is more likely controlled by dynamin in an actin and myosin II- dependent manner. So that activating dynamin makes the fusion pore open for either a longer period of time or/and much wider, alternatively, the inhibiting of dynamin is likely to limit the opening of the fusion pore, limiting the time that pores may take to open or the size of the pore itself. However, we could speculate that any secretion seen in the presence of these inhibitors are most likely to be due to other actin-dependent mechanisms that do not depend on actin polymerization in particular, and may perform the opening of the fusion pore, but not as significantly. To further understand this relationship, more study on how dynamin and actin inhibition impacts the kinetics of the fusion pore is required.

4.2.1 Examples of actin-dependent mechanisms

Actin is a large complex protein and these drugs (Cytochalasin B and Latrunculin B) inhibit actin polymerization only (Berberian et al. 2009; Kaksonen et al. 2006). However, other actin-dependent mechanisms or signalling pathways that do not necessarily depend on actin in its polymerized state may also mediate cell secretion. Some examples of such signalling pathways are Arp2/3 (Chan et al. 2010), Cdc42 (Taunton et al. 2000; Yang et al. 2012) and Rho pathways (de Matos et al. 2014; Stamnes 2002). First, the actin-related protein 2/3 (Arp2/3) complex promotes the nucleation of a new F-actin filament at the side of an F-actin mother filament, which is therefore crucial for regulating cytoskeletal networks, filament organizations, and signalling pathways (Amano et al. 2010; Rotty et al. 2013). Second, the GTPase, Cdc42 and their effector N-WASP, have been shown to control *de novo* actin polymerization at the exocytosis site. During the membrane trafficking process, actin dynamics are moderated by the interaction between dynamin and N-WASP (Koestler et al. 2013; Pang & Südhof 2010). Third, Rho-kinase is also important for regulating the contractility of actomyosin motor fibrils that regulates cell contractions, by small GTP-ases from the p21 Rho family, and is thought to be the main regulator of myosin activity that determines when and where in the cell myosin is active (Chan et al. 2010). Due to these other signalling pathways, some secretion is seen even when both dynamin and actin/myosin II are inhibited, however, it is difficult for us to confirm this from the amperometric records only, thus cell visualising experiments may help to further understand this process.

4.2.2 Dynamin is one component of a larger protein complex

Dynamin may represent a single component of a larger protein complex that regulates fusion pore expansion. Via its SH3-domain, dynamin directly binds to other proteins such as SNARE and other fusion accessory proteins that may influence fusion pore expansion (Galas et al. 2000; Okamoto et al. 1999; Robinson & Bonifacino 2001; Spiering & Hodgson 2011). The syntaxin/Munc-18 complex is critical for the localization of core complexes for SNARE-dependent fusion (Zhou et al. 1996). Munc18 proteins are thought to also play a role in vesicle translocation and docking, as well as post-docking and the rearrangement of the cytoskeletal functions (Lynch et al. 2008; Papadopulos et al. 2015; Robertson et al. 2014). Studies using either live cell imaging or confocal imaging verify that Munc18-2 with syntaxin 3 govern granule translocation during the degranulation process, which is thought to directly influence fusion (Liashkovich et al. 2015; Min et al. 2007; Robertson et al. 2014). Besides directly promoting fusion, there is also the possibility that Munc18-2 plays a role in joining the fusion apparatus with the microtubule cytoskeleton as it has been shown that Munc18-2 labelled vesicles form an alignment along microtubules (Shieh 2010). Whether or not the syntaxin/Munc-18 complex is involved in this dynamin-actin regulation of the fusion pore is a significant question and a novel area of future research in this field.

4.3 Actin/myosin II interplay with clathrin

Clathrin-mediated endocytosis (CME) is important for recruiting various adaptor and scaffold proteins such as dynamin and actin cytoskeletons to the neck of the budding

endocytic vesicle (Brochetta et al. 2014). Clathrin inhibition by Pitstop 2 showed a significant decrease in spike parameters and PSF signal, the PSF events were very quick and too small to analyse, which indicated clathrin involvement in regulating exocytosis (Jackson, 2013). To investigate this further we wanted to see if inhibiting actin would actually block the effect of Pitstop 2 inhibition on vesicle release. In these experiments we saw that the actin inhibitors (Cytochalasin B and Latrunculin B) and the myosin II inhibitor (Blebbistatin) disturbed the inhibitory effect of Pitstop 2 on granule secretion. However, there was still a significant decrease in all cases in spike frequency and in catecholamine release. Interestingly, there was an increase in the spike rise/decay time, which may potentially reflect the stability of the fusion pore (McMahon & Boucrot 2011). These findings lead us to assume that the combination of these drugs may have one or more of the following disturbing effects on exocytosis: the recycling and loading of vesicles, membrane tension, pore formation or its stability (Kaksonen et al. 2006; Zhou et al. 1996). To support the theory in regards to inhibiting vesicle recycling process, a recent study used quantitative fluorescence microscopy and showed that during endocytosis, when actin polymerization was blocked by Cytochalasin D (similar to Cytochalasin B) dynamin II was stabilized at the endocytic sites (Miklavc et al. 2015), therefore interrupting the vesicle scission process and recycling. The budding of clathrin-coated vesicles at the plasma membrane and the short actin filaments capped with gelsolin were found to trigger the GTPase activity of dynamin and promote its oligomerization into rings and helixes (Grassart et al. 2014; Gu et al. 2014; Gu et al. 2010). In turn, dynamin oligomer precedes the fission process for endocytosis and displaces gelsolin from the barbed thus regulating actin oligomerization. This

indicated that clathrin and F-actin polymerization are required in both early stages to recruit dynamin 2 in to rings and in the later stages for vesicle scission by dynamin 2 and that this interaction impacts the dynamics of PSF signals.

Endocytosis requires clathrin function in order to recruit different proteins for vesicle recycling and the reloading process. The mechanism of Pitstop 2 is to inhibit the interaction between amphiphysin and clathrin and to bind to the N-terminal of the heavy chain of clathrin which inhibits CME. Any defect in CME or the actin/myosin II cytoskeleton will directly affect all proteins recruited by those processes for both endocytosis and exocytosis. Some example of proteins regulating endocytosis are; the BAR domain-containing proteins amphiphysin and endophilin that facilitate dynamin recruitment to the neck of clathrin-coated pits in CME (Grassart et al. 2014); the intersectin protein that is also recruited by CME and binds to dynamin in order to regulate endocytosis and therefore may regulate exocytosis as well (Meinecke et al. 2013; Neumann & Schmid 2013); In addition, some other clathrin adaptor proteins such as AP-2, AP180/CALM, and epsin contain actin-nucleating properties which also could be affected by the inhibition of actin (Di Rubbo et al. 2013; Morlot 2012). Usually, dynamin binds these proteins via PRD-SH3 domain, as they have membrane-bending properties, hence, if these proteins are not recruited to the sites of membrane-vesicle interface, dynamin likewise may not be able to take its functional shape to regulate the fusion pore and actin/myosin II polymerisation as well (Moshkanbaryans et al. 2014). To provide greater insight into how actin and

clathrin control fusion pore opening, further experiments such as testing if the effect of actin stabiliser blocks the effect of Pitstop 2 need to be undertaken.

Currently, there are no studies that describes clathrin's role in exocytosis, however, some assumption were proposed in regards to its ability to drive the fusion process (Kozlov et al. 2010; Stollar et al. 2012). Therefore, it will be beneficial to look at this in greater depth to ascertain if clathrin does play a more significant role in exocytosis than currently thought. We could however postulate that clathrin may have a direct mechanism to regulate fusion pore or an indirect one through recruiting various protein partners. Further studies such as determining the clathrin-binding proteins before and after treatment with clathrin inhibitor Pitstop 2, or detecting the size of fusion pore after treatment with Pitstop 2 will provide new insight in to the critical mechanics underlying the regulation of fusion pore.

4.4 Future directions

With experimental and therapeutic pharmaceutical drugs, there is always the possibility of off-target effects which need to be taken into account. The fact that Ryngo-1 and 3 have similar effects on spike parameters, and that Ryngo-1 activation was blocked by actin/myosin II inhibitors (Jackson et al. 2015), is very strong evidence to support the specificity of these novel molecules to target the GTP-ase activity of dynamin. Also, Dynole 34-2 and Dyngo-4a drugs showed the same inhibitory effect, which indicate the specificity of these drugs towards dynamin. In general, by looking at the effects of dynamin activators and inhibitors producing contrasting results on vesicle release, this limits the potential of non-specificity of

these drugs. An efficient way to test the specificity of these chemical effectors is by using transfection techniques to mutate interaction sites. An example of this would be using dynamin with a mutation where it binds with actin, which could then be deliberately transfected into the cells and then analysed by amperometry to ascertain whether there is any increase or decrease caused by dynamin activator/inhibitor in the spike area. Any chemical that has the essential chemical elements similar to the dynamin activator/inhibitor could be used to see whether they have the same effect on pore expansion, as long as they do not affect the GTP-ase activity of dynamin.

Another possible way to examine which part of the fusion pore is regulated by dynamin is to measure the fusion pore size indirectly using confocal microscopy. Fusion pore size limits the maximum rate of content efflux from a single vesicle. This can be measured by using different sized of fluorescently-labelled dextran molecules before and after treating cells with dynamin modulators and observing how they are affected (Kozlov & Chernomordik 2002). This procedure should provide a clear idea about how dynamin could control the expansion of the fusion pore. That is, if the drugs are making the pore wider, more of the large dextran molecules should get inside, however if there was no effect on the width then we should not see any changes and it maybe that dynamin works on fusion pore by open it for longer (some of this work has been done, however, the data are not usable since the drug was showing a background fluorescence). Other alternative ways to examine the role of dynamin and actin in FPE is by superimposing actin stabilizers such as Jasplakinolide (Kozlov & Chernomordik 2002) on dynamin inhibitors to see if it prevents the inhibition effect. If it does, then it is suggestive that these drugs are

having an effect on the G-domain of dynamin and may provide further information in regards to drug specificity and molecular mechanism.

As the findings of this study in regards to actin/myosin II and clathrin inhibition were interesting but difficult to explain, the specificity of Pitstop-2 needs to be addressed as well. One way to do this is applying a negative analogue (Pitstop 2-100) which has a very similar chemical structure to Pitstop -2, however; it does not have the same function (Söllner et al. 1993; Théry et al. 2009). Thus, any effects of this drug would indicate that we were getting an off-target effect in our experiments.

A limitation in our project using this technique was that we were only able to measure what was released, so we were not able to discover what part upstream of fusion pore opening was affected. Amperometry does not provide any insight as to what causes transportation, docking, or fusion pore opening; therefore, studies involving visualizing the cellular localization before and after applying the drugs would provide an insight into this.

4.5 Conclusion

This study investigated how during exocytosis, the quantal release of transmitter is affected by fusion pore expansion and the role that dynamin plays in this. How actin and myosin II are involved with dynamin and its control of the fusion pore expansion, as well as the role that clathrin played were also investigated. This study found that quantal transmitter release was changed significantly when dynamin was either inhibited or activated. As such, during dynamin activation, more transmitter is

released from vesicles while during dynamin inhibition less transmitter is released. This is a strong indicator that dynamin modulation directly modulates the amount of catecholamine released per fusion event by either making the fusion pore open wider or for longer periods of time. The opening of the fusion pore by dynamin is strongly assisted by the actin/myosin II cytoskeleton and the mechanism behind this process is unclear. It may be that these motor proteins provide enough force to accelerate or/and evacuate vesicle content. Also, even though clathrin is an endocytosis protein, clathrin has been found, for the first time, to play a role in exocytosis, possibly through controlling the recycling process of vesicles and by interacting with actin/myosin II proteins. In general, how dynamin regulates fusion pore expansion by cooperating with the actin cytoskeleton and clathrin, how these proteins interact with other binding partners including endophilin and amphiphysin needs to be further investigated. To reduce the likelihood that the indirect mechanisms could possibly alter the effect the drugs have on cells, experiments that deduce drug specificity using both control and transfection techniques need to be also undertaken.

The research conducted in this study has produced new findings regarding how fusion pore expansion and quantal transmitter release are regulated by dynamin and its binding partners actin/myosin II and clathrin, characterising procedures that likely occur in human cells. A question that has arisen from this study is whether or not other Ca^{2+} controlled secretory cells in the body, are regulated in the same manner during fusion pore expansion and transmitter release by dynamin, actin/myosin II and clathrin, and whether during synaptic vesicle exocytosis these proteins control the transmitter release at neuronal synapses or facilitate the release of insulin from beta cells. An idea for future studies in this field are to study what role dynamin has

in the various types of exocytosis and characterise this. The potential for dynamin to be a therapeutic target that reduces diseases of secretion such as diabetes and synaptopathies such as epilepsy is profound and worthy of further research.

The significance of this study is that the approach used, a combination of chemical dissection and single vesicle physiology, is world first and ground breaking and therefore is worthy of investment in future research. As hormone transmitter release is important to all higher order organisms, this study is able to provide outcomes that are unique, informative and beneficial in a variety of fields.

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