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HOW THE STIMULUS DEFINES THE DYNAMICS OF VESICLE POOL RECRUITMENT, FUSION MODE AND VESICLE RECYCLING IN NEUROENDOCRINE CELLS

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Keywords: exocytosis, endocytosis, secretion, calcium, immediately releasable pool, kiss-and-run, vesicle recycling.

Abreviations used: AP, action potential; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; IRP, immediately releasable pool; P/Q-KO, α1A deficient mice; PACAP, pituitary adenylate cyclase-activating polypeptide; RRP, ready releasable pool; SNARE, SNAP (Soluble NSF Attachment Protein) Receptor; synprint, synaptic protein interaction site; VIP, vasoactive intestinal polypeptide; VDCC, voltage dependent calcium channels; WT, wild type. and

Abstract

The pattern of stimulation defines important characteristics of the secretory process in neurons and neuroendocrine cells, including the pool of secretory vesicles being recruited, the type and amount of transmitters released, the mode of membrane retrieval and the mechanisms associated with vesicle replenishment. This review analyzes the mechanisms that regulate these processes in chromaffin cells, as well as in other neuroendocrine and neuronal models. A common factor in these mechanisms is the spatial and temporal distribution of the Ca^{2+} signal generated during cell stimulation. For instance, neurosecretory cells and neurons have pools of vesicles with different locations with respect to Ca^{2+} channels, and those pools are therefore differentially recruited following different patterns of stimulation. In this regard, a brief stimulus will induce the exocytosis of a small pool of vesicles that is highly coupled to voltage-dependent Ca^{2+} channels, whereas longer or more intense stimulation will provoke a global Ca^{2+} increase, promoting exocytosis irrespective of vesicle location. The pattern of stimulation, and therefore the characteristics of the Ca^{2+} signal generated by the stimulus, also influences the mode of exocytosis and the type of endocytosis. Indeed, low frequency stimulation favors kiss-and-run exocytosis and clathrin-independent fast endocytosis, whereas higher frequencies promote full fusion and clathrin-dependent endocytosis. This latter type of endocytosis is accelerated at high frequency stimulation. Synaptotagmins, calcineurin, dynamin, complexin, and actin remodeling, appear to be involved in the mechanisms that determine the response of these processes to Ca^{2+}.

Introduction

The release of hormones is a highly regulated process that requires to be adjusted to maintain the body's internal balance and its response to the environment. This regulation is particularly required for the release of catecholamines and neuropeptides from the adrenal chromaffin cells. These neuroendocrine cells are innervated by cholinergic terminals of the splanchnic nerve, which release acetylcholine and peptides such as the vasoactive intestinal polypeptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP), activating ionotropic and metabotropic receptors present in the plasma membrane of the chromaffin cells (Chowdhury et al., 1994). The activation of the ionotropic nicotinic receptor deals to a series of regulated events that occur in a perfect sequence: opening of voltage-dependent Na^+ channels and membrane depolarization, opening of voltage-dependent Ca^{2+} channels (VDCCs), and cytosolic Ca^{2+} signals that trigger the exocytotic release of the transmitters stored in the chromaffin granules (Weiss and Zamponi, 2012). On the other hand, activation of the metabotropic receptors, such as muscarinic, VIP and PACAP receptors, activates signaling pathways that regulate the exocytosis (Chowdhury et al., 1994). Interestingly, the firing frequency of the sympathetic nerves defines the characteristics of the secretory process, the pool of vesicles being recruited and the types and amount of transmitters released from the chromaffin cells (Elhamdani et al., 2001; Fulop et al., 2005; Voets et al., 1999). This is critical to keep the internal homeostasis under a resting condition, or for the fight-or-flight response during a stressful situation.

Under basal conditions the action potential (AP) firing rate of chromaffin cells is low, approximately 0.2-0.5 Hz, while under stress the system approaches 10-20 Hz (Brandt et al., 1976; Fulop et al., 2005; Holman et al., 1994). The intense stimulation of chromaffin cells provokes an important global Ca^{2+} increase (Fulop and Smith, 2006; Klingauf and Neher, 1997; Marengo and Monck, 2003), promoting exocytosis irrespective of the secretory vesicle location, mobilizing vesicles from upstream to downstream pools (Marengo, 2005; Voets et al., 1999) and triggering the release of the totality of the transmitters stored in the chromaffin granules (Elhamdani et al., 2001; Fulop et al., 2005). On the other hand, in rest conditions the firing frequency is low, Ca^{2+} does not accumulate markedly (Fulop and Smith, 2006), and therefore exocytosis would be limited to a group of vesicles that is closely coupled to VDCCs (Álvarez et al., 2008; Marengo, 2005; Oré and Artalejo, 2005; Voets et al., 1999). This group of

vesicles, that has also been described in other types of endocrine cells, such as pancreatic β and α cells (Barg et al., 2002; Barg et al., 2000; Ge et al., 2006), is generally known as the immediately releasable pool (IRP) (Horrigan and Bookman, 1994). Further, at low physiological frequencies chromaffin cells only release low-molecular-weight neurotransmitters through Ω-shape kiss-and-run fusion events (Elhamdani et al., 2001; Fulop et al., 2005; Fulop and Smith, 2006).

The maintenance of secretion at any range of frequencies requires the continuous refilling of releasable pools of vesicles at rates that match the exocytotic activity (Smith et al., 1998; Sorensen, 2004). As it was mentioned above, the global Ca^{2+} increase reached at high frequency stimulation promotes vesicle mobilization from upstream to downstream pools and, the consequent vesicle pool refilling (Voets et al., 1999). On the other hand, low physiological frequencies favors kiss-and-run fusion events (Fulop et al., 2005), suggesting that the replenishment of vesicles might be associated with a short local cycling mechanism. This review analyzes the mechanisms by which the stimulation pattern defines the mode of exocytosis, retrieval and replenishment of secretory vesicles in chromaffin and other neurosecretory cells.

Brief depolarization pulses promote the synchronic release of a small pool of vesicles

Different pools of vesicles have been described in neurons and neuroendocrine cells. They include the readily releasable pool (RRP), the slowly releasable pool (SRP) and the reserve pool (RP). These different pools are recruited with different stimulus patterns. While low to middle stimulation recruits vesicles from RRP, which is composed by fully primed vesicles, stronger stimulation might additionally recruit vesicles from the SRP and even from the RP, which is composed by unprimed vesicles (Ashery et al., 2000; Richards et al., 2000; Rizzoli and Betz, 2005; Voets et al., 1999). Furthermore, a fraction of vesicles is released during the stimuli (synchronous exocytosis), whereas another fraction is released between stimuli (asynchronous exocytosis). Brief single or low frequency stimulation favors the synchronous release, while an important asynchronous release is observed during prolonged single or high frequency stimulation (Marengo, 2005; Seward and Nowycky, 1996; Voets et al., 1999).

Synchronous synaptic transmission and neurosecretion is generally explained by the presence of a tight coupling between synaptic/secretory vesicles and VDCCs, which serves to tune the precise timing of Ca^{2+}-triggered fast release (Neher, 1998; Wadel et al., 2007). In neuroendocrine cells, as chromaffin cells, fast synchronous secretion is usually associated with the presence of the so called immediately releasable pool (IRP).

The Immediately Releasable Pool: Horrigan and Bookman (Horrigan and Bookman, 1994), working in isolated rat chromaffin cells, demonstrated for the first time the presence of IRP in neuroendocrine cells. Briefly, the application of single brief depolarization pulses, which activate VDCCs during a short period, revealed the existence of a small group of vesicles that are highly coupled to the stimulus. The exocytosis of IRP is basically synchronous with the stimulus and progressed with a first order kinetic behavior; according to the work of Horrigan and Bookman (1994), it has an initial rate of 680 fF/s and a time constant of 50 ms. These authors proposed two alternative hypotheses about the nature of IRP vesicles: (1) The IRP reflects a population of vesicles (maybe small synaptic-like vesicles) that are functionally different from the secretory vesicles that make up the RRP; (2) IRP is a subpopulation of RRP vesicles, but located close to Ca^{2+} channels (Horrigan and Bookman, 1994). Subsequent work favored the second hypothesis. First, in conventional flash photolysis experiments, the IRP was not detected as a different kinetic component (Ashery et al., 2000; Voets et al., 1999). Therefore, IRP seems to not be part of a pool of vesicles intrinsically faster than the RRP. Second, the application of brief depolarization pulses to deplete the IRP immediately before the flash provoked a decrease in the fast component of the exocytotic burst associated with the RRP. Such decrease in RRP was similar to the IRP absolute size (Voets et al., 1999). Third, BAPTA, in comparison to EGTA, reduced markedly the efficiency of Ca^{2+} entry to induce IRP exocytosis (Álvarez et al., 2013). Therefore, the IRP is commonly defined as a small group of readily releasable vesicles that are located in close proximity to VDCCs (Voets et al., 1999).

Estimations of the IRP size fluctuate between different chromaffin cell preparations and between different types of neurosecretory cells, ranging from less than 10 to almost 50 vesicles (Álvarez et al., 2008; Barg et al., 2002; Barg et al., 2001; Ge et al., 2006; Horrigan and Bookman, 1994; Marengo, 2005; Merrins and Stuenkel, 2008; Voets et al., 1999). Chromaffin cells in slices presented a more prominent exocytosis in response

to short depolarizations than isolated chromaffin cells in culture (Moser and Neher, 1997). Consistently, the co-localization between Ca^{2+} channels and vesicles was higher in slices in comparison to isolated cells (Lopez et al., 2007). Regarding the rate at which the IRP releases their vesicles, Horrigan and Bookman (1994) and Marengo (2005) have reported a value for cultured rat or bovine chromaffin cells of approximately 20 s^{-1}, whereas Voets et al. (1999) estimated a value of 150 s^{-1} in mouse adrenal slices. Finally, for mice isolated chromaffin cells in culture, Álvarez and collaborators estimated an intermediate value of 64 s^{-1} (Álvarez et al., 2013).

Ca^{2+} Channel Subtypes Coupled to IRP Exocytosis: How a discrete number of readily releasable vesicles are positioned close to VDCCs, forming the IRP? One possibility is that RRP vesicles and VDCCs are randomly distributed throughout the plasma membrane, and therefore some portion of vesicles would lie close to Ca^{2+} channels by chance. Alternatively, it is also possible that one or more particular types of Ca^{2+} channel are specifically coupled to IRP vesicles. The first scheme is obviously the simplest one, because it does not presume any specific interaction. This possibility was considered by several authors (Chow et al., 1994; Klingauf and Neher, 1997; Voets et al., 1999). In this scenario, all VDCC subtypes of chromaffin cells are expected to participate in the IRP release in proportion to their contribution to the whole voltage-dependent Ca^{2+} current (Engisch and Nowycky, 1996). However, there is evidence suggesting that the different VDCC subtypes expressed in chromaffin cells are not equally efficient in triggering exocytosis (Elhamdani et al., 1998; Lara et al., 1998; Wykes et al., 2007). Moreover, Segura et al. (Segura et al., 2000), using Montecarlo simulations, demonstrated that a random distribution of vesicles and channels does not explain the biphasic capacitance time course provoked by a train of short depolarizations (Horrigan and Bookman, 1994; Marengo, 2005; Voets et al., 1999). On the other hand, these authors found that this characteristic biphasic behavior can be modeled by assuming a non-uniform distribution of vesicles and channels, where some vesicles are forced to be attached to the channels, while the others lie far from them (Segura et al, 2000).

It was demonstrated that chromaffin cells present a heterogeneous population of VDCCs, including L, P/Q, N, R and T-type, that varies in their contribution depending on the biological preparation (cell culture or slices), experimental conditions and species (Albillos et al., 2000; Aldea et al., 2002; Artalejo et al., 1994; Hernandez-Guijo et al.,

1998; Lomax et al., 1997; Novara et al., 2004; Santana et al., 1999). Evidence on the differential efficiency of particular VDCC subtypes for triggering secretion was reported in different preparations (Albillos et al., 2000; Artalejo et al., 1994; Elhamdani et al., 1998; Wykes et al., 2007). A major efficiency of P/Q-type channels to trigger exocytosis was supported by many groups (Aldea et al., 2002; Chan et al., 2005b; Lara et al., 1998; Polo-Parada et al., 2006; Wykes et al., 2007). Using patch-clamp capacitance measurements and a paired pulse stimulation protocol (see Fig. 1A legend for details), Álvarez and colleagues (Álvarez et al., 2013; Álvarez et al., 2008) showed that IRP exocytosis in mouse chromaffin cells mostly depends on P/Q-type Ca^{2+} channels. This finding was supported on: (i) the suppression of IRP release by ω-agatoxin IVA (Fig. 1A); (ii) the almost complete abolishment of IRP in chromaffin cells from α1A deficient (P/Q-KO) mice (Fig. 1A); (iii) P/Q-KO cells completely lack the fast synchronous exocytosis usually observed at the beginning of a train of stimuli in wild type cells, but keep the delayed asynchronous exocytosis (Fig. 1B); and (iv) the Ca^{2+} current entry through P/Q-type VDCC resulted 8 times more efficient than L-type VDCC to release the equivalent of 50% of IRP (Álvarez et al., 2013; Álvarez et al., 2008). This scenario suggests the existence of a specific physical interaction between IRP vesicles and P/Q-type channels (Fig. 1D).

The putative coupling between P/Q-type Ca^{2+} channels and IRP vesicles may be explained by the existence of a molecular interaction between these channels and the proteins of the exocytotic machinery. Indeed, the P/Q-type Ca^{2+} channel contains a synaptic protein interaction (synprint) site in the intracellular loop that connects the II and III domains of the α_{1a} subunit (Mochida et al., 2003; Zamponi, 2003). This synprint site interacts with SNAP-25, syntaxin, synaptotagmin and the cysteine string protein (Mochida et al., 2003; Rettig et al., 1996; Yokoyama et al., 2005; Zamponi, 2003). It was postulated that this interaction is critical for fast and highly synchronized exocytosis at the presynapse (Rettig et al., 1997; Zamponi, 2003). Hence, a similar type of interaction between the P/Q-type channels and IRP vesicles might also occur in neuroendocrine cells. The following findings support this idea: (i) the synprint site is present in different splice variants of the P/Q α_{1a} subunit in bovine chromaffin cells, (ii) P/Q-type Ca^{2+} channels and the SNARE complex co-immunoprecipitate with a monoclonal antibody against SNAP-25, and (iii) α_{1a} and SNAP-25 co-localize at the plasma membrane of chromaffin cells (Andres-Mateos et al., 2005). Recently, to study

the molecular basis of the functional coupling observed between the IRP and P/Q-type Ca^{2+} channels in mouse chromaffin cells, Álvarez and collaborators (2013) transfected mice chromaffin cells in culture with a plasmid containing the synprint sequence. In such condition, the efficiency of the Ca^{2+} current to promote exocytosis was reduced to a similar level as that obtained when P/Q-type channels were blocked with ω-agatoxin IVA. More importantly, the size of IRP exocytosis was markedly reduced in cells expressing the synprint in comparison with control cells (Fig. 1C). Therefore, the authors concluded that the synprint site of α_{1A} subunit is an important factor for the establishment of the functional coupling between the IRP and P/Q-type Ca^{2+} channels (Álvarez et al., 2013) (see Fig. 1D and E).

It is worth to mention that the synprint sequence is also present in N-type Ca^{2+} channels (Mochida et al., 1996). Therefore, it is possible that chromaffin cells expressing this type of Ca^{2+} channels can also have a contribution of N-type Ca^{2+} current in the triggering of the exocytosis component highly coupled to VDCC, as it was shown in other preparations like synaptic terminals and the pheochromocytoma cell line MPC 9/3L (Harkins et al., 2004; Mochida et al., 1996). Moreover, other types of VDCC, lacking the synprint sequence, also contribute to the rapid exocytosis evoked by short depolarizing pulses in chromaffin cells. In this regard, Albillos and collaborators found in perforated patch experiments a Ca^{2+} current, apparently mediated by R-type channels, which was highly efficient in triggering exocytosis (Albillos et al., 2000). On the other hand, the C-termini of P/Q- and N-type Ca^{2+}-channels have also been implicated in targeting Ca^{2+}-channels to the presynaptic active zone (Catterall et al., 2005). Maximov and collaborators demonstrated by *in vitro* and *in vivo* assays a specific association of the synaptic modular adaptor proteins Mint1-1 and CASK with the cytosolic carboxyl terminus of the long splice variants of $\alpha 1_B$ and $\alpha 1_A$ subunits of the N- and Q- type Ca^{2+} channels (Maximov et al., 1999; Maximov and Bezprozvanny, 2002). This molecular association probably recruits these types of VDCCs to a macromolecular signaling complex assembled at the presynapse. Additionally, using yeast two-hybrid screens, Kaeser and collaborators identified a direct interaction between the central PDZ-domain of the active-zone protein Rim and the C-termini of N- and P/Q-type Ca^{2+} channels (Kaeser et al., 2011). Moreover, the knockout of Rim decreased the presynaptic localization of VDCCs, inhibited neurotransmitter release, and reduced the size of the RRP (Han et al., 2011; Kaeser et al., 2011; Kaeser et al., 2012). Therefore, Rim appear

to regulate fast transmitter release by organizing high presynaptic density of VDCCs with docked vesicles at active zones (Han et al., 2011). The addition of both full-length or N-terminal Rim1 to permeabilized or intact chromaffin cells enhanced secretion, apparently through a mechanism involving the binding to a 14-3-3 protein (Sun et al., 2002b, 2003). There is also data suggesting that the disruption of Mint1-Munc18-1 binding decreases secretion in chromaffin cells (Graham et al., 2011). In pancreatic islet beta cells, CASK knockdown reduced the anchoring of insulin vesicles to cell membranes and insulin release (Wang et al., 2015).

Finally, it was also postulated a role for complexins in the coupling between VDCCs and secretory or synaptic vesicles. Besides the classical role of complexins in "clamping" the SNARE complex and inhibiting spontaneous membrane fusion (Giraudo et al., 2006; Huntwork and Littleton, 2007; Rizo and Xu, 2015; Schaub et al., 2006), recent work suggests that these proteins also have a positive regulatory role on exocytosis (Cai et al., 2008; Dhara et al., 2014; Rizo and Xu, 2015; Xue et al., 2008). Robert Chow and colleagues showed in mouse neuromuscular junctions that the lack of complexin 1 significantly reduces and desynchronizes Ca^{2+}-triggered synaptic transmission (Lin et al., 2013). The same authors also found that complexin 2-null adrenal chromaffin cells present a decreased and desynchronized evoked release in response to short depolarization. They also identified a significant reduction in the vesicle pool close to the VDCCs, which was associated with the IRP. They proposed that complexin is involved in coupling the vesicle-associated SNARE complex with VDCCs, which serves to fine-tune the precise timing of Ca^{2+}-triggered fast release. These authors related this effect of complexin with the previously proposed role of synaptotagmin in coupling the vesicle-associated SNARE complex with VDCCs (Young and Neher, 2009). They proposed that complexin stabilizes the binding of synaptotagmin to the SNARE complex, which in turn promotes the coupling of vesicles to VDCCs.

The stimulus pattern influences the mode of exocytosis

The first evidences that the frequency of the stimulus does not only determine the number of exocytotic events, but also the amount of transmitter molecules released from a single vesicle (quantal size) came from the experiments of Elhamdani et al. (2001). By

monitoring single exocytotic events in chromaffin cells using amperometry, these authors found that the frequency of APs, determines the amount of catecholamine released per individual fusion events. Thus, at low frequencies (0.25 Hz) the quantal size was more than 2 fold smaller than that observed at high frequency (10 Hz). The authors ruled out that the effects on the quantal size were due to the recruitment of different sized vesicles, since the quantal size increased continuously and not abruptly (Elhamdani et al., 2001). Similar results, also in chromaffin cells, were later observed by Fulop et al. (2005). They reported that the quantal size of amperometric spikes induced by APs applied at 0.5 Hz was more than 2-fold smaller than the one obtained at 15 Hz stimulation. They also noted that the area of the amperometric spikes at 0.5 Hz was very close to the value of the area of the pre-spike current (usually called foot) of the spikes recorded at 15 Hz. The foot current reflects the slow release of catecholamine through the fusion pore (Chow et al., 1992), a narrow channel formed during the fusion of the vesicle with the plasma membrane (Lindau and Alvarez de Toledo, 2003). This fusion pore can either close back or expand to a larger pore (Alés et al., 1999). Its premature closure gives rise to exocytotic events with an incomplete release, whereas its full expansion allows the complete release of the vesicle content (Albillos et al., 1997). Hence, Fulop and collaborators (2005) proposed that at low firing rates (0.5 Hz), corresponding to the sympathetic tone, the catecholamines are partially released through a restricted fusion pore that closes back. Whereas under high firing stimulation (15 Hz), that mimics an acute stress condition, the fusion pore totally expands leading to the complete release of the vesicular catecholamines. These two mechanisms of exocytosis are usually named “kiss-and-run” and “full fusion”.

The mode of exocytosis defines the type and amount of transmitter being released: The kiss-and-run and full fusion mechanisms were proposed for the first time in 1973 by two different groups, Ceccarelli and colleagues, and Heuser and Reese. Both groups stimulated frog neuromuscular junctions in the presence horseradish peroxidase for labeling recycled synaptic vesicles, and analyzed the nerve terminal ultrastructure using electron microscopy. However, they applied different stimulation protocols. After stimulating the motor nerve at 10 Hz, Heuser and Reese (Heuser and Reese, 1973) observed a depletion of synaptic vesicles accompanied first by an increased in the plasma membrane surface and the amount of coated vesicles, and later by the appearance of irregular membrane-walled cisternae. Several minutes after stimulation,

the latter structures disappeared for being replaced by new synaptic vesicles. On the other hand, when Ceccarelli and colleagues (Ceccarelli et al., 1973) stimulated the terminal nerve at 2 Hz, observed many synaptic vesicles labeled with horseradish peroxidase, but they did not find evidences of vesicle depletion, or an increase of coated vesicles and large organelles. Hence, the latter authors proposed that after fusion the synaptic vesicles rapidly reform from the plasma membrane, and that the reformed synaptic vesicles are able of storing and releasing transmitters again. They called this mechanism kiss-and-run. On the other hand, Heuser and Reese suggested that during exocytosis the synaptic vesicles merge with the plasma membrane, and then are recovered as coated vesicles that, forming first intermediate cisternae, recycled into new synaptic vesicles. Today it is well-known that after full fusion the secretory vesicle membrane is retrieved by clathrin-mediated endocytosis (McMahon and Boucrot, 2011). In this mechanism the GTPase dynamin catalyzes the separation of the clathrin-coated vesicle from the plasma membrane (Gonzalez-Jamett et al., 2014), then the vesicle is decoated and fuses with endosomes (Cardenas and Marengo, 2010).

The kiss-and-run was later demonstrated in neuroendocrine cells using a technique called patch-amperometry, which was developed by Alvarez de Toledo and Lindau, and that combines the measurements of the plasma membrane capacitance changes and the catecholamine release (Dernick et al., 2005). By using this technique in bovine chromaffin cells, Albillos et al. (1997) found that some vesicles release a small amount of catecholamines through a transient fusion pore that does not expand. Later, also using patch-amperometry in rat chromaffin cells, Ales and collaborators (Ales et al., 1999) observed another type of transient fusion event, in which the fusion pore conductance increases before reclosing and thus entirely releasing the intravesicular catecholamines. By using evanescent field microscopy and intravesicular peptides labeled with fluorescent proteins, Almers and collaborators also described two types of transient fusion events. One of the transient fusion events allows the release of only small molecules, but retains the bigger ones, such as neuropeptide Y and tissue plasminogen activator; the other one allows the release of intermediate size molecules, such as neuropeptide Y, but retains the biggest, the tissue plasminogen activator (Perrais et al., 2004). The latter mode of exocytosis was called “cavicapture”, since the cavity of the vesicle is recovered intact (Taraska et al., 2003). Interestingly, the selective release of transmitters according their molecular size is determined by the frequency of the

stimulus. Thus, low frequency stimulation (0.5 Hz) allows the release of catecholamines, but retains higher molecular-weight peptides, such as chromogranins, whereas at high frequencies (15 Hz) all the transmitters are released (Fulop et al., 2005).

Mechanisms that determine the mode of exocytosis: As aforementioned, kiss-and-run is the dominant exocytosis mechanism under low frequency stimulation, whereas full-fusion events predominate during high frequency firings (Elhamdani et al., 2001; Fulop et al., 2005), hence suggesting that cytosolic Ca^{2+} concentrations determine the behavior of the fusion pore, and then the mode of exocytosis. The following findings support this idea: First the Ca^{2+} concentration in the extracellular solution determines the quantal size (Elhamdani et al., 2001) and the mode of exocytosis (Alés et al., 1999); second, manipulations of cytosolic Ca^{2+} levels also determine the mode of exocytosis, thus high cytosolic Ca^{2+} levels promote full fusion events (Elhamdani et al., 2006) and increase the quantal size (Wang et al., 2006); and third, high cytosolic Ca^{2+} concentrations promote the expansion of the fusion pore (Scepek et al., 1998), whereas in low Ca^{2+} levels, the fusion pore seems unable to expand giving rise to incomplete release events (Wang et al., 2006).

It is widely accepted that synaptotagmin is a fundamental Ca^{2+} sensor during exocytosis. Synaptotagmins are vesicular membrane proteins with two C-terminal C2 domains, C2A and C2B, that bind Ca^{2+} with different affinities and appear to play different roles during exocytosis (Segovia et al., 2010). It has been described at least 17 synaptotagmin isoforms, which exhibit different tissue expression and affinity for Ca^{2+} and phospholipids (Moghadam and Jackson, 2013). Reportedly, synaptotagmins bind to SNARE proteins and phospholipids in a Ca^{2+}-dependent way (Chapman, 2008), but the different synaptotagmin isoforms appear to contribute differently to the fusion pore expansion and mode of exocytosis. For instance, Zhang and collaborators reported that the expression of synaptotagmin I in PC12 promotes kiss-and-run exocytosis, whereas the expression of synaptotagmin VII favors full fusion (Zhang et al., 2011). Accordingly, chromaffin cells from a mouse expressing a mutant synaptotagmin VII with point mutations that abolish Ca^{2+} binding to its C2B domain display an increased proportion of kiss-and-run events (Segovia et al., 2010). These different types of synaptotagmins display different activation kinetics and affinity for Ca^{2+}, having synaptotagmin I fast activation and low affinity for Ca^{2+}; conversely synaptotagmin VII displays a lower kinetics and higher affinity (Bhalla et al., 2005; Xu et al., 2007). In

neurons, synaptotagmin I is essential for synchronous release, whereas synaptotagmin VII operates during delayed asynchronous release (Bacaj et al., 2013). In adrenal chromaffin cells, synaptotagmin I and VII have overlapping role in the Ca^{2+}-triggered exocytosis (Schonn et al., 2008). However, synaptotagmin I mediates the exocytosis induced by short depolarizations (Voets et al., 2001), whereas the contribution of synaptotagmin VII to mild stimulation appears to be insignificant (Schonn et al., 2008). In addition, in PC12 cells synaptotagmin I localizes in vesicles with an average size of 108 nm, whereas synaptotagmin VII localizes in vesicles that are significantly larger, with average size of 148 nm (Zhang et al., 2011). This fact, together with the role of synaptotagmins in determining the mode of exocytosis, could explain why the frequency of the stimulus determines the quantal size in chromaffin cells.

In addition to activate synaptotagmins, Ca^{2+} also controls fusion pore dynamics through other mechanisms, among them the cortical actin meshwork. Indeed, Ca^{2+} concentrations that trigger exocytosis promote both the formation of new actin filaments and disruption of the preexisting cortical actin network (Gasman et al., 2004; Olivares et al., 2014; Trifaro et al., 2000). This actin remodeling facilitates different steps of the secretory process, including the motion of the vesicles to exocytotic sites (Giner et al., 2005) and the formation of active exocytotic sites (Gabel et al., 2015). Moreover, the actin remodeling also controls the expansion of the fusion pore (Berberian et al., 2009; Olivares et al., 2014) by a mechanism that include the participation of the motor protein myosin II (Berberian et al., 2009; Doreian et al., 2008; Neco et al., 2008) and the mechano-GTPase dynamin (Gonzalez-Jamett et al., 2010; Jackson et al., 2015; Samasilp et al., 2012). They, together, provide the membrane tension that controls the fusion pore dynamics (Bretou et al., 2014). Ca^{2+} regulates actin remodeling in chromaffin cells via diverse types of Ca^{2+}-regulated proteins, such as Src kinases and annexin 2, which promote actin assembly (Olivares et al., 2014; Gabel et al., 215), and the actin severing protein scinderin and the phospholipase Cη2 that promote F-actin disassembly (Lejen et al., 2001; Yamaga et al., 2015). Interestingly, the formation of new actin filaments and the actin meshwork disruption appear to display different sensitivity to Ca^{2+} (see Fig. 2). Indeed, a moderate Ca^{2+} concentration promotes actin polymerization, whereas a higher Ca^{2+} level is required to disrupt the cortical actin meshwork (Olivares et al., 2014). Thus, the F-actin formation induced by a moderate stimulus would favor fusion pore expansion, but the vesicle would not collapse in the plasma membrane, whereas disruption of the actin meshwork induced by a strong

stimulus would favor the full fusion of the vesicle with the plasma membrane (Doreian et al., 2008). This model is depicted in Figure 2.

Besides to act in concert with F-actin, expanding the fusion pore, it has been proposed that the GTPase dynamin promotes the closure of an already expanded fusion pore. This idea is supported by the fact that the disruption of dynamin GTPase activity increases the quantal size (Elhamdani et al., 2001; Gonzalez-Jamett et al., 2010; Gonzalez-Jamett et al., 2013; Graham et al., 2002) and hinders the rapid reuptake (cavicapture) of secretory vesicles (Holroyd et al., 2002; Tsuboi et al., 2004). Elhamdani et al. (2001; 2006) propose that this mechanism of exo/endocytosis is promoted by intermediate stimulation frequencies. However, additional evidences are required to demonstrate this role of dynamin.

The stimulus pattern also influences the form of compensatory endocytosis

In order to ensure the membrane homeostasis, vesicle recycling and transmitter release reliability, the cells retrieve the vesicle membrane after exocytosis through endocytosis. Electrophysiological measurements of membrane capacitance in chromaffin cells have shown that the mechanism of compensatory endocytosis is determined by the frequency of APs. In this regard, stimulation of adrenal tissue slices with an individual AP or at the sympathetic basal firing rate (0.5 Hz) promotes a rapid endocytosis that is independent of clathrin and calcineurin, a Ca^{2+}-dependent phosphatase that activates clathrin-dependent endocytosis (Chan et al., 2003; Chan and Smith, 2001, 2003). These authors reported that this form of endocytosis is inhibited at intermediate frequencies (2-6 Hz). However, high frequency stimulation (15 Hz) induces a different form of compensatory endocytosis that depends on calcineurin (Chan et al., 2003; Chan and Smith, 2001). Two different mechanisms of endocytosis were also reported by Artalejo and collaborators in cultured calf chromaffin cells (Artalejo et al., 2002). When they applied a train of 10 depolarizations of 50 ms at 2 Hz observed a rapid endocytosis that depends on calmodulin and dynamin-1, but it is independent of clathrin (Artalejo et al., 2002). Whereas, a more sustained stimulus composed of 29 depolarizations of 75 ms applied at 0.25 Hz resulted in a slow endocytosis that depends on dynamin-2 and clathrin (Artalejo et al., 2002). Both groups of authors indicate that these two forms of endocytosis are activated by different ranges of cytosolic Ca^{2+} (Chan et al., 2003;

Elhamdani et al., 2006). Indeed, when Elhamdani et al. (2006) increased the Ca^{2+} concentration from 10 to 210 μM in the patch pipette, observed a reduction in the kinetics of endocytosis. On the other hand, Chan and collaborators estimated that the fast endocytosis induced by basal frequencies is inhibited by cytosolic Ca^{2+} with a K_{inh} of 605 nM, while the endocytosis induced by high frequencies is activated by Ca^{2+} with a K_{act} of 1.46 μM (Chan et al., 2003).

In neurons, the kinetics of the endocytosis is also determined by the stimulation pattern, although with some differences respect to chromaffin cells. For instance, at the calyx of Held a single AP or APs applied at frequencies ≤ 2 Hz, promotes a very fast endocytosis, which is dramatically slowed down at higher AP frequencies (Sun et al., 2002a). Likewise, the increase of single stimulus duration from 2 to 50 ms slowed down endocytosis (Wu and Wu, 2014). On the other hand, 10 pulses of 20 ms depolarization at 1 - 10 Hz, or single depolarization prolonged to 200 ms, promotes a new rapid endocytotic component (Wu and Wu, 2014). These authors proposed that the pattern of the cytosolic Ca^{2+} signal defines the kinetics of endocytosis. Thus, the endocytosis is triggered and facilitated by a large, transient Ca^{2+} increase localized at a micro/nano domain, but hindered by a prolonged, small and global Ca^{2+} signals (Wu et al., 2014; Wu and Wu, 2014).

Different authors have proposed that rapid and slow endocytosis correlate with the mode of exocytosis, being rapid endocytosis associated with the kiss-and-run exocytosis, whereas slow endocytosis with full fusion (Elhamdani et al., 2006; Wu et al., 2014). These two mechanisms would impact the kinetics of vesicle recycling, as well as the economy of the cell, since a slow recycling process implies the synthesis and refilling of new vesicles.

The stimulus pattern also influences the replenishment of vesicles after depletion

The maintenance of secretion under AP firing requires the continuous refilling of releasable pools of vesicles at rates that match the exocytotic activity (Smith et al., 1998; Sorensen, 2004). In this regard, high frequency stimulation of chromaffin cells provokes an important global Ca^{2+} increase (Fulop and Smith, 2006; Klingauf and Neher, 1997; Marengo and Monck, 2003), that triggers exocytosis of secretory vesicles irrespectively of their location, and mobilizes vesicles from upstream to downstream

pools (Marengo, 2005; Voets et al., 1999). On the other hand, in rest conditions the firing frequency is low, Ca^{2+} does not accumulate markedly (Fulop and Smith, 2006), and exocytosis would be limited to a group of vesicles that are closely coupled to VDCC, i.e. the IRP (Álvarez et al., 2008; Horrigan and Bookman, 1994; Marengo, 2005; Oré and Artalejo, 2005; Voets et al., 1999).

How the IRP is refilled in order to maintain exocytosis during repetitive stimulation? In mouse chromaffin cells, it has been proposed that IRP is refilled directly from upstream vesicle pools in a sequential scheme (Chan et al., 2005a). Different factors may regulate this process, as cytosolic Ca^{2+} (Marengo, 2005; Voets et al., 1999), neural cell adhesion molecule (Chan et al., 2005a) and protein kinase C (Voets et al., 1999). A second possibility is that a fast endocytotic process, which is directly coupled to the exocytosis induced by APs at low frequencies, might be the first step of a short cycle of vesicle replenishment (Chan and Smith, 2001, 2003). Indeed, at low physiological frequencies, chromaffin cells release catecholamines through Ω-shape kiss-and-run fusion events (Fulop et al., 2005; Fulop and Smith, 2006), suggesting that the replenishment of vesicles could be associated with a short local cycling mechanism. Additionally, in bovine chromaffin cells, it was reported that kiss-and-run is directly associated with fast endocytosis (Elhamdani et al., 2006). Therefore, it is possible that application of AP_{ls} at low frequencies promotes a kiss-and-run like process, which might results in rapid replenishment of releasable vesicles. Fast endocytosis and/or fast recycling processes were also documented in other systems as cultured hippocampal neurons (Deak et al., 2004; Klingauf et al., 1998), calyx of Held (Wu and Wu, 2009), auditory hair cells (Cho et al., 2011), and synaptic terminals of retinal bipolar neurons (von Gersdorff and Matthews, 1994). However, other studies performed in neurons argue against a major contribution of kiss-and-run in overall endocytosis (Balaji and Ryan, 2007; Fernandez-Alfonso and Ryan, 2004; Granseth et al., 2009). Consistently, Ling-Gang Wu and collaborators (Wu et al., 2014; Wu and Wu, 2009), working in the calyx of Held, reported that rapid endocytosis does not recycle vesicles within the RRP. Based on that finding, they proposed an alternative explanation about the importance of fast endocytosis on rapid vesicle recycling. Rapid endocytosis may rapidly restore the structure of release sites after exocytosis. In other words, the clearance of the release sites by endocytosis, by removing exocytosed vesicle membranes and proteins, would facilitate the vesicle replenishment. In addition, rapid endocytosis would recycle

vesicles in a recycling pool beyond the RRP to prevent vesicle exhaustion (Wu and Wu, 2009).

Final Remarks

Accumulated data from different laboratories indicate that the pattern of stimulation defines the pools of vesicles being recruited, the mode of exocytosis and the type of endocytosis in neuroendocrine cells. The two latter determine, respectively, the types and amount of transmitters released, and the mechanisms associated with vesicle replenishment. In this regard, high frequency stimulation promotes the massive full fusion of vesicles, irrespectively of their location, followed by clathrin-dependent endocytosis and vesicle replenishment through vesicle mobilization from upstream to downstream pools. On the other hand, low frequency stimulation with action potentials or very brief stimuli triggers the secretion of vesicles that are highly coupled to Ca^{2+} channels, apparently through a kiss-and-run mechanism of fusion, which is followed by a clathrin-independent fast endocytosis process. This fast endocytosis would facilitate the rapid replenishment of releasable vesicles. This tight stimulus-secretion coupling and fast recycling could help chromaffin cells to maintain a basal secretory tone with little metabolic cost (Voets et al. 1999; Oré and Artalejo 2005).

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Figure Legends:

Figure 1: *Highly synchronous exocytosis in chromaffin cells is explained by the interaction between vesicles and channels at the molecular level.* **A**. Typical recordings of the membrane capacitance change in response to a dual 10 ms pulse protocol in chromaffin cells from a wild type (WT) mouse in control conditions (left) or in the presence of 200 nM ω-Agatoxin IVA (AGA; middle), or from P/Q-type channel knock-out (P/Q-KO) animals (right). The two pulses (p1 and p2, from -80 to 0 mV) were given 300 ms apart and induced identical Ca^{2+} currents (see Álvarez et al, 2008). Upper (Bmax) and lower (Bmin) bounds for IRP size can be calculated according to the equations (Voets et al, 1999): $B_{min} = \Delta C_{m1} + \Delta C_{m2}$; $B_{max} = \frac{\Delta C_{m1} + \Delta C_{m2}}{1 - (\Delta C_{m2} / \Delta C_{m1})^2}$; where ΔC_{m1} and ΔC_{m2} represent the capacitance responses to the first and second depolarization, respectively. The Bmin and Bmax values for the individuals experiments represented in the figure are below the records. Note that the P/Q-KO or the pharmacological blocking of P/Q-type Ca^{2+} channels practically abolishes IRP exocytosis (averages values of Bmin and Bmax were 21±2 and 31±3; 4±1 and 6±1; and 5±1 and 6±1, for control, PQ-KO and AGA respectively, see Álvarez et al, 2008). **B**. Average exocytotic response of WT (n=15, black) and P/Q-KO (n=7, gray) chromaffin cells in response to trains of ten 50 ms square depolarizing pulses (2 Hz). Notice that the P/Q-KO showed a clear reduction of exocytosis associated with the first pulses; but, on the other hand, the delayed capacitance increase, showing an important asynchronous component, was still prominent (Álvarez et al., 2008). **C**. Typical recordings of the membrane capacitance change in response to a dual 10 ms pulse protocol at control conditions (Syn^-), and in cells expressing a free synprint peptide (Syn^+). The stimulation protocol and the formula for the calculation of Bmin and Bmax (both are represented below the records) were identical than those described in panel A. The averages values of Bmin and Bmax were 17±2 and 22±2; and 7±2 and 9±1 fF, for Syn^- and Syn^+ respectively (see Álvarez et al., 2013). **D**. This cartoon represents an IRP vesicle (orange sphere on the left), which is located in close apposition to a P/Q-type Ca^{2+} channel, and therefore exposed to a narrow Ca^{2+} gradient (dark gray) generated when the cell is stimulated with a brief depolarization. This vesicle is physically associated with a hypothetical interaction site (light green bar) in the P/Q-type Ca^{2+} channel

molecule. Another vesicle, which is located distant from Ca^{2+} channels, is only exposed to a larger Ca^{2+} gradient (light gray) provoked by a longer depolarization or a train of depolarizations. **E**. This cartoon summarizes the interaction between synprint and the t-SNARE proteins syntaxin and SNAP-25. The synprint, which is located in the cytosolic loop between the II and the III domains of α_{1A} subunit of the P/Q-type calcium channel, is represented in light green. Records in panels A and B were extracted from Álvarez et al, 2008; and records in panel C were extracted from Álvarez et al, 2013. The cartoon in D was modified from Álvarez and Marengo (Álvarez and Marengo, 2011).

Figure 2: *Actin remodeling controls fusion pore dynamics and the mode of exocytosis*. (**A**) Actin polymerization and F-actin disruption have different Ca^{2+} sensitivity. At a free Ca^{2+} concentration of 0.1 μM, the actin network is not disrupted (red) and few new actin filaments (green) are formed. At 1 μM free Ca^{2+}, new actin filaments are observed, and the actin network is still undisturbed. At 10 μM free Ca^{2+}, new actin filaments are also observed, but the actin network is disrupted. For measuring the formation of new actin filaments, the experiments were performed in permeabilized chromaffin cells incubated in 0.1, 1 or 10 μM free Ca^{2+} and Alexa Fluor 488 G-actin. For analyzing the integrity of the cortical actin network, the cells were kept in the aforementioned Ca^{2+} concentrations and then stained with phalloidin-rhodamine B. The left panel shows representative confocal images. Scale bar = 10 μm. The right panel shows the quantification of the new (green) and total (red) cortical actin fluorescence intensity. Data are means ± SEM. *$p<0.05$. This figure was adapted from Olivares et al., 2014. (**B**) The model show actin remodeling and catecholamine release through the fusion pore during different types of cell stimulation. (**i**) Docked vesicles are surrounded by a meshwork of filamentous actin (Gabel et al., 2015). A mild stimulus that slightly increases the cytosolic Ca^{2+} concentration does not disrupt the preexistent actin meshwork (red) and does not promote a significant actin polymerization. In this condition the fusion pore does not expand and closes back, giving rise to small amperometric spikes (see the red spike). These events are observed with the application of low-frequency stimulation of 0.5 Hz (Doreian et al., 2008). (**ii**) A middle stimulus induces a Ca^{2+} rise that promotes the formation of new actin filaments (green), but is not enough to significantly disrupt the preexistent actin meshwork (Olivares et al., 2014). The Ca^{2+}-induced actin polymerization promotes expansion of the fusion pore

(Berberian et al., 2009; Olivares et al., 2014), but the integrity of the preexisting actin meshwork prevent the collapse of the vesicle in the plasma membrane (Doreian et al., 2008). The green spike is an example of amperometric spikes with partial release of catecholamines. These types of events are observed under middle stimulation of 7 Hz (Elhamdani et al, 2001). (**iii**) A robust Ca^{2+} rise disrupts the actin meshwork, allowing the vesicle to collapse in the plasma membrane, and giving rise full fusion exocytosis (Doreian et al., 2008; Olivares et al., 2014). The purple spike shows an amperometric event with a large quantal size. These types of events are observed in chromaffin cells under high frequency stimulation of 15 Hz or treated with the actin disrupting agent cytochalasin-D (Doreian et al., 2008; Olivares et al., 2014).

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Figure 1

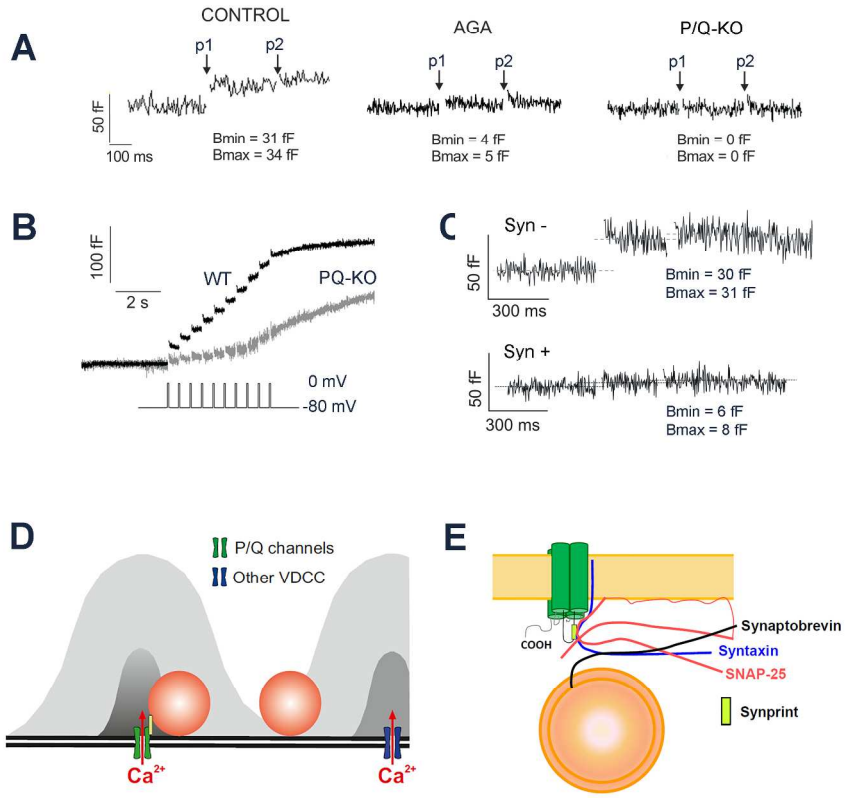

Figure 1: Highly synchronous exocytosis in chromaffin cells is explained by the interaction between vesicles and channels at the molecular level. A. Typical recordings of the membrane capacitance change in response to a dual 10 ms pulse protocol in chromaffin cells from a wild type (WT) mouse in control conditions (left) or in the presence of 200 nM ω-Agatoxin IVA (AGA; middle), or from P/Q-type channel knock-out (P/Q-KO) animals (right). The two pulses (p1 and p2, from -80 to 0 mV) were given 300 ms apart and induced identical Ca2+ currents (see Álvarez et al, 2008). Upper (Bmax) and lower (Bmin) bounds for IRP size can be calculated according to the equations (Voets et al, 1999): Bmin = ΔCm1 + ΔCm2; ; where ΔCm1 and ΔCm2 represent the capacitance responses to the first and second depolarization, respectively. The Bmin and Bmax values for the individuals experiments represented in the figure are below the records. Note that the P/Q-KO or the pharmacological blocking of P/Q-type Ca2+ channels practically abolishes IRP exocytosis (averages values of Bmin and Bmax were 21±2 and 31±3; 4±1 and 6±1; and 5±1 and 6±1, for control, PQ-KO and AGA respectively, see Álvarez et al, 2008). B. Average exocytotic response of WT (n=15, black) and P/Q-KO (n=7, gray) chromaffin cells in response to trains of ten 50 ms square depolarizing pulses (2

Hz). Notice that the P/Q-KO showed a clear reduction of exocytosis associated with the first pulses; but, on the other hand, the delayed capacitance increase, showing an important asynchronous component, was still prominent (Álvarez et al., 2008). C. Typical recordings of the membrane capacitance change in response to a dual 10 ms pulse protocol at control conditions (Syn-), and in cells expressing a free synprint peptide (Syn+). The stimulation protocol and the formula for the calculation of Bmin and Bmax (both are represented below the records) were identical than those described in panel A. The averages values of Bmin and Bmax were 17±2 and 22±2; and 7±2 and 9±1 fF, for Syn- and Syn+ respectively (see Álvarez et al., 2013). D. This cartoon represents an IRP vesicle (orange sphere on the left), which is located in close apposition to a P/Q-type Ca2+ channel, and therefore exposed to a narrow Ca2+ gradient (dark gray) generated when the cell is stimulated with a brief depolarization. This vesicle is physically associated with a hypothetical interaction site (light green bar) in the P/Q-type Ca2+ channel molecule. Another vesicle, which is located distant from Ca2+ channels, is only exposed to a larger Ca2+ gradient (light gray) provoked by a longer depolarization or a train of depolarizations. E. This cartoon summarizes the interaction between synprint and the t-SNARE proteins syntaxin and SNAP-25. The synprint, which is located in the cytosolic loop between the II and the III domains of α1A subunit of the P/Q-type calcium channel, is represented in light green. Records in panels A and B were extracted from Álvarez et al, 2008; and records in panel C were extracted fro

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Figure 2

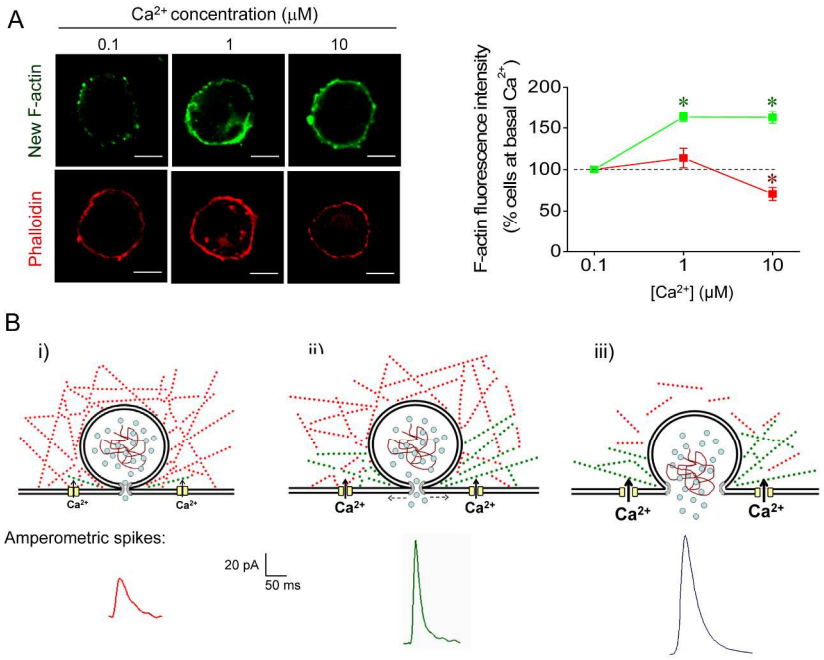

Figure 2: Actin remodeling controls fusion pore dynamics and the mode of exocytosis. (A) Actin polymerization and F-actin disruption have different Ca2+ sensitivity. At a free Ca2+ concentration of 0.1 μM, the actin network is not disrupted (red) and few new actin filaments (green) are formed. At 1 μM free Ca2+, new actin filaments are observed, and the actin network is still undisturbed. At 10 μM free Ca2+, new actin filaments are also observed, but the actin network is disrupted. For measuring the formation of new actin filaments, the experiments were performed in permeabilized chromaffin cells incubated in 0.1, 1 or 10 μM free Ca2+ and Alexa Fluor 488 G-actin. For analyzing the integrity of the cortical actin network, the cells were kept in the aforementioned Ca2+ concentrations and then stained with phalloidin-rhodamine B. The left panel shows representative confocal images. Scale bar = 10 μm. The right panel shows the quantification of the new (green) and total (red) cortical actin fluorescence intensity. Data are means ± SEM. *p<0.05. This figure was adapted from Olivares et al., 2014. (B) The model show actin remodeling and catecholamine release through the fusion pore during different types of cell stimulation. (i) Docked vesicles are surrounded by a meshwork of filamentous actin (Gabel et al., 2015). A mild stimulus that

slightly increases the cytosolic Ca2+ concentration does not disrupt the preexistent actin meshwork (red) and does not promote a significant actin polymerization. In this condition the fusion pore does not expand and closes back, giving rise to small amperometric spikes (see the red spike). These events are observed with the application of low-frequency stimulation of 0.5 Hz (Doreian et al., 2008). (ii) A middle stimulus induces a Ca2+ rise that promotes the formation of new actin filaments (green), but is not enough to significantly disrupt the preexistent actin meshwork (Olivares et al., 2014). The Ca2+-induced actin polymerization promotes expansion of the fusion pore (Berberian et al., 2009; Olivares et al., 2014), but the integrity of the preexisting actin meshwork prevent the collapse of the vesicle in the plasma membrane (Doreian et al., 2008). The green spike is an example of amperometric spikes with partial release of catecholamines. These types of events are observed under middle stimulation of 7 Hz (Elhamdani et al, 2001). (iii) A robust Ca2+ rise disrupts the actin meshwork, allowing the vesicle to collapse in the plasma membrane, and giving rise full fusion exocytosis (Doreian et al., 2008; Olivares et al., 2014). The purple spike shows an amperometric event with a large quantal size. These types of events are observed in chromaffin cells under high frequency stimulation of 15 Hz or treated with the actin disrupting agent cytochalasin-D (Doreian et al., 2008; Olivares et al., 2014).
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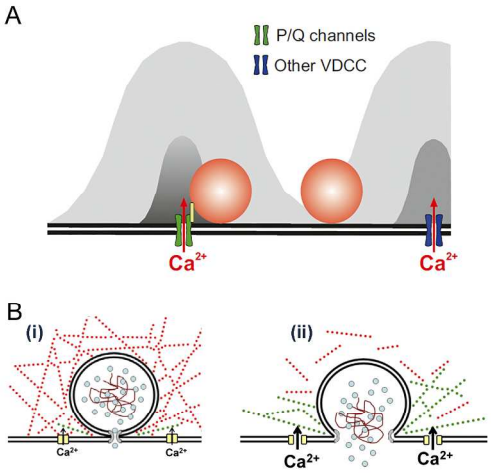

A. In chromaffin cells some secretory vesicles (orange sphere) are physically associated to the synprint site (yellow) in the P/Q-type Ca^{2+} channel molecule (green), and hence they are exposed to high Ca^{2+} levels (dark gray). These vesicles are immediately released upon brief stimulation. On the other hand, vesicles located distant from Ca^{2+} channels are exposed to smaller Ca^{2+} levels (light gray), and they undergo exocytosis only when the global cytosolic Ca^{2+} increased upon long-lasting stimulation. **B.** Docked vesicles are surrounded by an F-actin meshwork, which is remodeling by stimuli that induce exocytosis. **(i)** A small cytosolic Ca^{2+} signal slightly remodels the F-actin meshwork. In this condition the fusion pore does not expand and closes back, allowing the slow release of only small transmitters. **(ii)** Conversely, a robust Ca^{2+} signal disrupts the preexistent actin meshwork (red), but also promotes the formation of new actin filaments (green). This F-actin remodeling favors fusion pore expansion and release of all intravesicular content.

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