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INVITED REVIEW

How does the stimulus define exocytosis in adrenal chromaffin cells?

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Abstract The extent and type of hormones and active peptides secreted by the chromaffin cells of the adrenal medulla have to be adjusted to physiological requirements. The chromaffin cell secretory activity is controlled by the splanchnic nerve firing frequency, which goes from approximately 0.5 Hz in basal conditions to more than 15 Hz in stress. Thus, these neuroendocrine cells maintain a tonic release of catecholamines under resting conditions, massively discharge intravesicular transmitters in response to stress, or adequately respond to moderate stimuli. In order to adjust the secretory response to the stimulus, the adrenal chromaffin cells have an appropriate organization of Ca^{2+} channels, secretory granules pools, and sets of proteins dedicated to selectively control different steps of the secretion process, such as the traffic, docking, priming and fusion of the chromaffin granules. Among the molecules implicated in such events are the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, Ca^{2+} sensors like Munc13 and synaptotagmin-1, chaperon proteins such as Munc18, and the actomyosin complex. In the present review, we discuss how these different actors contribute to the extent and maintenance of the stimulus-dependent exocytosis in the adrenal chromaffin cells.

Keywords Exocytosis · Chromaffin cells · Voltage-dependent Ca^{2+} channels · Vesicle pools · Catecholamines

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Introduction

Adrenaline is the principal hormone released to the blood circulation during the acute response to stress. The key actions of this hormone comprise increase of heart rate and blood pressure, redistribution of blood to skeletal muscles, dilation of bronchia and pupils, and increase of blood glucose level. All these physiological changes prepare our body for a fight-or-flight response [29].

The biggest reservoir of adrenaline in the body is the medulla of the adrenal gland. Specifically, the functional unit of this tissue, the chromaffin cell, is in charge of producing and storing adrenaline. Indeed, the adrenal chromaffin cell (ACC) is equipped with the enzyme battery for the production of this hormone, including the enzymes tyrosine hydroxylase, L-aromatic amino-acid decarboxylase, dopamine-beta-hydroxylase, and phenylethanolamine-*N*-methyl-transferase, which respectively catalyze the synthesis of L-DOPA, dopamine, noradrenaline, and adrenaline from an initial precursor, tyrosine [70]. ACCs also produce enkephalins and other peptides, such as chromogranins and tissue plasminogen activator, which are packed in the secretory vesicles together with adrenaline, noradrenaline, small amount of dopamine, ATP, ascorbate and Ca^{2+} [38].

In response to stress, the splanchnic nerve releases acetylcholine into the adrenal medulla activating cholinergic receptors in the surface of the chromaffin cells. Then, the concomitant depolarization triggers the opening of voltage-dependent

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Ca^{2+} channels (VDCCs), with the consequent increase of cytosolic Ca^{2+} and activation of Ca^{2+} sensors and enzymes involved in the release of transmitter [28]. The most immediate response is the fusion of the chromaffin granules with the plasma membrane, an event that provokes the direct connection of the granular lumen with the extracellular space, allowing the release of the intragranular molecules [131].

The extent of the transmitter release depends on the characteristics of the cytosolic Ca^{2+} signal [46]. More specifically, the magnitude and duration of the Ca^{2+} signals regulate the chromaffin granule distribution among the different pools and their mobilization, docking, priming and final fusion with the plasma membrane. In this present review, we focus our discussion on the molecular mechanisms that determine how the stimulus defines the fate of the secretory vesicle and its exocytosis.

The stimulus pattern defines the shape and duration of the Ca^{2+} signal

In neuroendocrine cells, such as ACCs, exocytosis is triggered by a Ca^{2+} increase in the sub-membrane area that develops after the activation of VDCCs [3, 14, 103, 135]. Using imaging techniques, it was shown that sub-membrane Ca^{2+} gradients in ACCs appear rapidly (< 5 ms) upon membrane depolarization and dissipate over several hundred milliseconds after membrane repolarization [83, 88, 95]. These Ca^{2+} distributions can be accounted by the entry of this cation, and its consequent diffusion, binding to buffers and mitochondrial uptake [47, 69, 83, 84, 95, 100].

Are the sub-membrane Ca^{2+} gradients homogeneously distributed around the cell periphery? Monck and coworkers measured the generation of micrometer size Ca^{2+} hotspots in the periphery of ACCs in response to depolarization pulses [88]. Later on, using evanescent field imaging of sub-membrane [Ca^{2+}], Becherer et al. [16] were able to measure 0.35 μm Ca^{2+} microdomains associated to the activation of VDCCs. These microdomains are localized in a 200-nm thin layer of cytosol adjacent to plasma membrane, a region where docked chromaffin granules are also situated. Modeling studies predicted that Ca^{2+} microdomains occur immediately adjacent to Ca^{2+} channels [118]. This is a particularly important point because ready releasable chromaffin granules can distribute closer or further away to these Ca^{2+} entry points, having in consequence different release probabilities. Klingauf and Neher [69] modeled the Ca^{2+} diffusion in the vicinity of a Ca^{2+} channel pore in spherical cells, such as ACCs, stimulated with short depolarizations. From these studies, they concluded that Ca^{2+} microdomains, that are close to the Ca^{2+} channel mouth, rise within microseconds, reaching values of several tens of micromolar. On the other hand, at longer distances from the channel pore (i.e., 150–300 nm), cytosolic Ca^{2+} levels take some milliseconds to reach few micromolar values. Finally, and similarly to experimental data, the dissipation of these gradients is finished in more than 100 ms. Therefore, from experimental and modeling data, we can expect that Ca^{2+} gradients affect chromaffin granule exocytosis in a temporal and spatial fashion.

In physiological conditions, ACCs are stimulated by repetitive firing of action potentials, in a range of frequencies that goes from 0.5 Hz at rest to approximately 15 Hz in stress conditions [19, 61, 64, 67]. Because the cytosolic Ca^{2+} signals induced by single short depolarizations decrease in few hundred milliseconds, the application of repetitive stimulation at basal physiological frequencies is not expected to provoke significant residual Ca^{2+} accumulation [79, 83]. On the contrary, high frequencies promote an important residual Ca^{2+} buildup [28] that affects locations far from Ca^{2+} channels [69]. It is also important to consider the appearance of burst firing in chromaffin cells. Only a 14% of ACCs display spontaneous burst firing at resting condition, but a sustained membrane depolarization during stress can lead to a prominent burst of action potential firing, which provokes a large Ca^{2+} entry [134]. The occurrence of such bursts depends on the expression or degree of activation or inactivation of voltage-gated Na^{+} and K^{+} channels [85, 134]. These are important issues when considering the recruitment of releasable chromaffin granules as we will discuss later.

Chromaffin granule pools and kinetic components of exocytosis

Each ACC contains thousands of chromaffin granules, but they do not comprise a homogeneous population. On the contrary, chromaffin granules are highly heterogeneous in location and fusion readiness [13, 133]. Flash photolysis of caged Ca^{2+} compounds combined with patch-clamp measurements of cell membrane capacitance has revealed various kinetic components of exocytosis, which are related to secretory granule pools with different fusion readiness [13, 96, 120, 121]. The rapid increase in cytosolic Ca^{2+} produced after photolysis leads to an exocytotic burst followed by a sustained phase of exocytosis. In turn, the burst can be resolved into two kinetically distinct components associated with two separate pools of chromaffin granules: a slowly releasable pool (SRP) and a fully mature readily releasable pool (RRP) [13, 121, 138, 140]. Formerly, it was proposed that the exocytosis of SRP and RRP were activated independently by different Ca^{2+} sensors, but having RRP a kinetics approximately one order of magnitude faster than SRP [121, 138, 139]. However, recently, Walter et al. [142] challenged this concept and proposed that the lower Ca^{2+} sensitivity attributed to SRP was related just to a priming step between this pool and RRP [76, 121].

The slower and sustained phase of exocytosis induced by flash photolysis of caged Ca^{2+} has been associated with a large reserve pool composed of chromaffin granules that have to complete the maturation reactions required to become releasable-competent [60, 140]. Based on electron microscopy observations, the reserve pool was divided in a group of

chromaffin granules morphologically docked to the plasma membrane, but not primed (unprimed pool, UPP), and a larger group of non-docked chromaffin granules located at more than 200 nm from the membrane (depot pool, DP) (Fig. 1) [13]. More recent reports indicate that only a fraction of UPP is functionally docked (i.e., docked granules that are fusion competent) to the plasma membrane [125]. The molecular mechanisms implicated in the functional docking are discussed later in other sections of this review.

As the result of the type of experiments described above, the exocytosis of secretory granules is represented as a linear sequence of reversible maturation reactions followed by a irreversible last step of fusion, which, as we discuss later, is tightly controlled by cytosolic Ca^{2+} concentrations [120, 140] (Fig. 1). Thus, chromaffin granules of DP are docked, primed to SRP, and a further priming step, which was proposed to be Ca^{2+} dependent, finalizes the maturation process to RRP [76, 140, 142]. Finally, exocytosis finishes with the fusion of chromaffin granules with the plasma membrane.

The sequential model described above was proposed on the basis of the exocytotic components triggered by a spatially homogeneous Ca^{2+} increased, as promoted by flash photolysis. Therefore, it does not consider possible differences in chromaffin granule localization with respect to natural Ca^{2+} sources. When cells are electrically stimulated and Ca^{2+} enters the cytosol through VDCCs, exocytosis will depend not only on the priming state of chromaffin granules but also on their proximity to Ca^{2+} channels [45, 94]. A train of repetitive brief stimuli provokes a typical exocytotic pattern, composed by an initial phase of fast exocytosis, synchronous with stimuli, followed by a second phase of mostly asynchronous and sustained exocytosis [62, 82, 140]. This pattern suggests the presence of at least two populations of releasable chromaffin granules located at different positions with respect to the Ca^{2+} source. The delayed component of asynchronous exocytosis is proposed to be associated to chromaffin granules located far from VDCCs, and the early synchronous exocytosis to a fraction of ready releasable chromaffin granules that are located very close to VDCCs. Accordingly, Klingauf and Neher [69] found that the rising phase of cytosolic Ca^{2+} concentration, back-calculated from the secretory response induced by short depolarizations [25], best fits with the simulated gradients at 30 nm from the VDCC pore, explaining the release of 8% of RRP. Similarly, Segura and collaborators [116], using Monte Carlo simulations, demonstrated that the typical biphasic exocytosis during train stimulation can be modeled by assuming a non-uniform distribution of chromaffin granules and channels, where some chromaffin granules are forced to be

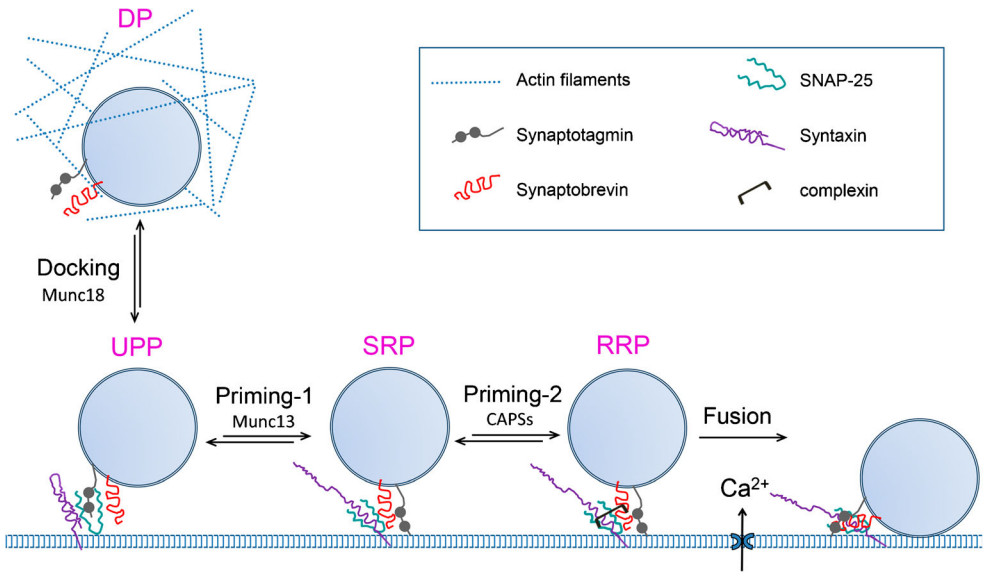

Fig. 1 Steps conducting to exocytosis of chromaffin granules. In resting chromaffin cells, chromaffin granules are retained in the cortical F-actin network. At this stage, they conform a depot pool (DP), the biggest reserve of chromaffin granules of the cell. Upon stimulation, the cortical F-actin network is rearranged to direct the arrival of secretory granules to the plasma membrane. Once chromaffin granules arrive to the cell periphery, they are functionally docked to the plasma membrane by a process that depends on the formation of a complex between synaptotagmin-1, SNAP-25, and syntaxin in its closed conformation. This complex is stabilized by Munc18-1. These functionally docked chromaffin granules constitute an unprimed pool (UPP) that requires to undergo a priming process to become fusion competent. It has been proposed that this priming process comprise at least two steps. In the first step (priming-1), Munc13 favors the opening of syntaxin. Chromaffin granules in this priming state constitute a slow releasable pool (SRP). In the second priming step (priming-2), CAPSs stabilize the open conformation of syntaxin. In this open state, syntaxin is able to initiate the assembling with synaptobrevin and SNAP-25. However, a premature assembly of SNARE proteins is prevented by complexin. Chromaffin granules in this second priming state constitute a ready releasable pool (RRP). Finally, the completed SNARE assembly and consequent chromaffin granule fusion with the plasma membrane is triggered by the entry of Ca^{2+} and by the binding of this cation to synaptotagmin-1

attached to the channels, while the others lie far from them. These and many other results support the presence of an immediately releasable pool (IRP) of chromaffin granules located close to VDCCs [7].

The concept of IRP, to define a group of secretory granules rapidly released upon stimulation in neuroendocrine cells, was introduced by the first time by Horrigan and Bookman [62]. The application of single brief depolarization pulses to isolated rat ACCs revealed the fast and synchronous exocytosis of a small group of chromaffin granules. The exocytosis of this pool increased exponentially with the duration of the stimulus, saturating at approximately 100 ms. Initially, Horrigan and Bookman [62] proposed two alternative hypotheses about the nature of IRP: (1) this pool comprises a population of chromaffin granules functionally distinct from RRP or (2) it is a subpopulation of RRP chromaffin granules located closer to VDCCs. Later experiments using combined electrical stimulation, flash photolysis of caged Ca^{2+} compounds and capacitance measurements, as well as fast Ca^{2+} buffers and modeling, favored the second hypothesis [5, 69, 140]. Nowadays, the IRP is commonly defined as a small group of readily releasable chromaffin granules that are located in close proximity to VDCCs [21, 107]. Although ACCs have a heterogeneous population of VDCCs, including L, P/Q, N, R, and T-type [2, 12, 45, 78, 99], many investigators reported a major efficiency of P/Q-type channels to trigger exocytosis [22, 72, 148]. Alvarez et al. [5, 6] showed that IRP exocytosis in mouse ACCs was almost completely inhibited in the presence of the P/Q-type Ca^{2+} channel blocker ω-agatoxin IVA, and the same was observed in knockout-P/Q-type Ca^{2+} channel cells, concluding that P/Q-type Ca^{2+} channel is eight times more efficient than L-type channels in inducing the exocytosis of IRP. Direct interactions between Ca^{2+} channels and soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins via the synaptic protein interaction (synprint) site and indirect interactions via complexin have been proposed as possible mechanisms that may establish this tight coupling between chromaffin granules and VDCCs [5, 74]. Estimations of the IRP size and rate of exocytosis fluctuate between different ACC preparations, ranging from 15 to more than 30 chromaffin granules and 20 to 150 granules/s, respectively [5, 62, 82, 140].

The stimulus pattern defines the recruitment and maintenance of releasable chromaffin granule pools

The stimulation pattern defines the temporal and spatial distribution of the cytosolic Ca^{2+} signals in ACCs. On the top of that, ready releasable chromaffin granules are distributed heterogeneously with respect to Ca^{2+} sources. Therefore, we can predict that the frequency of the applied stimuli will affect significantly the recruitment of chromaffin granules. Voets and collaborators [140] showed that 10 ms square depolarizations applied at 20 Hz induce first a fast exocytotic component representing the complete release of IRP, followed by a delayed and bigger component of exocytosis. The fact that IRP is depleted at the beginning of the train suggests that this pool does not significantly contribute to maintain the exocytosis during sustained high frequency stimulation. In addition, the replenishment of IRP after its depletion is too slow to allow significant participation of this pool at these stimulation frequencies [89, 90]. Therefore, the exocytosis in such conditions has to be dependent mainly on the release of RRP granules that do not colocalize with VDCCs and probably also on the transference of chromaffin granules from upstream pools [21]. Both these processes are favored by the buildup of residual cytosolic Ca^{2+} during the stimulation trains [21, 82, 84, 138, 140]. This sustained exocytosis is mostly asynchronous because Ca^{2+} has to diffuse from the channel mouth to far locations, and it is potentiated along the train because of local buffer saturation [69, 150]. This situation corresponds to what happens in stress conditions, where ACCs fire action potentials at high frequencies and the consequent accumulation of residual cytosolic Ca^{2+} promotes massive exocytosis irrespective of the location of chromaffin granules with respect to VDCCs [33]. Moreover, a burst mode of action potential firing during stress leads to a major Ca^{2+} entry and increased catecholamine release than simple trains at constant frequency [134].

But what happens under resting conditions, when the firing frequency is low (approximately 0.5 Hz), and Ca^{2+} does not accumulate significantly between stimuli? At first, it sounds reasonable to expect that synchronous IRP exocytosis might be important at basal low frequencies. IRP chromaffin granules are closely coupled to VDCCs, and therefore, Ca^{2+} microdomains around channels should be sufficient to induce their release [7, 16, 69]. It was reported that IRP recovers exponentially after total depletion with a time constant of 7–10 s [89, 90]. This replenishment rate is still too slow to sustain a significant participation of IRP even at low frequencies. Two different research lines resulted recently in two hypotheses that might explain chromaffin granule exocytosis at basal frequencies. First, Lefkowitz et al. [73] proposed a mechanism by which spontaneous asynchronous exocytosis triggered by Ca^{2+} release from intracellular stores may contribute to basal secretion during action potential waveforms applied at 0.5 Hz. Second, Moya-Díaz et al. [90] studied the mechanism of replenishment of a group of chromaffin granules released by a single action potential. This exocytosis triggered by action potential-like stimulus (ETAP) represents the 40% IRP and is replenished with a time constant of 0.7 s. This kinetics is fast enough to maintain synchronous exocytosis at action potential frequencies up to 0.5 Hz. The authors also provided an explanation with respect to the mechanisms of ETAP replenishment. ETAP was consistently followed by a fast endocytosis process that completely compensated the preceding exocytosis with a time constant of approximately 0.5 s. The

inhibition of this fast endocytosis with the intracellular application of a monoclonal antibody against dynamin or a peptide containing the recognition motif for SH3 domain-containing proteins in the dynamin-1 proline-rich domain severely delayed ETAP replenishment. Additionally, an effect in the same direction was found after RRP depletion. Therefore, they proposed a model where both the transfer of chromaffin granules from the ready releasable pool and fast endocytosis allow rapid ETAP replenishment during low stimulation frequencies. The mechanisms proposed by Lefkowitz et al. [73] and by Moya-Díaz et al. [90] are not mutually exclusive, and it is possible that both contribute to the maintenance of secretion during basal firing frequencies in ACCs.

Mechanisms that regulate the arrival of chromaffin granules at the plasma membrane

After their biogenesis and assembled in the endomembrane system [68], newly formed chromaffin granules are transported along microtubules to the F-actin-rich cell cortex, where they undergo a maturation process [112].

In resting ACCs, matured secretory granules are retained in the cortical F-actin network by a process dependent on the motor protein myosin VI. It was demonstrated that myosin VI associates to chromaffin granules in a Ca^{2+}-dependent manner, and consequently its knockdown in PC12 cells significantly reduced the number of granules in the cell cortex without affecting granule biogenesis. Importantly, myosin VI knockdown did not affect exocytosis during the first 15 min of stimulation with high extracellular K^+, but significantly impacted the long-term maintenance of Ca^{2+}-triggered exocytosis [124]. Then, this myosin isoform plays a role in keeping a pool of chromaffin granules tethered to actin filaments in the cell cortex. Additionally, synapsin II might also regulate the granules association to cortical actin in ACCs. Synapsins are a family of proteins that anchor synaptic vesicles to actin filaments in a manner dependent on its phosphorylation status; thus, dephosphorylated synapsins bind synaptic vesicles and maintain a reserve pool, whereas synapsin phosphorylation by the Ca^{2+}/calmodulin-dependent protein kinase II disrupts the anchoring of synaptic vesicles to actin filaments [117]. In ACCs synapsin II phosphorylation is induced by secretagogues [42], and its deletion increases the extent of catecholamine release [136]. The latter authors proposed that synapsin II might play a role as a negative regulator of catecholamine release. However, the involvement of this protein and its potential cooperation with myosin VI in maintaining a pool of chromaffin granules associated to the cortical actin filaments remains uncertain.

Upon stimulation of ACCs, the cortical F-actin network is rearranged to direct the arrival of secretory granules to the plasma membrane. This actin rearrangement comprises both Ca^{2+}-dependent actin depolymerization and polymerization [102]. The breakdown of cortical actin filaments is catalyzed by scinderin, an actin filament-severing protein activated by high cytosolic Ca^{2+} concentrations [35], whereas actin polymerization is promoted by the actin nucleation promoting factors N-WASP and cortactin and regulated by Ca^{2+}-activated kinases such as ERK1/2 and Src kinases [48, 54, 102]. Actin depolymerization permits vesicles to cross the cortical actin barrier towards the plasma membrane [137], whereas the formation of new actin filaments facilitates vesicles mobilization to exocytosis sites [51, 105].

It was proposed that Ca^{2+}-induced F-actin rearrangement allows the formation of channel-like structures perpendicular to the plasma membrane that serve as pathways for the conduction of chromaffin granules to exocytotic sites [50, 51]. An essential motor protein that drives granule conduction through the F-actin trails is myosin II. Indeed the knockdown of this myosin isoform abolishes the movement of chromaffin granules towards the plasma membrane [104]. This mechanism depends on myosin II phosphorylation, since overexpression of an unphosphorylatable form of myosin regulatory light chain restricts the movement of chromaffin granules [93]. Interestingly, myosin II also seems to work regulating the tension of the F-actin cortex in order to facilitate the movement of chromaffin granules towards the plasma membrane.

The Ca^{2+}-induced F-actin rearrangement also plays an important role in the organization of active exocytotic sites. Indeed L- and P-Q-type Ca^{2+} channels, as well as components of the fusion machinery, such as syntaxin-1 and SNAP-25, are clustered at the borders of F-actin cage-like structures, where maximal Ca^{2+} signals and granule exocytosis take place [127, 128]. An important role in the formation of active exocytotic sites is also played by annexin A2. Annexins are a group of proteins that bind, in a Ca^{2+}-dependent manner, acidic phospholipids [49]. They also remodel actin filaments [49]. In stimulated ACCs, annexin A2 migrates from the cytosol to the plasma membrane, where it bundles actin filaments and promotes the formation of phosphatidylinositol 4,5-bisphosphate (PIP2) microdomains and protein clusters of the fusion machinery, such as SNAP-25 [44, 132]. PIP2 microdomains are required for efficient docking and fusion of chromaffin granules, and their disruption interrupts exocytosis in ACCs [130]. The expression of an annexin A2 mutant that fails to bundle actin filaments impairs the formation of plasma membrane lipid microdomains and decreases the number of exocytotic events in ACCs [44]. Then, the F-actin bundling induced by annexin A2 appears to be critical for the formation of active exocytotic sites in ACCs.

In summary, in ACCs, the actomyosin cortex plays an important role in retaining a pool of chromaffin granules away from the plasma membrane. Then upon stimulation, the rearrangement of the actomyosin cortex allows the correct secretory granule conduction to annexin A2-organized membrane microdomains, where exocytosis takes place.

Docking, priming, and fusion of chromaffin granules

The exocytosis of chromaffin granules is preceded by two processes, first a process known as docking by which the granules are tethered to the plasma membrane and a second process called priming that makes granules fusion competent. Fusion is then accomplished by the complete assembly of a complex composed by the transmembrane vesicle protein synaptobrevin, the plasma membrane protein syntaxin, and SNAP-25, a palmitoylated plasma membrane protein. These proteins are known as SNAREs, a term derived from soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors, as they were discovered as receptors for SNAPs, a group of soluble proteins that associate to the *N*-ethylmaleimide-sensitive factor [119]. Although the SNARE complex is considered the basic machinery that drives membrane fusion in different cellular compartments, other proteins, including Ca^{2+} sensors, are required to catalyze in a Ca^{2+}-dependent manner priming and fusion of the granules docked to the plasma membrane.

The docking process in chromaffin cells

Once chromaffin granules arrive to the cell periphery, they are docked to the plasma membrane by a process that depends on plasma membrane proteins syntaxin and SNAP-25 and the vesicular protein synaptotagmin-1, but it is independent of Ca^{2+} and synaptobrevin [18]. In ACCs, deletion of syntaxin or SNAP-25 abolishes the docking process [30, 31], but the simultaneous deletion of synaptobrevin-2 and its homologue cellubrevin does not impair the tethering of chromaffin granules at the plasma membrane.

Synaptotagmin-1 [31, 87] is a transmembrane vesicle protein that has two C2 domains (C2A and C2B) that bind Ca^{2+} and anionic phospholipids, such as phosphatidylserine and phosphoinositides [26, 123]. Synaptotagmin-1 also binds a polyacidic region of the SNARE complex formed by syntaxin-1 and SNAP-25 through a polybasic patch in its C2 domains [20, 149]. Chromaffin granule docking is drastically impaired in cells lacking synaptotagmin-1, and such phenotype is not reverted by the expression of a full-length synaptotagmin-1 mutant with reduced affinity for SNAP-25 [31]. Then, the tethering of chromaffin granules to the plasma membrane requires at least the association of synaptotagmin-1 to SNAP-25.

Munc18-1, a member of the Sec1/Munc18-like protein family that binds to syntaxin [126], also plays a critical role during the docking process. It has been proposed that it acts favoring the formation of a compatible acceptor complex of 1:1 syntaxin/SNAP-25 for docking [27, 31, 80]. In the absence of Munc18-1, syntaxin tends to form an unproductive complex with SNAP-25, in a ratio of two syntaxin molecules with one SNAP-25 molecule [27]. Accordingly, ACCs lacking Munc18-1 exhibit a drastic reduction of docked granules and Ca^{2+}-induced exocytosis [141]. Moreover, a Munc18 mutant with a reduced association to syntaxin also attenuates chromaffin granule docking [58]. Interestingly, SNAP-25 overexpression reverts granule docking reduction in Munc18-1deficient ACCs [31], suggesting that the excess of SNAP-25 molecules also favors the formation of the acceptor intermediate 1:1 syntaxin/SNAP-25 complex that allows functional docking [63]. Instead, the 2:1 syntaxin/SNAP-25 complex arrests morphologically docked chromaffin granules in a fusion incompatible conformation [63].

Together, the aforementioned findings show that the docking process relies on a complex composed by the vesicular protein synaptotagmin-1 and the plasma membrane SNARE proteins SNAP-25 and syntaxin, and stabilized by Munc18-1.

The priming process of chromaffin granules

The priming process is dependent on Ca^{2+} and includes the participation of three distinct types of Ca^{2+} sensors: synaptotagmin-1, the Munc13 isoforms Munc13-1 and ubMunc13-2, and the Ca^{2+}-activated dependent activator protein for secretion (CAPS)-1 and -2. All these proteins have C2 domains that bind Ca^{2+} and PIP2 [66, 110], and their deletions significantly disrupt the release of the RRP in ACCs [81, 91, 98]. However, they exhibit different Ca^{2+} sensitivities [66, 110] and appear to act at different steps of the priming process. For instance, it has been proposed that Munc13-1 and ubMunc13-2 catalyze an initial priming step that allows the formation of SRP [81], whereas CAPS1 and CAPS2 appear to act on a later step [76, 98], and their deletion induces a selective loss of the RRP [77].

Munc13s and CAPSs appear to promote different conformational changes in the SNARE complex. In the absence of Munc13, syntaxin is in a closed conformation, which is stabilized by Munc18-1 [27], and in where the syntaxin N-terminal Habc region folds back onto the motif that binds the other SNARE proteins [34]. However, the binding of Munc13 to syntaxin favors its open conformation [80], allowing the syntaxin SNARE motif to bind synaptobrevin and SNAP-25, and thus initiate the assembly of the SNARE complex. Once syntaxin is in an open conformation, it is then stabilized by CAPSs [106].

Besides to be involved in the docking process, synaptotagmin-1 also catalyzes granule priming. Both docking and priming are mediated by synaptotagmin-1 C2AB domains and different binding regions in SNAP-25 [87]. Replacement of acidic amino acids either at N-terminal or at C-terminal SNARE motif of SNAP-25 impairs synaptotagmin-1 binding to SNAP-25 and chromaffin granule docking. However, only mutations in the N-terminal SNARE motif impair priming, as observed by loss of RRP and SRP [87]. The authors propose that the docking process depends on

poorly defined and promiscuous electrostatic interactions that become more specific and highly defined during the priming process; at this latter level, synaptotagmin-1 stabilizes the primed granules [87]. Then, considering the above mentioned findings, it can be reasoned that the priming process is carried out in various stages, with Munc13s catalyzing an initial priming step that allows the formation of SRP [81], CAPSs refilling and/or maintaining RRP [77], and synaptotagmin-1 stabilizing the priming state [87]. At this point, the chromaffin granules are compatible for fusion. But, what does it prevent fusion at resting Ca^{2+} levels? Here, complexin-2 plays a critical role in preventing premature granule fusion in ACCs.

The fusion of chromaffin granules with the plasma membrane

The fusion of the chromaffin granule with the plasma membrane is accomplished by the complete assembly of the SNARE complex. However, the premature assembly of this complex at resting Ca^{2+} is avoided by complexin-2.

Complexins are a family of small cytosolic proteins that bind to the SNARE complex [75] and clamp it in a prefusion state, preventing premature vesicle fusion at low Ca^{2+} concentrations [114]. ACCs express complexin-2 [9], which appears to play a dual role during exocytosis. In one hand, complexin-2 seems to increase the Ca^{2+} sensitivity for exocytosis via its N-terminal domain, and on the other hand, it prevents premature fusion of chromaffin granules at submicromolar Ca^{2+} concentrations through its C-terminal domain [32]. It seems that first complexin increases the affinity of synaptotagmin-1 for Ca^{2+}, and then, when cytosolic Ca^{2+} increases as a consequence of VDCCs activation, the Ca^{2+} binding to synaptotagmin-1 releases the complexin clamp, allowing the complete assembly of the SNARE complex [52, 114]. This dual role of complexin-2, which seems to be crucial for an efficient synchronous release, has also been observed with complexin-1 in neuronal synapses [86] and reconstituted vesicles [71]. However, additional experiments are required to fully understand the conformational changes occurring in synaptotagmin-1, the SNARE complex, and complexin-2 during this process.

The core of the SNARE complex is formed by four α-helices, with synaptobrevin contributing with one α-helix, SNAP-25 with two α-helices, and syntaxin with one [122]. This complex is closed in a zipper-like manner from the cytosolic regions of the SNARE proteins towards their transmembrane domains, thus bringing the vesicle and plasma membranes closely together and lowering the energy barrier for membrane fusion [101]. SNARE-mediated fusion occurs very slowly in absence of Ca^{2+}, synaptotagmin-1 and PIP2, but it is strongly stimulated in their presence, as observed in fusion assays using liposomes [145]. Mutations in the synaptotagmin C2B that disrupt Ca^{2+} binding inhibit liposome fusion [145]. In addition, Brewer and coworkers [20], using a lanthanide nuclear magnetic resonance approach, have demonstrated that basic residues in the synaptotagmin-1 C2B domain bind to a polyacidic region formed by syntaxin-1 and SNAP-25 in the SNARE complex. As proposed by these authors, this interaction releases the complexin clamp that prevents the zippering of SNARE complex, and therefore the fusion of both membranes. In ACCs, mutations in SNAP-25 that impair synaptotagmin-1 association to the SNARE complex significantly delay fusion kinetics [87].

Recently, Reinhard Jahn and collaborators proposed that synaptotagmin supports exocytosis through a mechanism which is independent of its binding to the SNARE complex [108]. This mechanism implies the initial binding of the synaptotagmin C2B domain to PIP2 via its polybasic patch; then, a rise in cytosolic Ca^{2+} levels enhances the affinity of both C2AB domains to PIP2 by neutralizing negative charges at the Ca^{2+}-binding sites and thus promoting the penetration of synaptotagmin into the plasma membrane [108]. It, however, remains unclear whether the binding of synaptotagmin to PIP2 is competitive or synergistic with its association with the SNARE complex. In this regard, a recent work of Wang and collaborators indicates that synaptotagmin-1 can simultaneously bind anionic phospholipids and the SNARE complex. Importantly, disruption of these two simultaneous associations completely abolishes fusion [144].

The chromaffin granule fusion pore

Electrophysiological recordings in ACCs have demonstrated that the fusion process is initiated with the formation of a high conductance channel that allows the outflow of catecholamines from the lumen of the chromaffin granule to the extracellular medium [1, 4]. It has been estimated that this channel, which has been called "fusion pore," has an initial diameter of a few nanometers [1]. Recent experiments using reconstituted membranes and nanodisks indicate that the fusion pore has a structure composed of both lipids and transmembrane domains of the SNARE proteins [15]. Its initial formation requires at least of two SNARE complexes [15, 147], but its diameter enlarges with increasing amounts of complexes [147]. In ACCs, mutations in the transmembrane domains of synaptobrevin-2 or syntaxin significantly alter fusion pore conductance [23, 59], supporting the idea that the transmembrane domains of these SNAREs form part of the fusion pore. The conductance of the fusion pore is also influenced by mutations in SNAP-25 [39, 40] or in the N-terminal and juxtamembrane region of synaptobrevin [41, 97], suggesting that tightening the SNARE assembly also defines the diameter of the initial fusion pore.

The fusion pore can remain open for a variable period of time before it becomes more dilated or can expand to allow the complete vesicle collapse into the plasma membrane [4]. In ACCs, the fusion pore expansion is influenced by Ca^{2+} [4, 10,

11], synaptotagmin-1 and its isoform synaptotagmin-7 [111, 115], and complexin-2 [32], indicating that molecules that modulate SNARE assembly regulate fusion pore dynamics. However, the fusion pore expansion is also influenced by F-actin [17, 54, 102] and myosin II [17, 92], which reportedly contribute to pull open the Ω shaped membrane profile formed by the vesicle during exocytosis [146].

The fusion pore can also close back resulting in the partial release of catecholamines [4]. This mechanism known as kiss-and-run is still unclear, but it is the predominant form of exocytosis at low frequency stimulations [36, 37, 43]. It appears that high Ca^{2+} concentrations avoid fusion pore closure [143]. It has been also proposed that dynamin, a GTPase that catalyzes membrane fission during endocytosis [55], promotes kiss-and-run exocytosis [24, 37, 53, 57] and rapid vesicle recycling [90]. However, dynamin has also reportedly been involved in favoring fusion pore expansion [8, 56] and stability [65, 129] and in facilitating full fusion of chromaffin granules [65, 113]. These divergent roles played by dynamin seem to depend on the intensity of the stimuli, the status of dynamin phosphorylation, and its interaction with the actomyosin cytoskeleton [56, 65, 113].

The diameter of the fusion pore also determines the amount and types of molecules released. Thus, a narrow fusion pore limits the outflow of catecholamines, but a more dilated fusion pore allows the passage of bigger molecules such as neuropeptide Y, and the full collapse of the granule on the plasma membrane permits the release of all intragranular transmitters [43, 109]. Therefore, considering that the transmitters stored in the chromaffin granules act on diverse types of receptors and tissues, the regulation of fusion pore dynamics and the mode of exocytosis could have important physiological consequences.

Concluding remarks

The release of hormones and active peptides from ACCs relies on a highly regulated sequence of events that leads to maintain a contingent of releasable chromaffin granules and finally to trigger their fusion. However, ACCs must respond to different physiological situations, such a massive response to stress, maintain a tonic release of transmitter under basal conditions, as well as to respond to moderate external stimuli. Therefore, the series of events conducting to the transmitter release should be sensitive to the magnitude and shape of the Ca^{2+} signals produced under such different situations. To support these actions, ACCs are equipped with a set of molecules and cellular elements that control the shape and spatial pattern of the Ca^{2+} signal and with Ca^{2+}-sensitive proteins that define the proper recruitment, priming, and fusion of chromaffin granules. As discussed in this article, several of those molecules and their contribution to the secretory process have been identified. However, it remains to be further investigated how different Ca^{2+}-sensitive proteins, including synaptotagmins, Munc13s, and CAPSs, are coordinated and contribute to the exocytosis triggered by different types of stimuli. Future studies should be addressed to resolve these issues.

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