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REVIEW

The intravesicular cocktail and its role in the regulation of exocytosis

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Abstract

The accumulation of neurotransmitters within secretory vesicles (SVs) far exceeds the theoretical tonic concentrations in the cytosol, a phenomenon that has captivated the attention of scientists for decades. For instance, chromaffin granules can accumulate close to molar concentrations of catecholamines, along with many other products like ATP, calcium, peptides, chromogranins, ascorbate, and other nucleotides. In this short review, we will summarize the interactions that are currently believed to occur between the elements that make up the vesicular cocktail in the acidic environment of SVs, and how they permit the accumulation of such high concentrations of certain components. In addition, we will examine how the vesicular cocktail regulates the exocytosis of neurotransmitters.

Keywords: adrenal, chromaffin, chromogranins, secretion, vesicular ATP.

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Exocytosis is a fundamental process in neurotransmission, a phenomenon that involves secretory vesicles (SVs) fusing with the plasma membrane. Therefore, SVs are one of the main elements in neural communication and hormone secretion. A SV is essentially a lipid bilayer enclosing an aqueous phase that incorporates the proteins necessary to provoke exocytosis in response to a rise in intracellular calcium. SVs are formed in the Golgi apparatus, incorporating both membrane and cargo proteins that are synthesized in the endoplasmic reticulum. However, SVs can also arise from endocytosis (reuse) or by budding from early endosomes. The mechanisms underlying the biogenesis and sorting of SVs have been studied extensively, and these mechanisms are far from the topic of this paper, particularly since several excellent reviews dealing with this issue are available elsewhere (Thiele and Huttner 1998; Tooze 1998; Hook and Metz-Boutigue 2002; Gondre-Lewis *et al.* 2012).

While it is clear that SVs constitute a heterogeneous collection of cell organelles, the usual classification distinguishes two main groups of SVs: synaptic vesicles that mediate fast synaptic transmission; and large dense core vesicles (LDCVs) that typically contain monoamines and/or neuropeptides. The latter usually mediate slow modulatory responses. Although both types of vesicle require Ca^{2+} to fuse with membranes and share many common proteins involved in membrane fusion, there are several differences between SVs and LDCVs. Indeed, the general criteria used to distinguish between them involve their size and content, the presence of an electron dense matrix, the cell type, as well as their fusion kinetics, indicating that distinct molecular mechanisms drive SV- and LDCV-mediated secretion (Malosio *et al.* 2004; Crivellato *et al.* 2008). The main characteristics of both types of SV are summarized in Table 1.

Discovering the components of the vesicular cocktail

The bovine adrenal medulla is a generous source of granules that can be purified and as such, it has been useful to characterize the composition and structure of secretory organelles (Blaschko and Welch 1953; Hillarp *et al.* 1953; Sjostrand and Wetzstein 1956). Indeed, the results obtained from this tissue constitute the reference that served for the later characterization of SVs from other tissues.

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Abbreviations used: CgA, chromogranin A; CgB, chromogranin B; Cgs, chromogranins; LDCV, large dense core vesicles; SVs, secretory vesicles; VMAT, vesicular monoamine transporter; VNUT, vesicular nucleotide transporter.

Table 1 General characteristics of large dense core vesicles and synaptic vesicles

	Synaptic vesicles	Dense core vesicles
EM appearance		
	Taken from (Watanabe *et al.* 2013)	N. Dominguez, E. Santos, L. Castañeyra, J. D. Machado and R. Borges, unpublished
Diameter (nm)	40	150–300
Cell types	Neurons	Neuroendocrine and neurons
Location	Synaptic	Synaptic and extra-synaptic
Cargo	Acetylcholine, ATP, GABA, glutamate, glycine, monoamines	Active peptides, ascorbate, ATP, calcium, monoamines neurotrophic factors
Filling mechanisms	Vesicular -$\Delta\Psi$ and -ΔpH dependent transporters like VMAT, VGLUT, or VNUT	Golgi network and vesicular-$\Delta\Psi$/pH dependent transporters
Matrix composition	Proteoglycans	Chromogranins, heparan sulfate, proteglycans
Trigger for exocytosis	Ca^{2+}	Ca^{2+}
Dysregulation of vesicle fusion and associated diseases	Epilepsy, Parkinson's	Cognitive disorders, diabetes, Obesity

It might now be surprising to know that for decades the scientific community did not accept the fact that natural occurring amines (catechols, histamine, or serotonin) are stored in granules and some curious experiments were required for this to be recognized. For example, it was demonstrated that, contrary to lysed vesicles, the injection of intact vesicles into the blood of a cat had no effect on its heart rate (Blaschko *et al.* 1955). Moreover, once methods for granule preparation became available, the near molar concentrations of catecholamines they contained astonished scientists. Based on the average content of purified bovine chromaffin granules, the vesicular catecholamine concentration was first estimated to be around 600 mM (Winkler and Westhead 1980), although it was later reduced to around 400 mM by monitoring single vesicle secretion using electrochemical techniques: single-cell amperometry (Schroeder *et al.* 1992) or cyclic voltammetry (Pihel *et al.* 1994). Years later, with the advent of patch-amperometry, the intravesicular catecholamine concentration could be measured directly, quantifying it as $\approx$ 1 M in bovine (Albillos *et al.* 1997) and $\approx$ 0.8 M in mouse chromaffin cells (Montesinos *et al.* 2008).

SVs were described as 'perfect osmometers' as they can shrink or swell in response to the tone of the cytosol (Morris *et al.* 1977). The effects of osmolarity have been studied using amperometry, quantifying the effects of changes in osmolarity on the kinetics of exocytosis (Borges *et al.* 1997). Indeed, this notion received support from elegant experiments demonstrating how the clear halo of vesicles swells and shrinks when exposed to L-DOPA (see below).

Once the presence of large amounts of chromogranin A (CgA) in chromaffin granules had been described (Blaschko *et al.* 1967), it was suggested that its colligative properties could serve to bind catechols thereby reducing the intravesicular osmotic pressure. This catecholamine/CgA interaction was later confirmed *in vitro* using a membrane osmometer (Helle *et al.* 1985) and subsequently, *in vivo* using mice lacking chromogranins (Cgs-KO, see Diaz-Vera *et al.* 2012).

A few years earlier, large amounts of ATP had been described in the chromaffin granules of the bovine adrenal medulla (Hillarp *et al.* 1955). Moreover, ATP had been found in the buffer escaping from perfused adrenal glands. It was thus concluded that ATP is not used solely as an intravesicular energy source but rather probably behaves as a passive aggregating agent (Blaschko *et al.* 1956). At this time it was also noted that ATP seems to maintain a relatively fixed molar ratio with catecholamines in function of the sedimentation coefficient of granule fractions. Indeed, the vesicular concentration of ATP might be as high as 120–200 mM (Winkler and Westhead, 1980) in a fixed 1 : 4 stoichiometry, although this remains a little controversial (see for instance Terland *et al.* 1979; Bolstad *et al.* 1980; Caughey and Kirshner 1987). The real importance of vesicular ATP as transmitter resides in its universal distribution. Indeed, virtually all SVs from every known animal species contain ATP and as such, phylogenetically speaking secreted ATP can be considered to be the very first neurotransmitter (Borges 2013).

The last component of the cocktail found to be present in millimolar concentrations was calcium, the importance of

which has frequently been ignored. However, 30% of the total chromaffin cell volume is occupied by ≈ 20 000 granules (Plattner *et al.* 1997) and the free Ca^{2+} concentration in the granules has been estimated to be 40–80 μM in PC12 cells (Moreno *et al.* 2005) and 50–100 μM in chromaffin cells (Santodomingo *et al.* 2008). Moreover, granules can accumulate 20–40 mM of total Ca^{2+}, more than 99% in a bound form. This contrasts strikingly with the concentration of ≈ 100 nM Ca^{2+} found in the cytosol, creating a Ca^{2+} concentration gradient of nearly one-million fold across the granule membrane. Therefore, chromaffin granules contain far more Ca^{2+} than any other organelle, accounting for ≈ 60% of the total calcium in the chromaffin cell (Yoo 2010). Nevertheless, the contribution of this vesicular Ca^{2+} regulating the exocytosis remains controversial.

The advent of molecular biology, as well as the refinements in microscopy and proteomics permitted the lipids and proteins in many types of SVs to be better characterized (Takamori *et al.* 2006). Evaluating such studies is beyond the scope of this short review, it should be noted that extensive information is currently available elsewhere (Wegrzyn *et al.* 2007; Gupta *et al.* 2010). While the importance of the proteins involved in the exo/endocytosis is unquestionable, there has perhaps been insufficient interest in revealing the mechanism involved in the accumulation of neurotransmitters in SVs, even though quantal transmission depends on these.

The inner milieu of chromaffin secretory vesicles is used to concentrate solutes

The inner pH of SVs has been reported to be around 5.5 (Johnson and Scarpa 1976; Pollard *et al.* 1979), which is maintained thanks to a vacuolar V-ATPase that pumps H^+ toward the vesicle (Nelson & Harvey 1999). This H^+ creates both pH (ΔpH) and electrical gradients (Δψ) toward the cytosol. These gradients constitute the electromotive forces for the carrier exchangers (secondary active transmitter transporters), as witnessed with ionophores such as nigericin or valinomycin. Hence, the amine and acetylcholine transporters (vesicular monoamine transporter and VAChT) or Ca^{2+} all use the pH gradient, exchanging two protons, and their accumulation is blocked by nigericin (Fon *et al.* 1997; Camacho *et al.* 2008). In contrast, ATP and glutamate carriers are affected by valinomycin, indicating that they preferably use the electrical potential (ψ, see Sawada *et al.* 2008; Van Liefferinge *et al.* 2013).

The bi-compartmental nature of the intravesicular milieu of chromaffin SVs

The presence of a proteic matrix in chromaffin granules influences the storage and release of soluble components. Although the precise nature of the matrix is still unknown, it is likely to be formed by aggregates of the major components with the granins, thereby generating a functional compartment that can enter into exchange with the free fraction. Using cyclic voltammetry to analyze the pre-spike feature – the spike foot, the concentration of free amines during fusion – pore formation was shown to be about 34 mM, roughly 10% of the estimated vesicular concentration (Schroeder *et al.* 1996).

Indeed, the actual kinetics of catecholamine release during single exocytotic events strongly suggested a bicompartimental structure, with the presence of a functional matrix that binds catecholamines and a free, soluble fraction. This latter element was thought to be represented by the clear halo that surrounds the matrix of the granules when they are observed by electron microscopy. Mathematical modeling also implied that the matrix-associated fraction is responsible for the major part of vesicular storage (Schroeder *et al.* 1996). Using targeted aequorins to intravesicular proteins (Santodomingo *et al.* 2008) revealed the presence of two functional compartments similar to what was described above for catecholamines.

Although it should be borne in mind that owing to the fixation regimes followed up for electron microscopy artifacts could cause some morphological distortion, the clear halo surrounding chromaffin granules seems likely to correspond to the free fraction. In fact, exposure to the catecholamine precursor L-DOPA provokes the swelling of chromaffin granules, which only affects the clear halo surrounding vesicular matrix (Colliver *et al.* 2000). Hence, while the free concentration of amines (present in the halo) would appear to remain constant, the matrix can still bind solutes, albeit with a lower exchange rate.

Once chromogranins knockout mice were available, it became evident that the lack of CgA accelerated exocytosis, which was a consequence of an impaired capacity to retain amines (Montesinos *et al.* 2008). Moreover, the ability of SVs to take up newly synthesized amines was largely impaired in CgA-KO cells. This latter effect was also reproduced in the chromogranin B-KO mouse (Diaz-Vera *et al.* 2010) and in cells of the double KO (CgA&B-KO, see Diaz-Vera *et al.* 2012). These data assigned a double role to the protein matrix in the adsorption of catecholamines. On the one hand, it is a reservoir that accumulates amines as the vesicle's cargo increases when chromogranins are overexpressed (Dominguez *et al.* 2014) and on the other hand, de-adsorption from the matrix slows quantum catecholamine release.

Studying the interaction of solutes inside SVs, in vitro approaches

In conjunction with the progressive description of novel SV components, several attempts have been made to understand whether their chemical interactions could explain the huge

accumulation of neurotransmitters. Chromogranins are currently considered as a saturable high capacity and low affinity buffer, given that CgA seems to bind 32 mol adrenaline per mol with a Kd of 2.1 mM (Videen *et al.* 1992). Indeed, chromogranins also bind Ca^{2+} (50 mol per mol) with a Kd of 1.5–4 mM depending on the type of granin (Yoo and Albanesi 1990; Yoo 2010). The ability of the chromogranins to interact with each other and form dimers or hetero-tetramers has also been studied to explore their interaction with Ca^{2+} (Yoo and Lewis 1996). However, a similar interaction with soluble species like catecholamines and ATP is likely to occur, as the presence of multiple dibasic groups in their structure increases their ability to concentrate solutes (Yoo and Albanesi 1990; Yoo and Lewis 1996; Park *et al.* 2002).

In the early eighties, a series of elegant experiments demonstrated the non-ideal osmolarity of the ATP/catechol interaction (Kopell and Westhead 1982; see also Borges 2013). ATP and catechols form weak complexes *in vitro* that precipitate and can be purified by ultracentrifugation (Da Prada *et al.* 1971; Berneis *et al.* 1973). Indeed, the interaction of these two species can be confirmed by nuclear magnetic resonance (NMR: (Weiner and Jardetzky 1964; Granot 1978), infrared spectroscopy and microcalorimetry (Weder and Wiegand 1973), providing further evidence that this interaction might be behind the stability of these vesicles. This conclusion might be extended to other biological amines like 5-HT, tyramine, and histamine, assigning a more general role to vesicular ATP. However, acetylcholine seems not to interact with ATP and based on NMR studies, it appears that the indol or catechol rings may be involved in this interaction (Weder and Wiegand 1973). Besides ring stacking (purine and catechol) there are electrostatic interactions between the positive chain of the amines and the negatively charged phosphate chain of the nucleotide moiety. These weak interactions (estimated Kd in the mM range; Granot and Fiat 1977) suggest that these complexes are extremely dynamic so as not to impede the release of the CA during exocytosis (Weder and Wiegand 1973). In Fig. 1(a), our current view of how the main vesicular components associate is represented and Fig. 1(b) focuses on the proposed association between ATP and adrenaline).

The possible role of vesicular Ca^{2+} in the aggregation of soluble products within the secretory vesicle is still not fully clear. In a classic study, vesicular Ca^{2+} was considered as a cross-linking agent between ATP and catechols (Weder and Wiegand 1973), although there remains certain controversy regarding this interpretation (Pletscher *et al.* 1973; Granot and Fiat 1977). However, the behavior of divalent/monovalent cations on a granule matrix was later confirmed using optical methods (Curran and Brodwick 1991) and amperometry (Pihel *et al.* 1996). As such, while divalent cations promote the shrinkage of the matrix, monovalent cations promote its swelling.

The interaction of vesicular components, in vivo approaches

The interaction of soluble compounds on isolated chromaffin granules has been studied using NMR, producing similar results to those obtained with artificial mixtures *in vitro* (Daniels *et al.* 1974, 1978; Sharp and Sen 1978). Granins (CgA, chromogranin B, and secretogranin II) are acidic soluble glycoproteins that are in part responsible for the characteristic electron dense core of SVs. Granins are quantitatively the main components of LDCVs, accounting for more than 80% of the soluble intravesicular protein. All granins consist of single-polypeptide chains and the two chromogranins bind calcium, catecholamines and ATP with low affinity but high capacity. For instance, a single CgA can bind 32–93 Ca^{2+} ions (Yoo and Albanesi 1990). Our studies of mouse strains lacking chromogranins demonstrated their crucial role in the accumulation of vesicular amines (Montesinos *et al.* 2008; Diaz-Vera *et al.* 2010, 2012), which seem to account for the 50% reduction in amine content. However, the predicted interaction between CgA and catecholamines only accounts for less than 20% of the total (Sen and Sharp 1980). However, this discrepancy can be explained if we consider the inner cocktail as a functional mixture, which also involves ATP and Ca^{2+} (Machado *et al.* 2010).

Despite the data obtained *in vitro* regarding the formation of complexes between ATP and amines, little information exists about the role of intravesicular ATP in the storage of other neurotransmitters and in their exocytosis. Chromaffin granules behave as 'ATP sinks' and vesicular ATP accounts for $\approx$ 75% of the entire cellular ATP content (Bevington *et al.* 1984; Corcoran *et al.* 1986a). However, the turnover of vesicular ATP seems to be very slow in chromaffin granules when measured using radioactive nucleotides (Corcoran *et al.* 1986b). Pharmacological inhibition of ATP production through the application of cyanide and deoxyglucose successfully reduces the cytosolic ATP, yet these agents prove to have virtually no effect on the vesicular ATP content, even when treatments were prolonged over several days. This resistance to change suggests that this molecule can be considered as metabolically inert, scarcely entering into dynamic exchange with the cytoplasmic ATP (Corcoran *et al.* 1986a).

The carrier that promotes the transport of ATP toward the SV is the vesicular nucleotide carrier – vesicular nucleotide transporter (VNUT) – (or Slc17a9: (Sawada *et al.* 2008). VNUT has been characterized using drugs like Evans' blue or 4,4′-diisothiocyano-2,2′-disulphonic stilbene acid, as well as by modulating its expression in isolated cells and in VNUT-KO mice (Sakamoto *et al.* 2014). In all cases, diminished transporter expression/activity causes a drastic reduction in ATP exocytosis. In this regard, we are currently conducting studies to determine the role of ATP in the accumulation of catecholamines in SVs (Estévez-Herrera *et al.* 2012). Although it has yet to be proved under physiological stimuli, the existence of large concentrations

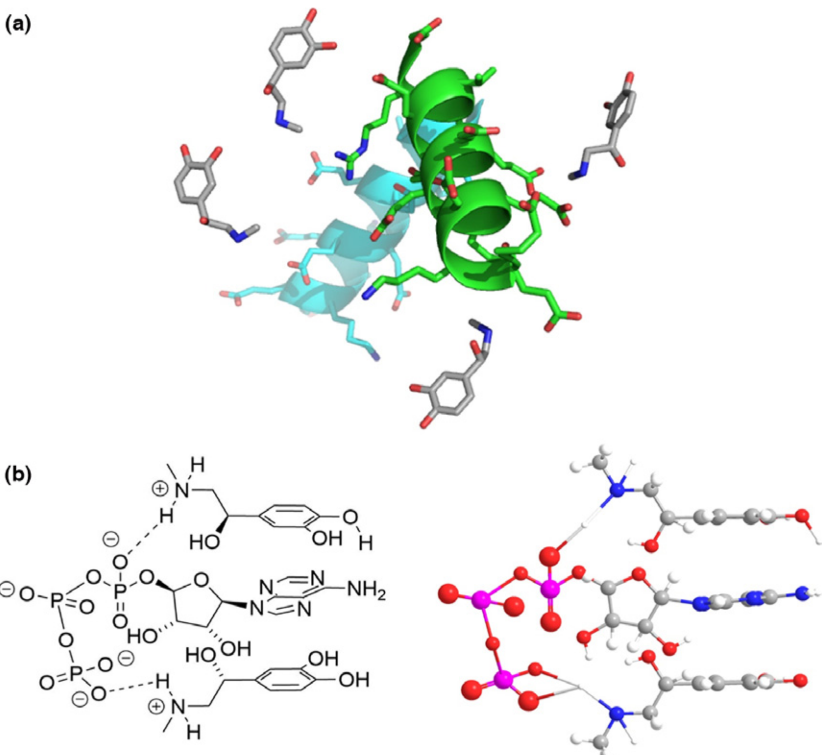

Fig 1. Theoretical models of the interaction of some of the major intravesicular components. (a) Chromogranins and catechols. The presence of high concentration of catechols surrounding a vesicular protein core facilitates the association of both adrenaline and chromogranins probably through amine groups. In the example, a glutamic-rich sequence of mouse chromogranin A (KQEE-KEEEEEEEAVARE) is assumed to adopt an α-helical conformations and two such sequences are displayed. At acidic pH adrenaline molecules are adsorbed to the protein through their ammonium groups. The model was created by sequence alignment from known 3D structures (3CAZ, doi10.2210/pdb3caz/pdb and 3WY9, doi10.2210/pdb3wy9/pdb) using Clustal Omega (EMBL-EBI, 2013). (b) Adrenaline and ATP. One ATP binds two adrenaline molecules by electrostatic interaction between the phosphates and the ammonium salts. In addition, weak stacking of catechol and adenosine rings and hydrogen bonds contribute to facilitate the association. Calcium has been deliberately ignored even though its role in the folding of granins. Data adapted from (Granot and Rotman 1978).

of the two essential factors to trigger exocytosis (Ca^{2+} and ATP) across the granule membrane raises the exciting possibility that these vesicular components participate in secretion.

Thus, the overall picture emerging from over 50 years of research is that of a dynamic interaction between a protein matrix – chromogranins that associate with biogenic amines, ATP and Ca^{2+}, and that account for 90% of these constituents – and a free fraction in which these elements are in equilibrium with the matrix. The acidic pH, and probably other components of the vesicular cocktail not mentioned here (Mg^{2+}, other nucleotides, ascorbate, peptides), will likely contribute to reinforce the concentrating power of LDCVs. Indeed, it is striking that despite the long-standing interest of scientists, the true nature of the vesicular cocktail is still far from clear. In addition, it should be considered that the failure of vesicular transporters to maintain a stable and adequate inner concentration of ATP, pH and Ca^{2+} could underlie neurological, endocrine, immunological and cardiovascular diseases, opening the way to an exciting period of future research.

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