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Article Addendum

On the role of intravesicular calcium in the motion and exocytosis of secretory organelles

José D. Machado,[1,*] Marcial Camacho,[2] Javier Alvarez[3] and Ricardo Borges[1]

[1]Unidad de Farmacología; Facultad de Medicina; Universidad de La Laguna Tenerife; La Laguna, Spain; [2]Department of Molecular and Human Genetics; Baylor College of Medicine; Houston, TX USA; [3]Instituto de Biología y Genética Molecular (IBGM); Valladolid, Spain

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Secretory vesicles of sympathetic neurons and chromaffin granules maintain a pH gradient towards the cytosol (5.5 vs. 7.2) promoted by the V-ATPase activity. This gradient of pH is mainly responsible for the accumulation of amines. The secretory vesicles contain large amounts of total Ca^{2+}, but the free intragranular [Ca^{2+}], the mechanisms for Ca^{2+} uptake and release from the granules and their physiological relevance regarding exocytosis are still matters of debate.

We have recently shown that disruption of the pH gradient of secretory vesicles slowed down exocytosis. Fluorimetric measurements, using the dye Oregon green BAPTA-2, showed that the V-ATPase inhibitor bafilomycin A1 directly released Ca^{2+} from freshly isolated vesicles. Accordingly, vesicle alkalinization released Ca^{2+} from the granules to the cytosol, measured with fura-2 in intact chromaffin cells. Using TIRFM in cells overexpressing the EGFP-labeled synaptobrevin (VAMP2-EGFP) protein, we have then shown that the Ca^{2+} released from the vesicles to the cytosol in the presence of bafilomycin, dramatically increased the granule motion of chromaffin- or PC12-derived granules, and triggered exocytosis (measured by amperometry).

We conclude that the gradient of pH of secretory vesicles might be involved in the homeostatic regulation of the local cytosolic Ca^{2+} around the vesicles and in two of the major functions of secretory cells, vesicle motion and exocytosis.[1]

Most neurotransmitters and hormones are stored in secretory vesicles that release their contents to the extracellular media after a stimulus.

As exocytosis and vesicle motions are well-established Ca^{2+}-dependent mechanisms and large concentrations of Ca^{2+} are stored in secretory vesicles, a considerable effort has been placed to address a physiological role(s) of vesicular Ca^{2+} in their own motion and exocytosis.

In this brief we will discuss recent data about the homeostasis of intravesicular Ca^{2+}, which have provided strong evidence that it may represent a crucial source able to create a specific microdomain of Ca^{2+} in the vicinity of granule membrane, the exact location to control both the granule motion and exocytosis.

The Secretory Organelle

Secretory granules from chromaffin cells are large dense core vesicles similar to those present in many other neuroendocrine cells and in sympathetic neurons.[2] Chromaffin granules are extremely efficient concentrating soluble transmitters such as catecholamines 500–1000 mM[3,4] together with other components as ATP 125–300 mM,[5] ascorbate 10–30 mM,[6,7] peptides and chromogranins, forming a condensed protein matrix (~180 mg/ml).[8] In addition they concentrate H^+ to create an acid media and, the main reason of this report, large amounts of Ca^{2+}. The mechanisms followed to get these large concentrations of solutes in spite of the large osmotic forces created have intrigued researchers for decades.

Chromaffin granules maintain a pH gradient across their membranes of about two orders of magnitude, ≈5.5 inside and ≈7.3 in the cytosol. This gradient is held stable by the activity of a specific H^+-ATPase (V-ATPase). This vesicular H^+ gradient is used as antiporter to accumulate catecholamines, by the vesicular monoamine transporter VMAT2,[9] and Ca^{2+} through the H^+/Ca^{2+} antiport, although most of Ca^{2+} accumulation in the vesicles appears to occur via a SERCA-type Ca^{2+} ATPase[10,11] (Fig. 1). The presence of a vesicular matrix composed by the aggregation of the components of the vesicular cocktail with proteins has been proposed as the chelating method to reduce the osmotic forces[12] that permit the accumulation of solutes at high concentrations.[13] Therefore, most of the intravesicular solutes are not free but associated to the matrix, where the main proteic components are chromogranins, whose pK_a is also around 5.5.[12,14] Therefore, it is plausible that intravesicular pH can regulate the ability of chromogranin A to form aggregates[15] and that the regulation of vesicular pH could play an important role in the dynamics of vesicular Ca^{2+} and catechols.[11,16,17]

*Correspondence to: José D. Machado; Unidad de Farmacología, Facultad de Medicina; Universidad de La Laguna; La Laguna E-38071 Spain; Tel.: +34.922.319346; Fax: +34.922.655995; Email: jdmacha@ull.es

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Bi-compartmental Storage of Ca^{2+}

The idea that intravesicular Ca^{2+} could be involved in the exocytotic process was first postulated by Borowitz in 1967.[18] Nevertheless, this idea has not received fully acceptance by the scientific community. Endoplasmic reticulum has been classically considered as the main source of Ca^{2+}, mainly because the mobilization of Ca^{2+} from stores by $InsP_3$ was first discovered in this organelle. More recently, the involvement of other cell structures like mitochondria, nucleus and Golgi in the uptake, release and cytosolic redistribution of Ca^{2+} have also been proven.[19-21] Therefore secretory vesicles are still frequently ignored and considered as a simply non-functional sink for Ca^{2+}. The main argument, with little experimental support, has been that vesicular Ca^{2+} is sequestered into the vesicular matrix from where it experiences little turnover. In spite of the new data that contradicts this assumption let us to show here some numbers.

About 30% of the total a chromaffin cell volume is occupied by around 20,000 granules.[22] The recent development of targeted aequorins to the inner side of secretory vesicles has directly confirmed that Ca^{2+} is distributed in two fractions; the chelated Ca^{2+} which is estimated to be about 40 mM,[23] and the free fraction which was calculated to be around 50–100 μM.[11,23,24] The free fraction is in equilibrium with the Ca^{2+} bound allowing a rapid recovery after an acute depletion. Chromaffin granules contain far more Ca^{2+} than any other organelle, accounting for about 60% of the total in chromaffin cells.[23,25] Even considering that this cation is crucial for processes that take place 'just across their membrane' like vesicle movement or exocytosis, the old hypothesis of Borowitz is still receiving little attention.

Mobilization of Vesicular Ca^{2+}

The disruption of pH gradient using protonophores[26] or weak bases[27-29] has been used to induce the alkalinization of granules that causes the release of Ca^{2+} and catecholamines towards the cytosol.[29] This effect is shared by clinically relevant drugs like the hypotensive agent hydralazine,[30] amphetamines[31] or β adrenergic blockers.

Other stimuli like histamine, caffeine or depolarization mobilize the free Ca^{2+} fraction.[11,24] Targeted aequorine data suggest that intravesicular Ca^{2+} kinetics follows a bi-compartmental model where the total amount of free [Ca^{2+}] is nearly three orders of magnitude smaller than bound calcium. This explains the rapid recovery of free Ca^{2+} after the depletion of the free compartment with SERCA inhibitors (BHQ, cyclopiazonic acid) or pH-disrupting agents.[11,24] In addition, both $InsP_3$-induced and Ca^{2+} induced Ca^{2+} release (CICR) are present and functional in chromaffin and PC12 secretory vesicles. The main problem to demonstrate whether the intravesicular Ca^{2+} is actively participating in granule motion and exocytosis, under physiological conditions, is the difficulty in differentiating this Ca^{2+} from the Ca^{2+} arriving from other sources. All known secretagogues increase free cellular Ca^{2+} by activating its entry from external media and/or promoting its release from internal stores. Nevertheless, the vesicular alkalinization observed upon the activation of several second messenger routes will contribute also to the mobilization of vesicular Ca^{2+} and catecholamines; this latter effect was recently demonstrated using single cell amperometry. It seems plausible that the pH gradient across the vesicular membrane could be the necessary link between physiological stimuli and the regulation of Ca^{2+} and catecholamines release from the secretory vesicles.

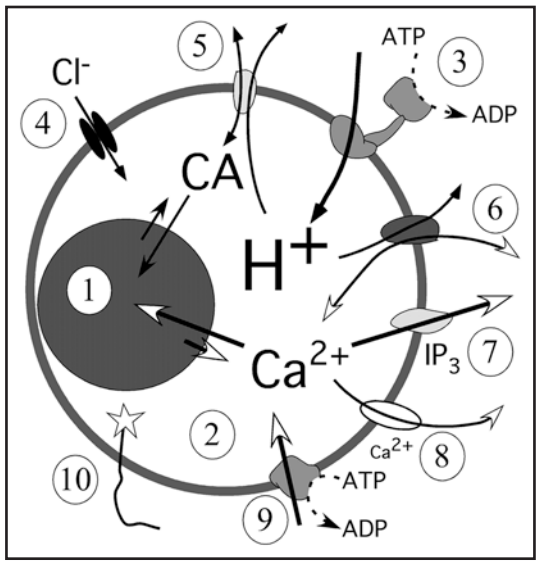

Figure 1. Mechanism used for Ca^{2+} (and catecholamines, CA) turnover in chromaffin secretory organelles. The relative sizes for the granule matrix (1) and the free compartment (2) have been change for clarity. The H^+ are pumped towards the vesicle lumen by an ATP dependent (V-ATPase, 3). Protons maintain the pH and the potential gradients with the help of Cl^- channels which acts as counter ions (4) to keep the $\Psi \approx -80$ mV. Catecholamines (5) and Ca^{2+} (6) use H^+ as antiporters to be accumulated inside vesicles, both carriers can work also in the reverse mode. The IP_3 receptors (7) release Ca^{2+} as a response to intracellular IP_3 whereas CICR (8) amplifies the Ca^{2+} signaling by releasing Ca^{2+} a response that is modulated by ryanodine and caffeine. The SERCA (9), not described yet in chromaffin granules will be the Ca^{2+} pump; this pump is blocked by thapsigargin. In these studies the luminal terminal of VAMP (10) (synaptobrevin) has been modified to place a Ca^{2+} sensor (low Ca^{2+}-affinity aequorine) or pH sensor (EGFP).

Besides the role of other known organelles, in future, cell stimulation by different mechanisms, either mediated by $InsP_3$ receptors, ryanodine receptors or plasma membrane Ca^{2+} channels should take into account that they also induce vesicular Ca^{2+} release. In addition, other stimuli that activate guanylate cyclase or adenylate cyclase, which alkalinize the vesicular lumen, might also mimic these mechanisms.

Taking into account the poor diffusion of Ca^{2+} through the cytosol,[32] we consider it highly plausible that vesicular Ca^{2+} could be playing a relevant physiological role in the granule's approach to the membrane[33,34] and in their own exocytosis. The physiological relevance of the Ca^{2+} release from secretory vesicles will require further investigation.

An additional support to this argument was found using bafilomycin A1, a potent and highly specific inhibitor of the H^+-ATPase, to study the effects of vesicle alkalinization and the release of vesicular Ca^{2+} to cytosol. This Ca^{2+} increases the lateral motion of chromaffin granules and triggered exocytosis. Although bafilomycin is not a physiological stimulus, these results revealed a novel mechanism for releasing vesicular Ca^{2+}, which is controlled by the pH gradient.

In summary, the recent data from our laboratories and other have demonstrated that: (i) secretory vesicles from PC12,[24] and chromaffin cells[11] accumulate Ca^{2+} under two different and exchangeable conditions: free (≈50–100 μM) and bound Ca^{2+} (≈40 mM), (ii) the vesicular pH is closely associated with the modulation of the kinetics and quantal characteristics of the exocytosis of catecholamines,[29] (iii)

secretory granules possess mechanisms for fast uptake and release of Ca^{2+} and (iv) Ca^{2+} release from the granule can participate in their own movement and exocytosis.[1]

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