# Physiological Mini Reviews

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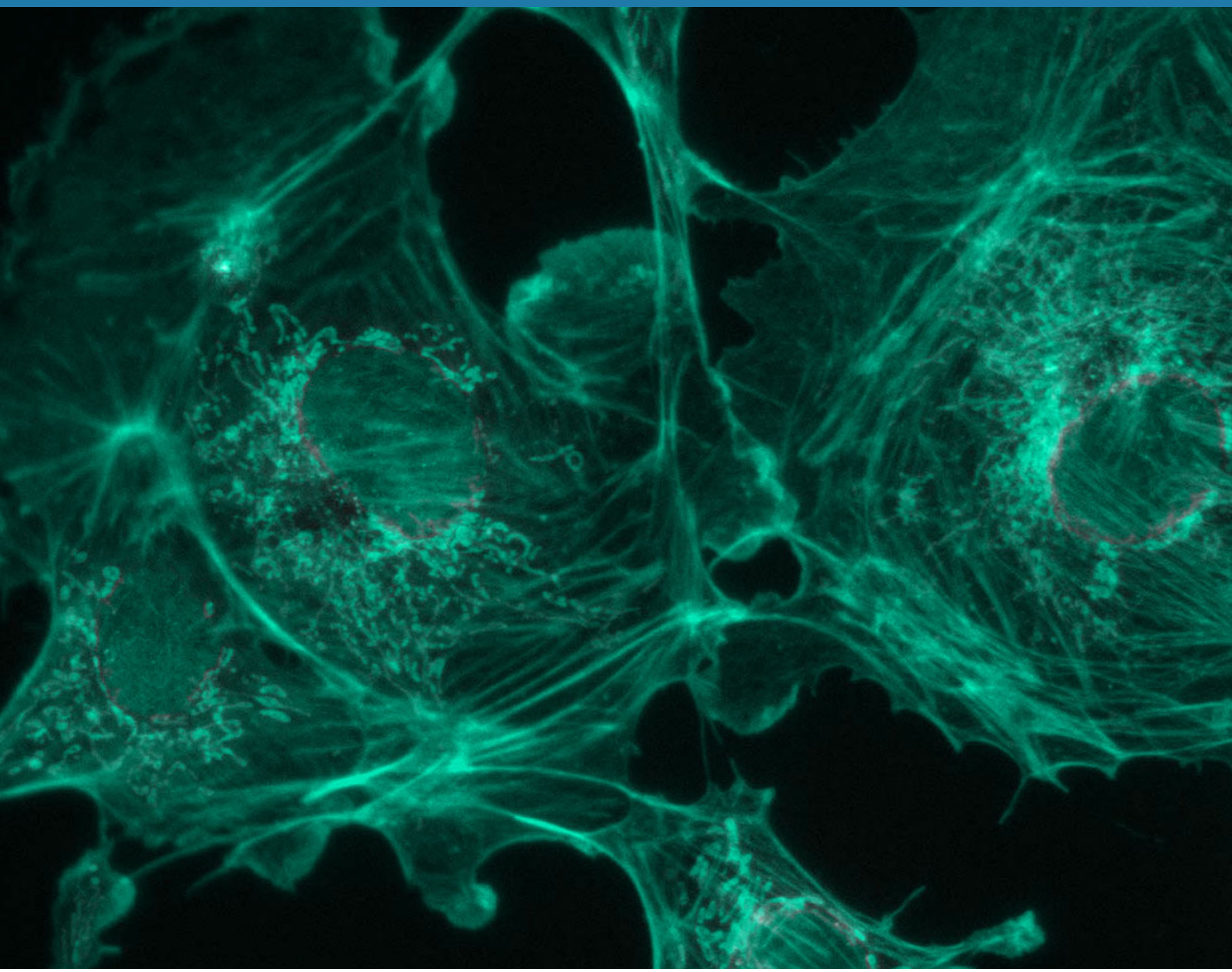
Volume



**Vol. 11**, July - August 2018
ISSN 1669-5410 (Online)
pmr.safisiol.org.ar

Physiological Mini Reviews

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# Physiological Mini-Reviews

**[ISSN 1669-5410 (Online)]**
Edited by **the Argentinean Physiological Society**

Journal address: Centro de Investigaciones Cardiovasculares y Cátedra de Fisiología y Física Biológica.
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# THE IMMEDIATELY RELEASABLE POOL OF SECRETORY VESICLES IN ADRENAL CHROMAFFIN CELLS

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## ABSTRACT

The exocytosis of hormone-filled vesicles in adrenal chromaffin cells is triggered by localized $Ca^{2+}$ gradients that develops after the activation of voltage-dependent $Ca^{2+}$ channels. To reach the fusion competent state the vesicles go through a variety of maturation steps, including the mobilization through the cytoskeleton, the docking to membrane, and the priming of the exocytotic molecular machinery. However, the fusion readiness of vesicles will also depend on their proximity to the $Ca^{2+}$ source. The immediately releasable pool is a small group of ready–to-fuse vesicles, whose fusion is tightly coupled to $Ca^{2+}$ entry through channels. Recent work indicates that such a coupling is not produced by a random distribution between parts, but is the result of a specific interaction of secretory vesicles with P/Q-type $Ca^{2+}$ channels. The immediately releasable pool is able to sustain with high efficiency the secretion triggered by brief depolarizations applied at low frequencies, like action potentials at basal conditions in adrenal chromaffin cells.

**Keywords:** exocytosis; P/Q channels; endocytosis; calcium.
**Running Title:** The Immediately Releasable Pool.

## RESUMEN

La exocitosis de las vesículas secretorias en células cromafines adrenales es disparada por gradientes de $Ca^{2+}$ localizados que se desarrollan a partir de la activación de canales de $Ca^{2+}$ voltaje dependiente. Para poder fusionarse a la membrana, las vesículas deben pasar primero por una serie de etapas madurativas que incluyen el transporte por el citoesqueleto, el anclado a la membrana y el priming de la maquinaria exocítica. Sin embargo, la eficiencia con que la vesícula se fusiona depende también de su proximidad con la fuente de $Ca^{2+}$calcio. El pool inmediatamente liberable es un pequeño grupo de vesículas maduras cuya fusión está altamente acoplada a la entrada de $Ca^{2+}$ por canales. Trabajos recientes indican que este acople no se explica por una distribución aleatoria entre las partes, sino a través de una interacción específica de la vesículas secretorias con los canales de $Ca^{2+}$ de tipo P/Q. El pool inmediatamente liberable es capaz de sostener con alta eficiencia la secreción inducida por despolarizaciones cortas aplicadas a bajas frecuencias, tal como el disparo de potenciales de acción en condiciones basales en células cromafines.

**Palabras Clave:** exocitosis, canales P/Q, endocitosis, calcio

Original received: April 12, 2018; Accepted in its final form: May 2, 2018.

## Introduction

Adrenaline is the major hormone released to the blood during the acute response to stress. This hormone increases heart rate and blood pressure, facilitates the redistribution of blood to skeletal muscles, and promotes the dilation of bronchia, among other effects that prepare our body for a fight-or-flight response [1]. Adrenal chromaffin cells (ACC) are in charge of producing, storing and releasing adrenaline in addition to many active peptides like enkephalins, neuropeptide Y and chromogranins, which are packed together in secretory vesicles [2].

In response to stress, the splanchnic nerve releases acetylcholine, activating cholinergic receptors in the ACCs. Subsequently, the process of excitation-secretion coupling begins when action potentials are fired and activate voltage-dependent $Ca^{2+}$ channels (VDCC), and ends when $Ca^{2+}$ entry through VDCCs promotes the exocytosis of fusion competent vesicles [3]. To reach the fusion competent state vesicles have to go through a series of maturation steps, including mobilization through the cytoskeleton, detachment from cytoskeletal proteins, docking to plasma membrane and a series of priming reactions that result in totally matured releasable vesicles [4]. However, under physiological stimulation conditions, the fusion readiness of a vesicle not only depends on this maturation process, but also on the proximity between the vesicle and a $Ca^{2+}$ source [5]. This review is focused on the distribution of secretory vesicles respect to $Ca^{2+}$ sources and how this distribution affects the secretion kinetics; and particularly on the cellular mechanisms that determine the tight coupling of a small pool of vesicles, named immediately releasable pool (IRP), with VDCCs.

## Vesicle Pools in Chromaffin Cells

Experiments combining a rapid and spatially homogenous elevation of cytosolic $Ca^{2+}$ induced by the flash photolysis of caged-$Ca^{2+}$ compounds with simultaneous patch-clamp measurements of cell membrane capacitance revealed different kinetic phases of exocytosis [4, 6]. As the result of these type of experiments, the secretion process in ACCs and other neuroendocrine cells was represented as a linear sequence of reversible transitions between pools of vesicles with different levels of maturation (Figure 1): an immature reserve pool (RP), a slowly releasable pool (SRP), and a totally matured ready releasable pool (RRP) [6-9]. Finally, the vesicles of RRP are fused to plasma membrane through an irreversible reaction triggered by $Ca^{2+}$ binding to the vesicle protein synaptotagmin [10], and is accomplished by the complete assembly of the SNARE complex (composed by the transmembrane vesicle protein synaptobrevin and the plasma membrane proteins syntaxin and SNAP-25) [11]. These different pools are recruited differentially with different stimulus patterns. While low to middle stimulation induce the fusion of vesicles only from RRP, stronger stimulation recruit also vesicles from the SRP and even from the RP [6, 7, 12].

The sequential model described above was proposed on the basis of the exocytotic components measured after the induction of a spatially homogeneous $Ca^{2+}$ increase, as promoted by flash photolysis. Therefore, it does not consider possible differences in chromaffin vesicle 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 maturation state of chromaffin vesicles but also on their proximity to $Ca^{2+}$ channels [5]. It was observed that brief depolarizations (i.e. action potential) applied individually or at low frequencies trigger the release of just a small fraction of vesicles during the stimuli (synchronous exocytosis), whereas the application of a prolonged depolarization or action potentials at high frequencies promotes also the delayed release of an additional

fraction of vesicles (asynchronous exocytosis) [6, 13, 14]. Synchronous exocytosis is generally explained by the presence of a tight coupling between a small group of synaptic/secretory vesicles and VDCCs [5, 6] (Figures 1 and 2). On the other hand, asynchronous exocytosis is interpreted in part by the fact that most vesicles in neuroendocrine cells are not located in the microdomain of VDCCs and also because the release of upstream pools like SRP and RRP [6, 7]. In neuroendocrine cells, as ACCs, fast synchronous secretion is usually associated with the presence of the so called immediately releasable pool (IRP).

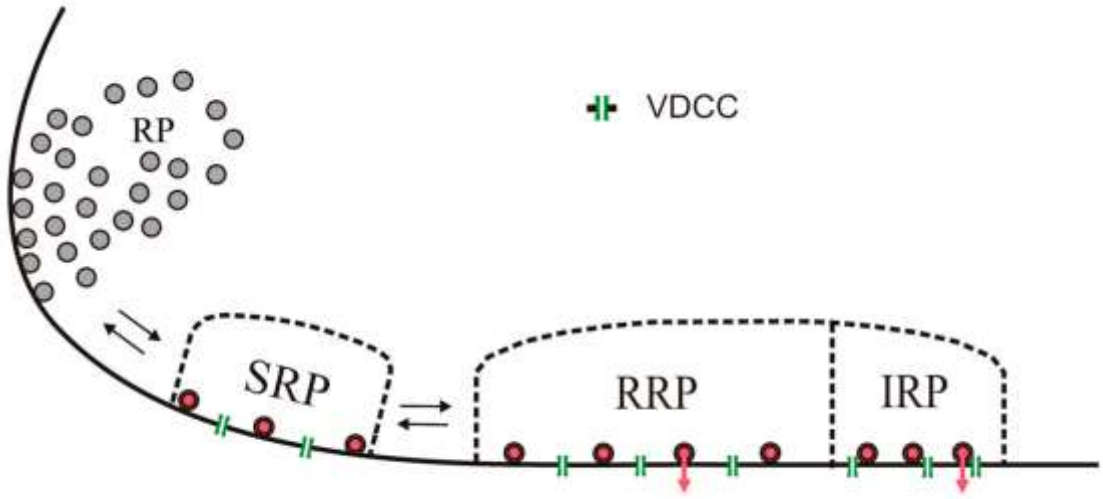


**Figure 1:** The scheme represents the different vesicle pools defined by kinetic analysis of capacitance measurements of exocytosis induced by flash photolysis of caged $Ca^{2+}$ and electrical stimulation (see text). RP: immature reserve pool; SRP: slowly releasable pool; RRP: readily releasable pool; IRP: immediately releasable pool. The horizontal black arrows represent forward and backward vesicle maturation reactions between pools; vertical downward red arrows represent irreversible fusion reactions for RRP and IRP respectively.

## The Immediately Releasable Pool

Horrigan and Bookman [15], working in isolated rat ACCs, 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. They showed that the exocytosis of IRP was synchronous with the stimulus and progressed with a first order kinetic behavior, with a time constant of 50 ms. These authors proposed two alternative hypotheses about the nature of IRP vesicles: (1) IRP reflects a population of vesicles functionally different from the secretory vesicles that make up the RRP; (2) IRP is a subpopulation of RRP vesicles, but located closer to $Ca^{2+}$ channels [15]. Subsequent work favored the second hypothesis. First, in conventional flash photolysis experiments, the IRP was not detected as a different kinetic component [6, 7]. Therefore, IRP seems to not be 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 [6]. Third, BAPTA, in comparison to EGTA, markedly reduced the efficiency of $Ca^{2+}$ entry to induce IRP exocytosis [16]. Therefore, the IRP is

commonly defined as a small group of readily releasable vesicles (Figure 2) that are located in close proximity to VDCCs [6].

Estimations of the IRP size fluctuate between different ACCs preparations and between different types of neurosecretory cells, ranging from less than 10 to almost 50 vesicles [6, 13, 15, 17-19]. ACCs in slices presented a more prominent exocytosis in response to short depolarizations than isolated ACCs in culture [20]. Consistently, the co-localization between $Ca^{2+}$ channels and vesicles was higher in slices in comparison to isolated cells [21]. Regarding the rate constant at which the IRP releases their vesicles, Horrigan and Bookman (1994) and Marengo (2005) have reported a value for cultured rat or bovine ACCs 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 ACCs in culture, Álvarez and collaborators estimated an intermediate value of 64 $s^{-1}$ [16].

## $Ca^{2+}$ Channel Subtypes Coupled to IRP Exocytosis

Two opposite hypothesis were proposed to explain why a fraction of readily releasable vesicles are located 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 lies close to $Ca^{2+}$ channels by chance. Alternatively, it is also possible that a particular type of $Ca^{2+}$ channel is specifically coupled to IRP vesicles. The first scheme is the simplest one, as it does not assume any specific interaction. This possibility was considered by several authors [6, 22, 23]. In this scenario, all VDCC subtypes (L, N, P/Q and R) present in ACCs are expected to participate in the IRP release in proportion to their contribution to the whole voltage-dependent $Ca^{2+}$ current [24]. However, there is evidence suggesting that the different VDCC subtypes expressed in ACCs are not equally efficient in triggering exocytosis [25-27]. Moreover, Segura and collaborators [28] predicted that a random distribution of vesicles and channels does not explain the biphasic capacitance time course provoked by a train of short depolarizations [6, 13, 15]. On the other hand, these authors found that this characteristic biphasic behavior can be simulated by assuming a non-uniform distribution of vesicles and channels, with some vesicles attached to the channels and the others located far from them (Segura et al, 2000).

Several investigators reported a major efficiency of P/Q-type channels to trigger exocytosis in comparison with other VDCC subtypes [25, 26, 29-31]. Using patch-clamp capacitance measurements, Álvarez and colleagues [16, 17] showed that IRP exocytosis in mouse ACCs mostly depends on P/Q-type $Ca^{2+}$ channels (Figure 2). This finding was supported on: (i) the suppression of IRP release by the specific blocker ω-agatoxin IVA; (ii) the almost complete abolishment of IRP in ACCs from α1A deficient (P/Q-KO) mice; (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; 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 [16, 17]. This scenario suggests the existence of a specific physical interaction between IRP vesicles and P/Q-type channels (Fig. 2).

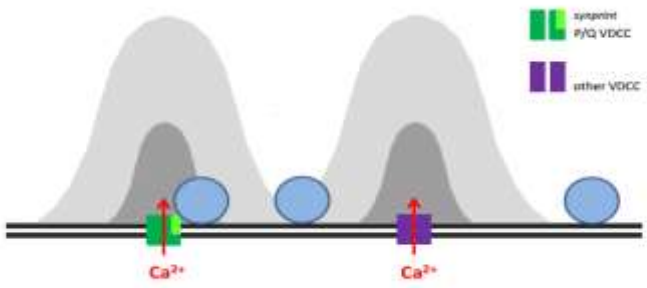


**Figure 2:** The scheme represents an IRP vesicle (blue sphere on the left) located in close physical association to a $Ca^{2+}$ channel (P/Q subtype in green), and therefore exposed to the narrow $Ca^{2+}$ gradient (dark gray) generated when the cell is stimulated with a brief depolarization. This vesicle is physically associated to synaptic interaction site (light green bar) in the P/Q $Ca^{2+}$ channel molecule. Another vesicle located distant from $Ca^{2+}$ channels (blue sphere in the center) is only exposed to the more prominent $Ca^{2+}$ gradient (light gray) provoked by strong stimulation, as a long depolarization. The scheme was modified from Cardenas and Marengo (2016).

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 $\alpha_{1a}$ subunit [32, 33]. This synprint site interacts with SNAP-25, syntaxin, synaptotagmin and the cysteine string protein [32-35]. It was postulated that this interaction is critical for fast and highly synchronized exocytosis at the presynapse [32, 36]. Hence, it is possible that a similar type of interaction between the P/Q-type channels and IRP vesicles might occur in ACCs. The following findings support this idea: (i) the synprint site is present in different splice variants of the P/Q $\alpha_{1a}$ subunit in bovine ACCs, (ii) P/Q-type $Ca^{2+}$ channels and the SNARE complex co-immunoprecipitate with a monoclonal antibody against SNAP-25, and (iii) $\alpha_{1a}$ and SNAP-25 co-localize at the plasma membrane of ACCs [37]. Recently, to study the molecular basis of the functional coupling observed between the IRP and P/Q-type $Ca^{2+}$ channels in mouse ACCs, Álvarez and collaborators (2013) transfected mice ACCs 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, and the size of IRP exocytosis was markedly reduced. Therefore, the authors concluded that the synprint site of $\alpha_{1A}$ subunit is an important factor for the establishment of the functional coupling between the IRP and P/Q-type $Ca^{2+}$ channels [16] (see Fig. 1).

It is worth to mention that the synprint sequence is also present in N-type $Ca^{2+}$ channels [38]. Therefore, it is also possible that ACCs expressing this type of $Ca^{2+}$ channels have a contribution of N-type $Ca^{2+}$ current in the triggering of IRP exocytosis, as it was shown in the pheochromocytoma cell line MPC 9/3L [38, 39]. Moreover, other types of VDCC lacking the synprint sequence also may contribute to the rapid exocytosis evoked by short depolarizing pulses in ACCs. In this regard, Albillos and collaborators found in perforated patch experiments that R-type $Ca^{2+}$ current was highly efficient in triggering exocytosis [40]. Additionally 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 [41]. Finally, Robert Chow and colleagues found that complexin 2-null adrenal ACCs present a decreased and desynchronized evoked release in response to short depolarization [42]. They also identified a significant reduction in the vesicle pool close to the VDCCs, which was associated with the IRP. They proposed that complexin stabilizes the binding of synaptotagmin to the SNARE complex, which in turn promotes the coupling of vesicles to VDCCs [43].

## Physiological Relevance of IRP

When ACCs are stimulated with prolonged depolarizations or trains of brief depolarizations (i.e. action potentials) at high frequencies there is a buildup of residual cytosolic $Ca^{2+}$, and exocytosis is mainly dependent on the release of RRP vesicles that do not colocalize with VDCCs and on the transference of chromaffin vesicles from upstream pools [6, 8, 13, 44, 45]. This exocytosis is mostly asynchronous because $Ca^{2+}$ has to diffuse from the channel mouth to far locations [22, 46] and/or vesicles have to follow various maturation steps that delay exocytosis. This situation corresponds to what happens in stress conditions, where ACCs fire action potentials at frequencies above 10 Hz, and the consequent accumulation of residual cytosolic $Ca^{2+}$ promotes massive exocytosis irrespective of the location of chromaffin vesicles respect to VDCCs [6, 47].

But what happens under physiological resting conditions, when the firing frequency is low and $Ca^{2+}$ does not accumulate significantly between stimuli? Since IRP vesicles are closely coupled to VDCCs, $Ca^{2+}$ microdomains around channels has to be sufficient to induce their release [22, 44, 48]. In consequence, it is reasonable to expect that synchronous IRP exocytosis should be important at basal low frequencies. However, it was reported that IRP is recovered exponentially after total depletion with a time constant of 7–10 s [20], what is too slow to sustain a significant participation of IRP even at low frequencies. However, the fraction of vesicles released by a single action potential-like stimulus (ETAP, which represents approximately the 40% of IRP) is recovered after depletion with a time constant of 0.7 s [49]. This kinetics is fast enough to maintain synchronous exocytosis at action potential frequencies up to 0.5 Hz, what is approximately the physiological firing frequency at rest in ACCs.

We also analyzed the mechanisms responsible of fast ETAP recovery. We found that ETAP is consistently followed by a fast endocytosis process that completely compensates 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 or a peptide interfering with dynamin action severely delayed ETAP replenishment. Additionally, an effect in the same direction was found after RRP depletion. Therefore, we proposed a model where both the transfer of chromaffin vesicles from RRP and fast endocytosis allow rapid ETAP replenishment and the maintenance of secretion during basal firing frequencies in ACCs [49]. We concluded that ETAP, which is an IRP fraction, is able to sustain with high efficiency the secretion triggered by the small and localized $Ca^{2+}$ gradients produced by action potentials at basal frequencies in adrenal ACCs.

**Acknowledgements:** This work has been supported by the grants PICT 0524-2014 from Agencia Nacional de Promoción Científica y Tecnológica (Argentina) and UBACyT 2014-2017 from Universidad de Buenos Aires (Argentina).

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