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EDITORIAL

Old and emerging concepts on adrenal chromaffin cell stimulus-secretion coupling

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Abstract The chromaffin cells (CCs) of the adrenal medulla play a key role in the control of circulating catecholamines to adapt our body function to stressful conditions. A huge research effort over the last 35 years has converted these cells into the *Escherichia coli* of neurobiology. CCs have been the testing bench for the development of patch-clamp and amperometric recording techniques and helped clarify most of the known molecular mechanisms that regulate cell excitability, Ca^{2+} signals associated with secretion, and the molecular apparatus that regulates vesicle fusion. This special issue provides a state-of-the-art on the many well-known and unsolved questions related to the molecular processes at the basis of CC function. The issue is also the occasion to highlight the seminal work of Antonio G. García (Emeritus Professor at UAM, Madrid) who greatly contributed to the advancement of our present knowledge on CC physiology and pharmacology. All the contributors of the present issue are distinguished scientists who are either staff members, external collaborators, or friends of Prof. García.

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The early studies

Adrenal chromaffin cells (CCs) together with the sympathetic nervous system are the main sources of catecholamines that our body mobilizes for the "fight or flight" response during fear, stress, exercise, or conflict conditions. During the response, the body is prepared to achieve maximal strength and awareness by increasing heart work and blood pressure. Vasodilation and vasoconstriction are regulated in a way that the skeletal muscles and the heart receive more blood while peripheral and gastrointestinal blood supply is attenuated. Glucose is mobilized from the liver while bronchioles and pupils dilate to improve respiration and increase visual acuity.

CCs contribute massively to the fight or flight response by mainly secreting adrenaline into the bloodstream after the release of acetylcholine (ACh) from preganglionic splanchnic fibers. W. Feldberg was the first to identify ACh as the primary neurotransmitter triggering adrenaline and noradrenaline release [29] while W. W. Douglas coined the term "stimulus-secretion coupling" to describe the release of catecholamines following the activation of nicotinic receptors by ACh [28]. This latter also identified Ca^{2+} as the main extracellular ion involved in the secretagogue action of ACh.

Chromaffin cell physiology has been widely studied since then. Early studies (1965–1981) focused on the role of nicotinic (*nAChR*) and muscarinic (*mAChR*) receptors in regulating the CC response to ACh [27, 68] (see the reviews by Albillos and McIntosh, Inoue and Kao, and Criado in this issue). Great interest was also dedicated to clarify how vesicle secretion was regulated by extracellular Ca^{2+} flows [5] and which molecules, besides adrenaline and noradrenaline, were packed in the large dense core (LDC) secretory granules and released during activity [45]. It was evident that chromaffin cells were excitable cells like neurons and thus able to generate action potentials (APs) sustained by voltage-gated Na^{+} and

K^+ channels [6, 11] and that most of the Ca^{2+} required for the exocytosis entered the cell through not yet fully identified voltage-gated Ca^{2+} channels [10, 12].

Of great interest during this period was also the identification of the cytoskeletal protein components (*f-actin* and *myosin*) that are the major constituents of cytoplasmic microfilaments along which LDC vesicles move from inside the cell, where they are stored, to the plasmalemma where they are docked and fused [32, 67]. Of enormous interest was also the first report on the existence of the intravesicular protein *chromogranin A* (CgA) in the mid-1960s [7]. The initial idea was that CgA served as a colligative agent for reducing the osmotic forces resulting from the large accumulation of solutes in large dense core vesicles. Later, chromogranin B and chromogranin C (*secretogranin II*) have been added to the list that currently includes nine members [26].

Thirty-five years of amazing discoveries (1982–2017)

Ion channels, receptors, neurotransmitters, and gap junctions regulating chromaffin cell activity

As for other neuroendocrine cells, adrenal chromaffin cells gained greatly from the advent of the patch-clamp technique [38]. The approach allowed to identify the gating properties of a large number of Na^+, K^+, and Ca^{2+} channels that regulate AP firing and catecholamine secretion [30]. Meanwhile, Na^+, K^+, and Ca^{2+} channels where identified by DNA sequencing and newly available blockers, in CCs, it was possible to establish the presence of a voltage-gated Na^+ channel (Nav1.7) [31], several voltage-gated (Kv) [50, 62] and Ca^{2+}-gated K^+ channels (SK, BK) [55], and a number of voltage-gated Ca^{2+} channels (L, N, and P/Q-type) [33, 69]. The Madrid group of Antonio García was particularly active in these studies. It was the first to identify the key role of L-type calcium channels (Cav1.2, Cav1.3) in controlling catecholamine secretion in cat CCs [34] (see the review by Nanclares et al. in this issue) and to uncover variable densities of P/Q- (Cav2.1) and N-type (Cav2.2) in bovine, cat, rat, mouse, and human CCs that contribute differently to secretion [33]. Rat and mouse CCs express also R-type channels (Cav2.3) sensitive to SNX-482 [3, 47] and T-type channels (Cav3.2) that are effectively coupled to the secretory apparatus [15, 16]. The role of each ion channel in CC excitability is still debated but it is now clear that their expression and regulation are key factors to set the neuron-like firing modes of CCs [69] (see the review by Lingle et al. in this issue).

Besides muscarinic receptors, CCs were found to express a large variety of G protein-coupled receptors (GPCRs) that are autocrinally activated by the released products of the same cells [17, 23, 33]. This action was of great interest to understand CC function and boosted a massive work of several laboratories that brought to the identification of α- and β-adrenergic (see the review by Artalejo et al. in this issue), δ-opioidergic, P2y-purinergic, and GABA receptors (see the review by Alejandre-García et al. in this issue). Most of them are effectively activated by the neurotransmitter molecules released by CCs (ATP, opioids, A, NA) and induce a marked depression of Ca^{2+} currents. Here also, the Madrid group was determinant in demonstrating that the purified content of secretory vesicles (*soluble vesicle lysate*) when applied on bovine CCs had a potent voltage-dependent depressive action on N- and P/Q-type Ca^{2+} currents (Cav2.1, Cav2.2) that was prevented by mixtures of broad spectrum opioidergic and purinergic antagonists [2, 33]. L-type channels (Cav1.2 and Cav1.3) are also autocrinally modulated by β1- and β2-AR but the action is voltage independent and can be either depressive or potentiating [18]. Apart from the L-type channel upregulation by β-AR stimulation, most pathways of Cav channel modulation serve as a negative feedback inhibition to regulate catecholamine secretion in CCs [23].

CCs express also GPCRs selective for the pituitary adenylate cyclase-activating polypeptide (PACAP), a 38-amino acid peptide [70] co-released with ACh from preganglionic splanchnic nerve fibers during sympathetic stimulation [20, 72, 73]. PACAP is able to sustain catecholamine release from CCs even during sustained depolarizations and to lead to CC gene transcription [63]. Since PACAP and its receptors are broadly expressed in the central nervous system, in particular in the hypothalamic-pituitary-adrenocortical axis, the neurotransmitter is proposed as a "master regulator" of stress signaling throughout the nervous system [65] (see the review by Eiden et al. in this issue).

Since the first morphological observations on adrenomedullary cells, it was evident that CCs are in contact with each other and grouped in clusters of either adrenergic or noradrenergic cells [22]. These functional units are differentially innervated [43] and electrically coupled by gap junctions [49] forming an excellent model for studying the molecular components of cell-to-cell communication (see the review by Guerineau in this issue).

Vesicle exocytosis viewed through cell capacitance changes, amperometric recordings, and fluorescence microscopy

The advent of patch-clamp techniques allowed also an impressive breakthrough into CC function, allowing a direct measurement of the Ca^{2+}-dependent neurosecretion during cell stimulation. By measuring the increase of cell surface as *membrane capacity changes* (ΔC) during exocytosis, it was possible to correlate Ca^{2+} entry to the amount of vesicles that fuse and release catecholamines during stimulation [56]. Alternating pulses of Ca^{2+} loading and ΔC measurements made it possible to determine, with high-time resolution, key parameters such as the amount of "release-ready" vesicles, the

probability of vesicle release, and the quantal size of single secretory events in bovine CCs [35, 39]. In mouse CCs, ΔC recordings combined with Ca^{2+}-uncaging experiments also allowed to obtain a clear picture of the vesicle distribution at rest and during stimulus-secretion coupling with high-time resolution. Erwin Neher's group could resolve the presence of four different pools of vesicles in dynamic equilibrium among each other (see the review by Neher in this issue). A *reserve* (*depot*) pool, containing many vesicles (2000 to 4000) in slow equilibrium with an *unprimed* pool (UPP) of 650 vesicles, a *slowly releasable* pool (SRP), and a *ready-releasable* pool (RRP) of about 100 vesicles each were close to the membrane [60, 71]. Movements from the UPP to the SRP and RRP lead to vesicle *docking*, *priming*, and *fusion* and terminate with the emptying of the vesicle content. All these sequential events are common to neurons and CCs (for a review, see [40] and Dhara et al. in this issue) and are regulated by the formation of the four-helix SNARE complex (*syntaxin*, *synatobrevin*, and *SNAP25*) and by the interaction of SNARE with the priming protein Munc13-1 and the Ca^{2+} sensor *synaptotagmin* [60]. In this regard, the studies on CCs using the fast time resolution of ΔC recordings in combination with KO mice models have been crucial in identifying the role of each molecular player on the sequence of events regulating stimulus-secretion coupling (see the review by Cardenas and Marengo in this issue).

Studies on CC function were further boosted by adapting electrochemical methods to measure the oxidation currents generated by specific released neurotransmitters. Using carbon fiber microelectrodes, it was possible to demonstrate that pressure ejections of ACh induce brief spikes of oxidative currents associated with the release of catecholamines in bovine CCs [44, 74]. Amperometric and voltammetric recordings broadened the present knowledge of cell exocytosis. Cyclic voltammetry allowed identifying the type of biological amines released (*adrenaline*, *noradrenaline*, *histamine*, *or serotonin*) while amperometry helped resolve bursts of quantal secretory events during stimulus-secretion coupling and to distinguish the kinetics of vesicle fusion (*foot*) and neurotransmitter release (*amperometric peak*) during single events (for a review, see [8, 25, 66]). Bovine and mouse CCs have been the ideal cells for studying the role that SNARE-related and cytoskeletal proteins play on the regulation of vesicle transport, priming, and fusion using either amperometric recordings alone [37, 54] or in combination with whole-cell capacitance and Ca^{2+}-uncaging measurements [9, 57, 64]. Amperometry has been determinant also in demonstrating many key biophysical and pharmacological properties of exocytosis. Among them, it is worth recalling the well-accepted evidence that (i) fusion pore is permeable to catecholamines [19], (ii) secretion occurs in spatially localized microareas of CCs (*hot spots*) [36, 61], and (iii) PKG, PKA, and PKC are effective modulators of exocytosis [37, 46]. Of interest are also recent observations that the quantal size is regulated by VMAT and autoreceptors [21, 33] and vesicular pH regulates the kinetics and quantal size of chromaffin cell granules [14]. Amperometry has been also successfully employed to correlate changes in the kinetics of exocytosis and quantal size with changes in the content of other soluble species co-stored with catecholamines that contribute to granule homeostasis (see the review by Borges et al. in this issue).

Since the early observations that actin and myosin are the main components of neurofilaments in CCs, the present view of cytoskeleton protein function has progressively evolved. In the 1980s, the presence of filamentous actin (*F-actin*) was simply interpreted as a peripheral cortical barrier of proteins preventing vesicle access to the secretory sites [4, 13]. At the beginning of this century, with the availability of more advanced immunofluorescence techniques, it became apparent that F-actin also participated in vesicle transport and fusion in addition to its original "retentive" role [41, 53, 59]. Recent evidence on adrenal gland slices suggests that F-actin has an even more complex function than expected and that the traditional 2D primary cell culture arrangement usually employed for these studies does not accurately mimic the 3D in vivo environment (see the review by Gutiérrez et al. in this issue).

Chromaffin cells for testing new materials for amperometric microdevices

Because of the many electrochemical active species stored in the secretory granules, CCs are still the favorite cell model to test novel approaches and materials for fabricating electrochemical devices and lab-on-chips currently used for drug screening (see the review by Gillis et al. and the research article by Huang et al. in this issue). From the time when electrochemical detectors were firstly implemented as sensors for HPLC to date, their sizes have been progressively reduced to allow on-cell recordings as conventional amperometry [66], microelectrode fabrication for patch amperometry [1], and intracellular electrochemistry [52]. This tendency persists and miniaturization is currently used for studying secretory vesicles inside living CCs (see the review by Cans in this issue).

Chromaffin cells for studying cardiovascular and neurodegenerative diseases

Although recent research has precisely defined the roles of chromogranins (Cgs) in the storage of catecholamines [26], these proteins are shown to possess new key physiological roles. Cgs are now recognized to be the precursors of several active peptides involved in the regulation of glycemia, blood pressure, or innate immunity (see the review by Helle et al. in this issue). Recent studies have shown that CCs can produce granules in animals lacking CgA [51], thus proving that CgA is not critical for granulogenesis. Indeed, Cgs are sufficient to

trigger functional granule production and sorting even in non-secretory cells [26]. Interestingly, Cgs are now widely used as clinical markers for cardiovascular, gastrointestinal, and inflammatory diseases [42]. Since the pioneering work of D. T. O'Connor [58], the presence of large plasma concentrations of Cgs is a key diagnostic and prognostic tool of several tumors (see the review by Corti et al. in this issue).

CCs are also widely used as a model system for studying diseases. The presence of abnormal blood catecholamines is still the most reliable test for the diagnosis of pheochromocytoma. In addition, CCs are considered paraneurons and as such have been exploited for studying hypersympathetic activity and hypertension as well as neurotoxic mechanisms and neuroprotective drugs [48] (see the review by de los Ríos et al. in this issue). CCs are currently used to investigate the altered neurotransmission mechanism induced by Parkinson's and Alzheimer's diseases [24].

Future perspectives on chromaffin cells in health and disease

Despite the great advances of our acquaintance on the biology, biochemistry, physiology, pharmacology, and pathology of chromaffin cells described above, there are still many critical issues that remain unsolved and require future work. They are all of great interest, particularly in the view of the critical role that CCs play in the control of circulating catecholamines and other hormones during physiological stress conditions. In addition to this, it is worth mentioning that with the increased availability of transgenic animal models, mouse CCs have further attracted the attention of researchers in using these cells as a model system for studying *stimulus-secretion coupling*. Indeed, in mouse CCs, it is possible to combine excellent voltage-clamp recording of ion currents with high-time resolution whole-cell capacitance measurements and amperometric spike detection to obtain simultaneously, on the same cell, Ca^{2+} current injection and secretory event recordings in the forms of the number of vesicles fused (capacitance) and quantal release of catecholamines (amperometry). A condition that is unlikely in most neuroendocrine cells or neuronal presynaptic terminals.

This special issue of *Pflügers Archiv* contains a collection of review articles plus an original article that covers nearly all the key issues described above on chromaffin cell function. All of them highlight the past drawbacks and scientific improvements and indicate new future perspectives worthy of investigation. The authors are all well-distinguished scientists who are working on chromaffin cells and are good friends of Antonio García to whom this issue is dedicated.

The contributors of this special issue and many other colleagues, who were not invited only due to space limitation, meet every 2 years since 1982, when the 1st International Symposium on Chromaffin Cell Biology (ISCCB) took place in Ibiza (Spain). The ISCCB meetings are a great occasion for presenting, discussing, and advertising the new findings on chromaffin and its "sister" cells. The group met last August in occasion of the 19th ISCCB in Sheffield (UK) (https://www.sheffield.ac.uk/isccb) and the next ISCCB meeting will take place on January 2020 at the Indian Institute of Technology in Madras (India). We are looking forward to meeting you there!

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