PFLÜGERS ARCHIV – EUROPEAN JOURNAL OF PHYSIOLOGY 470.1 (2018): 1-6

DOI: https://doi.org/10.1007/s00424-017-2082-z

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

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Abstract

The chromaffin cells (CCs) of 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 in the *E. coli* of neurobiology. CCs have been the testing bench for the development of patch-clamp and amperometric recording techniques and helped clarifying most of the molecular mechanisms that regulate cell excitability, Ca^{2+} signals associated to secretion and the molecular apparatus that regulates vesicle fusion. This special issue provides a state-of-the-art on the many well-know 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 UNAM, Madrid) who contributed greatly to the advancement of our present knowledge on the CCs physiology and pharmacology. All the contributors of the present issue are distinguished scientists that are either staff members, external collaborators or friends of Prof. García.

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 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 cells 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, 69) (see reviews by Albillos & McIntosh, Inoue & 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, beside 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+} used 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 constituent of cytoplasmic microfilaments along which LDC vesicles move from the cell inside, where they are stored, to the plasmalemma where they are docked and fused (32, 68). Of enormous interest was also the first report on the existence of the intravesicular protein *chromogranin A* (CgA) in the mid-sixties (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 9 members (26).

35 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, 63) and Ca^{2+}-gated K^+ channels (SK, BK) (51, 56) and a number of voltage-gated Ca^{2+} channels (L, N and P/Q-type) (33). 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 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 CCs 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 (70) (see 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 were autocrinally activated by the released products of the same cells (for a review see (17, 23, 33)). This action was of great interest to understand CCs function and boosted a massive work of several laboratories that brought to the identification of α- and β-adrenergic (see review by Artalejo et al. in this issue), δ-opioidergic, P2y-purinergic and GABA receptors (see 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 opioidergic and purinergic antagonists (2, 33). L-type channels (Cav1.2 and Cav1.3) were also autocrinally modulated by β1- and β2-AR but the action was voltage-independent and could be either depressive or potentiating (18). Apart from the L-type channel up-regulation 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 for the pituitary adenylate cyclase-activating polypeptide (PACAP) that is a 38-amino acid peptide (71) co-released with ACh from preganglionic splanchnic nerve fibers during sympathetic stimulation (20, 73, 74). PACAP is able to sustain catecholamine release from CCs even during sustained depolarizations and to lead to CCs gene transcription (64). Since PACAP and its receptors are broadly expressed in the central nervous system, in particular in the hypothalamic-pituitary-adrenocortical axis, it is proposed as a "master regulator" of stress signaling throughout the nervous system (66) (see review by Eiden et al. in this issue).

Since the first morphological observations on adrenomedullary cells it was evident that CCs are in contact one to 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 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 CCs 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 (57). Alternating pulses of Ca^{2+} loading and ΔC measurements it was possible to determine, with high-time resolution, key parameters such as the amount of "ready-*releasable*" 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 allowed also to obtain a clear picture of the vesicles distribution at rest and during stimulus-secretion coupling with high time resolution. Erwin Neher's group could resolve the presence of four different pool of vesicles in dynamic equilibrium among each other (see review by

Neher in this issue). A *reserve (depot) pool,* containing many vesicles (2,000 to 4,000) 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, close to the membrane (61, 72). 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* (61). 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 the "stimulus-secretion" coupling (see review by Cardenas & Marengo in this issue).

Studies on CCs function were further boosted by adapting electrochemical methods to measure the oxidation currents generated by specific released neurotransmitters. Using carbon fibre microelectrodes it was possible to demonstrate that pressure ejections of ACh induce brief spikes of oxidative currents associated to the release of catecholamines in bovine CCs (44, 75). 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 resolving 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, 67)). 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, 55) or in combination with whole-cell capacitance and Ca^{2+}-uncaging measurements (9, 58, 65). Amperometry has been determinant also to demonstrate 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 micro-regions of CCs (*hot spots*) (36, 62) 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 an 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 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 is progressively evolved. In the eighties, the presence of filamentous actin (*F-actin*) was simply interpreted as a peripheral cortical barrier of proteins preventing vesicles 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 participated also to vesicle transport and fusion in addition to its original "retentive" role (41, 54, 60). 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 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 electrochemistry devices and lab-on-chips currently used for drug screening (see the review by Gillis & Carabelli 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 (67), microelectrodes fabrication for patch amperometry (1) and intracellular electrochemistry (53). This tendency persists and miniaturization is currently used for studying secretory vesicles inside living CCs (see review by Cans in this issue).

Chromaffin cells for studying cardiovascular and neurodegenerative diseases

Although recent research has precisely defined the roles of 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 glycaemia, blood pressure or innate immunity (see review by Helle et al. in this issue). Recent studies have shown that CCs can produce granules in animals lacking Cgs (52), thus proving that Cgs are not critical for granulogenesis. Indeed, Cgs are sufficient to trigger functional granule production and sorting even in non-secretory cells (26, 42). Interestingly, Cgs are now widely used as clinical markers for cardiovascular, gastrointestinal, and inflammatory diseases. Since the pioneering work of D. T. O'Connor (59), the presence of large plasma concentrations of Cgs are key diagnostic and prognostic tools of several tumors (see 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 diagnostic of pheochromocytoma. In addition, CCs are considered paraneurons and as such have been exploited for studying hyper-sympathetic activity and hypertension as well as neurotoxic mechanisms and neuroprotective drugs (48) (see 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 knowledge 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 KO and KI animal models, mouse CCs have further attracted the attention of researchers to use 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 forms of 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 cover 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 worth of investigation. The authors are all well-distinguished scientists who are working on chromaffin cell physiology 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 for space limitation, meet every two years since 1982, when took place the 1st International Symposium on Chromaffin Cell Biology (ISCCB) 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 meet you there!

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