Chromaffin Cells of the Adrenal Medulla: Physiology, Pharmacology, and Disease

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ABSTRACT

Chromaffin cells (CCs) of the adrenal gland and the sympathetic nervous system produce the catecholamines (epinephrine and norepinephrine; EPI and NE) needed to coordinate the bodily "fight-or-flight" response to fear, stress, exercise, or conflict. EPI and NE release from CCs is regulated both neurogenically by splanchnic nerve fibers and nonneurogenically by hormones (histamine, corticosteroids, angiotensin, and others) and paracrine messengers [EPI, NE, adenosine triphosphate, opioids, γ-aminobutyric acid (GABA), etc.]. The "stimulus-secretion" coupling of CCs is a Ca^{2+}-dependent process regulated by Ca^{2+} entry through voltage-gated Ca^{2+} channels, Ca^{2+} pumps, and exchangers and intracellular organelles (RE and mitochondria) and diffusible buffers that provide both Ca^{2+}-homeostasis and Ca^{2+}-signaling that ultimately trigger exocytosis. CCs also express Na^+ and K^+ channels and ionotropic (nAChR and $GABA_A$) and metabotropic receptors (mACh, PACAP, β-AR, 5-HT, histamine, angiotensin, and others) that make CCs excitable and responsive to autocrine and paracrine stimuli. To maintain high rates of E/NE secretion during stressful conditions, CCs possess a large number of secretory chromaffin granules (CGs) and members of the soluble NSF-attachment receptor complex protein family that allow docking, fusion, and exocytosis of CGs at the cell membrane, and their recycling. This article attempts to provide an updated account of well-established features of the molecular processes regulating CC function, and a survey of the as-yet-unsolved but important questions relating to CC function and dysfunction that have been the subject of intense research over the past 15 years. Examples of CCs as a model system to understand the molecular mechanisms associated with neurodegenerative diseases are also provided. Published 2019. *Compr Physiol* 9:1443-1502, 2019.

Didactic Synopsis

Major teaching points

1. During stress, chromaffin cells (CCs) from the adrenal medulla release a surge of catecholamines (CAs) [epinephrine (EPI) and norepinephrine (NE)] into the bloodstream, which serves to prepare the different organs of the body for the fight-or-flight response, to escape from danger and survive.

2. Classically, the secretory response of CCs is triggered by a rapid elevation of the Ca^{2+} concentration in the cytosol, mainly contributed by Ca^{2+} entry via voltage-activated Ca^{2+} channels that open upon membrane depolarization as a consequence of the activation of postsynaptic cholinergic receptors by the neurotransmitter acetylcholine (ACh) that is released from splanchnic nerve terminals. Pituitary adenylate cyclase-activating polypeptide (PACAP) co-released with ACh at the adrenomedullary synapse contributes to CA secretion from the adrenal medulla during stress.

3. CCs express many types of Na^+, Ca^{2+}, and K^+ channels that are also able to generate spontaneous "neuronal-like" tonic and burst-firing patterns that can drive a sustained "nonneurogenic" release of CAs upon stimulation by various secretagogues.

4. The Ca^{2+} signal due to Ca^{2+} entry is subsequently modulated by Ca^{2+} uptake and release in mitochondria and endoplasmic reticulum (ER). Intracellular Ca^{2+} controls

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Published online, October 2019 (*comprehensivephysiology.com*)

DOI:10.1002/cphy.c190003

granule movement to the plasma membrane, exocytosis and endocytosis, and couples cell activity to adenosine triphosphate (ATP) generation.

5. CAs, chromogranins, ATP, GABA, opioids, and other peptides are tightly packaged in secretory granules (SGs) that release their contents by fusion with the plasmalemma. In this process (exocytosis) several soluble NSF-attachment receptor complex (SNARE) proteins participate in regulating the formation of a membrane fusion pore and its subsequent expansion to release the granule content.

6. CAs are preferentially released from newly synthetized granules. Older granules may act as CA donors for newly formed granule reloading.

7. Secretagogues of the adrenal medulla stimulate both CA/peptide synthesis and release via "stimulus-secretion-synthesis coupling." CC excitability, Ca^{2+} channel currents and the ensuing secretory responses are regulated further by several receptors for opioids, PACAP, ATP, GABA, and various other neurotransmitters.

8. Altered cell excitability, Ca^{2+}-handling, and exocytosis defects have been reported in CCs from rodent models of diseases such as hypertension, Alzheimer's disease, Huntington's disease (HD), autism, and amyotrophic lateral sclerosis, suggesting that the defective proteins of the disease also have an impact on CC function.

Introduction

The adrenal glands are endocrine organs that reside above the kidneys in mammals and, as such, have also been called "suprarenal" glands. The inner part of the adrenal is the medulla, a tissue originating from the neural crest, and surrounded by the cortex. Both cortex and medulla contribute critically to the body's responses to stress. At the beginning of the 19th century, Alfred Kohn coined the term "chromaffin" for adrenal medulla catecholamine-containing cells, which can be stained by a chemical reaction to chromium (113). Since then, CCs have provided one of the most successful models for the study of nerve cells, especially for the stimulus-secretion coupling process and its regulation (394).

During intense fear, metabolic stress, exercise, or struggle, adrenal CCs release into the blood stream EPI and NE necessary for regulating the "fight-or-flight" response (96). These potent chemical messengers bind to their molecular targets, the adrenergic receptors, distributed throughout the entire organism, and prepare it for maximal muscle performance and motor responsiveness by increasing heart pumping strength and blood pressure, producing vasodilation in the blood supply of skeletal muscles and heart, and vasoconstriction in the skin and gastrointestinal vasculature. At the same time, mobilization of glucose from the liver is enhanced, while bronchioles dilate to optimize blood oxygenation and pupils dilate to improve visual acuity. Released CAs also promote relaxation of smooth muscle in tissues not required for a stress response such as the intestinal tract or the uterus (570).

The adrenal medulla performs these tasks, by virtue of its unique functional organization: Preganglionic sympathetic nerve fibers originating from cell bodies in the thoracolumbar segments of the spinal cord bypass the celiac ganglion without making synapses and form the splanchnic nerve, which innervates CCs directly. These synaptic terminals release ACh, that upon binding to postsynaptic nicotinic receptors in CCs, induces a rapid membrane depolarization. This triggers action potentials (APs) that allow Ca^{2+} influx, followed by rapid elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Ca^{2+} elevation then triggers the fusion of SGs with the plasma membrane and the exocytotic release of CAs. The neurogenic control of CCs permits a continuum of CA output, from a firing rate of splanchnic nerve impulses of about 1 Hz, sufficient to maintain resting cardiovascular, respiratory, and metabolic functions (247, 249, 506), to nerve fiber discharges at maximum rates equal or higher than 10 Hz producing a massive release of CAs during stress (569).

The first synapse described electron microscopically was the splanchnicoadrenomedullary synapse (139). Earlier, the storage of CAs within SGs of adrenal medulla was characterized (299). The concept of exocytosis was developed from the observation of the secretion of a highly charged, >70 kDa protein, chromogranin A (CgA), unlikely to be released by diffusion across cell membranes, from the adrenal medulla following splanchnic nerve stimulation (280, 673). Ca^{2+}-dependence of exocytosis was also demonstrated in the adrenal medulla (176), culminating in Douglas's concept of stimulus-secretion coupling (172), analogous to the muscle stimulus-contraction coupling.

CCs resemble neurons in many ways. Besides their synaptic input, they receive chemical signals both from their neighbors (paracrine signal) and from themselves (autocrine signal). Some of them are electrically coupled (261). Their excitable properties originate from a surprisingly wide variety of ion channels, comparable to those of many central neurons (389, 394). Thus, far from being passive followers of their neurogenic input, these cells can decode and integrate different types of extra- and intracellular signals to generate complex patterns of electrical and cytoplasmic second messenger signaling that culminate in the regulated release by exocytosis of potent chemical messengers, which include CAs, opioids and other peptides, ATP, and GABA.

The unique properties of CCs derive from their embryological origin: they belong to both the nervous and the endocrine systems (16, 628). Their neuron-like phenotype has been particularly valuable over more than 30 years of research to understand excitability, nerve impulse generation, synaptic transmission, regulated neurotransmitter secretion, and the actions of drugs that regulate central nervous system (CNS)

function (70). CCs have also been crucial for understanding the basic mechanisms of intracellular Ca^{2+} dynamics and neurochemical transmission through secretory vesicles or CGs. The CGs were first isolated from the bovine adrenal medulla and their releasable cargo analyzed (215). Most of the vesicular proteins involved in the secretory machinery were first definitively characterized in CCs (86, 163, 301, 583, 601). CCs, together with the pheochromocytoma cell line (PC12) isolated from a rat tumor of adrenomedullary origin in 1976 (257), have been the major cell types employed in the development and refinement of patch-clamp and amperometric recording techniques. These two independent but complementary electrophysiological approaches have been indispensable in clarifying most of the now-known molecular mechanisms that regulate cell excitability, Ca^{2+}-dependent secretion, and the molecular apparatus that regulates vesicle fusion (394, 667).

Starting with the seminal work of Levi-Montalcini and Unsicker (16, 628), showing that CCs can transdifferentiate into sympathetic neurons when exposed to nerve growth factor (320, 325), adrenal CCs have extended our understanding of neural development. Much of our present knowledge of the physiology and pathophysiology of neuropeptide and monoamine neurotransmitters stems from studies in CCs. More recently, CCs have been valuable in studies of neurodegenerative processes, tumorigenesis, and drug development (157, 435). This article aims at providing the reader with a broad view of both classical and recent advances concerning the molecular mechanisms regulating the secretory function of these remarkable cells, as well as their concerted role within the adrenal medulla as an endocrine organ responsible for integrating physiological responses to a range of homeostatic challenges.

The sympathoadrenal medulla connection

It is recognized that in higher organisms the brain regulates the "milieu interieur" (60) and body homeostasis (95). This regulation ensures that under normal conditions, blood glucose, oxygen, electrolyte concentrations, body temperature, blood pressure, heart rate, respiration, and blood flow to vital organs do not vary much or for very long. To maintain the integrity of the organism, the brain controls the inner world via feedback-regulated systems. Cannon introduced three concepts focused on the adrenal medulla that are well known and widely accepted: homeostasis, the "fight-or-flight" response, and the functionally unitary nature of the sympathoadrenal system. The autonomic nervous system has two divisions, parasympathetic and sympathetic. The sympathetic nervous system (SNS) comprises a large variety of cells. Thus, sympathetic neurons form the pre- and paravertebral ganglia and their axonal noradrenergic nerve terminals innervate and regulate most organs and blood vessels through the release of NE. A second SNS component is the collection of CCs within the adrenal medulla that, upon ACh and PACAP release from the splanchnic nerve, trigger the secretion of NE and EPI. CCs are also located in paraganglia close to large blood vessels and along sympathetic nerves that innervate pelvic organs (296, 366, 437). Finally, paraganglionic cells also include chemoreceptive type I cells of the carotid body, which are peripheral oxygen sensors.

Unique to the SNS is that a quite low firing frequency (less than 1 AP per second), is sufficient to maintain constant cardiovascular, respiratory, and metabolic functions of the body. Full activation during stress occurs when nerve fibers discharge at maximum rates equal or higher than 10 Hz (2, 50, 155, 515). The splanchnic nerve innervates the adrenal medullary CCs and is cholinergic. Through the release of ACh at the cholinergic-CC synapse, the human adrenal medulla secretes about 0.05 $\mu g\,kg^{-1}\,min^{-1}$ NE and 0.2 $\mu g\,kg^{-1}\,min^{-1}$ EPI (i.e. about 75% EPI and 25% NE), similar to the ratios reported in adrenal glands of cats, dogs, and calves (576). Thus, NE release from sympathetic nerve terminals throughout the body, and NE plus EPI release from the adrenal medulla maintains the basal circulating CA levels at about 250 and 100 $pg\,mL^{-1}$, (1.25 and 0.55 nM), respectively, for NE and EPI. This dual mechanism of stimulation represents a safety mechanism to maintain body homeostasis. The sympathetic neurons releasing NE locally at tissues they innervate and the adrenal medulla CCs releasing NE and EPI into the circulation augment the metabolic rate of practically every cell in the organism (250, 268).

Classical and modern histochemical, morphological, and functional studies have shown the existence of separate populations of NE- and EPI-secreting CCs in the adrenal medulla of mammals (140, 300, 456). The activity of these two cell subtypes seems to be regulated by separate neuronal pathways to the adrenal medulla. Nerve terminals on NE CC cells are morphologically distinct from terminals on EPI cells (259). Double-virus transneuronal labeling shows that the secretory activity of CCs is regulated by a set of neurons at the brainstem and the hypothalamus (323). Cumulative evidence supports the view that NE and EPI release from their respective adrenal medullary CCs is tightly regulated at three different levels, namely the brain, the spinal cord, and the CCs themselves. Thus, burst-pattern stimulation of the trigeminal nucleus caudalis in the cat brain stem preferentially releases EPI (59). The selective release of EPI is also regulated by autonomic areas in the cerebral cortex (648), hypothalamus (228, 540), and medulla oblongata (388, 438). Furthermore, the stimulation of other regions of the hypothalamus (228, 540) and medulla oblongata (438) selectively regulates the release of NE. This occurs probably because distinct preganglionic neurons innervate NE and EPI CCs (457). In addition, separate populations of preganglionic neurons, originating in different segments of the intermediolateral column of the spinal cord, differentially innervate CC subtypes, with the more cephalic preganglionic outputs innervating EPI cells and the more caudal ones innervating NE cells (187).

Thus, the different secretion of NE and EPI seems to be regulated by the distinct innervation of NE and EPI cells and

by the different input patterns that each cell type receives from the brain during stress. This may lead to selective recruitment of different subtypes of voltage-gated Ca^{2+} channels (Ca_V) expressed by CCs (240, 417). Thus, in two studies, different stimulation patterns caused differential secretion of EPI or NE; in bovine CCs stimulated with KCl, $Ca_V2.1$ (P/Q-type) channels were associated more tightly with EPI release, while Ca_V1 (L-type) channels were linked to selective NE release (400). The type of cholinergic receptor targeted by ACh may also condition the selective amine release; while nAChRs mediate the release of both CAs, muscarinic receptors preferentially mediate the release of EPI (173).

The differential secretion of NE and EPI may find a physiological and pathophysiological explanation in the different stressors or pathological situations that disrupt the organism's homeostasis. During the "flight-or-fight" response described by Cannon, an alarming acute stressful conflict causes a massive discharge of the sympathoadrenal axis, with the ensuing a large surge of NE and EPI release. Pupils and bronchioles dilate; heart rate, myocardial contraction, and blood pressure increase to switch blood to skeletal muscles that must increase the vigor of their activity; glucose is mobilized from muscle and liver to increase glycemia and metabolic activity is augmented in practically all cells of the organism. In this way, the organism is prepared as a whole to fight or run away from danger, two highly coordinated physiological responses.

From a pathophysiological perspective, the differential secretion of NE and EPI may also occur as a response to specific stressors (252). For instance, hemorrhage in cat causes a preferential release of NE, a situation in which vasoconstriction is required; however, insulin-induced hypoglycemia causes a selective EPI release because of its stronger capability to mobilize glucose from the liver (225). Other studies in the rat also demonstrate a preferential EPI release during hypoglycemia (597, 645). This contrasts with the preferential release of NE observed in rats exposed to cold stress (646). Histamine releases preferentially EPI through the activation of H1 receptors (68). Muscarinic stimulation also preferentially releases EPI (164).

Synthesis, Storage, and Metabolism of CAs

Synthesis and storage of CAs

Winkler and colleagues first introduced the concept of the "secretory cocktail" of CCs: the complement of proteins, peptides, cations, ATP, and CAs that are released from the CG as the major effector function of the CC of the adrenal medulla. Here, we describe briefly CA and protein/peptide biosynthesis and granule storage, as a necessary prelude to the more elaborate description of how this secretory cargo is released from the CC of the adrenal medulla to exert the unique physiological function of this essential endocrine organ.

The CA content of the CG depends on two processes: CA biosynthesis and CA vesicular transport and storage. The story of the discovery of the biosynthetic pathways of CAs is one of the most fascinating in neuroscience that, in spite of the enormous technical limitations was started in the latest 1930s of the past century. The detailed description of all the steps involved in the transformation of the amino acid tyrosine in the three natural CAs, DA, NE, and EPI, is beyond the aim of this article. A comprehensive description of the enzymes, cofactors, and kinetics associated with CA biosynthesis can be found elsewhere (647).

The adrenal medulla is the paradigm for the full CA synthetic pathway from the amino acid tyrosine to the final major CA product of the adrenal medulla, EPI. The conversion of tyrosine to DA occurs via the sequential actions of tyrosine hydroxylase (importantly, the rate-limiting enzyme in CA biosynthesis) to produce L-dopa and aromatic amino acid decarboxylase (AADC, a.k.a. dopa decarboxylase) to produce DA. DA is transported into the storage vesicle by vesicular monoamine transporters (VMATs) (see below) and converted to NE by DBH within the SG. Sequestration of DA into the SG accomplishes two important cellular goals: presenting DA to dopamine-β-hydroxylase (DBH) for its conversion to NE and removal of DA from the cytoplasm, where its metabolism by monoamine oxidase (MAO) creates potentially toxic oxidation products which, in the brain, have been implicated in the neurodegeneration of Parkinson's disease (293). The only synthetic step that must occur inside the CG is the conversion of DA into NE as DBH is an intragranular protein, in fact, one of the intragranular proteins that, along with CgA, helped to first establish the concept of exocytosis itself (62, 283). Once DA is converted to NE, NE diffuses from the SG and is converted in the cytoplasm to EPI [in cells that express the cytoplasmic enzyme phenylethanolamine-*N*-methyltransferase (PNMT)], which is then transported back into the CG and stored mainly in an osmotically inert compartment prior to exocytotic release. The proportion of NE/EPI varies depending on the species where it is measured; for instance, the proportion of EPI found in rats is around 90%, whereas this ratio drops to ≈73% in dogs, mice, and cows and 60% in cats and goats. The CA content of the human adrenal medulla is about 80% EPI. As described earlier in this article, EPI and NE are found mainly in separate CCs, that is most NE in PNMT-containing CCs is converted to EPI.

Almost all CAs are stored in CGs. This process occurs via transport by the VMAT. VMAT is at the heart of CC function because it is responsible for accumulation of EPI and NE into CGs at the concentrations required for hormonal action at distant targets, following exocytotic secretion into the general circulation. The free catechol concentration in the cytosol is 50 to 500 μM, comprising 10% CAs (458), while the measured concentration of catechols in CGs is 0.8 to 1.0 M, depending on the mammalian species (9, 454, 455). Thus, the CG concentrates CAs more than 10,000-fold against their concentration gradient, a thermodynamic feat whose accomplishment defines the function of the CC.

Understanding how VMAT transports CAs began with the work of Scarpa, Johnson, and colleagues (329, 330).

CA uptake and storage in CGs is a two-step process in which CA transport into the CG is followed by its osmotic inactivation via complexation with other granule components including ATP, chromogranins, and Ca^{2+} (282, 517, 629). To study the first process in isolation, workers employed CG ghost granules lysed to release their contents, and then resealed as empty vesicles (330). Accumulation of CAs into these "ghosts" was the model system in which Scarpa and Johnson worked out the now-famous formula for CA transport via proton exchange, where the proton-motive force $\Delta p = \Delta\Psi - [2.30\ RT/F \times \Delta p\mathrm{H}]$, with proton-motive force generated by the vacuolar ATPase of the CG membrane (331). The laboratories of Schuldiner and Henry made seminal contributions to the pharmacological characterization of amine uptake in the granule "ghost" system, identifying the approximate molecular weight of the transporter via reconstitution and explaining the amine-depleting effects of reserpine and tetrabenazine based on their inhibition of CA transport. It was deduced that there were two forms of VMAT—now known as VMAT1 and VMAT2—based on the universal uptake-inhibition properties of reserpine, but the species and tissue-specific inhibition by tetrabenazine (for reviews, see Refs. 286 and 559). The uptake of quaternary amines such as the Parkinsonogenic MPP+ confirmed the likelihood of amine transport through the carrier as a positively charged species (559). All of these pharmacological properties were used to allow the eventual cloning of VMAT1 and VMAT2 cDNAs in *in cellula* expression systems (208, 393). VMAT1 and VMAT2 are expressed in all mammalian species, while *Caenorhabditis elegans* contains only one VMAT, with the properties of VMAT2 (uptake of histamine and inhibition by tetrabenazine). This suggests that the evolutionary selection of the properties of VMAT1 allows it to function as a selective transporter for serotonin, to the exclusion of histamine, in the enterochromaffin cells of the gut that supply serotonin for utilization as a neurotransmitter in the enteric nervous system (179). Although VMAT1 was originally characterized as the "endocrine" VMAT and VMAT2 as the "neuronal" VMAT (664), the CG contains VMAT1, VMAT2, or both VMATs, depending on species and (in the rat), on the state of stress of the organism. Immobilization stress causes an upregulation of VMAT2 expression in the rat adrenal medulla, mainly within EPI cells (616). VMAT2, however, is the exclusive transporter for CAs in the CNS (209, 210). VMAT is a structurally highly conserved protein across species—both isoforms possess twelve vesicular membrane-spanning domains, with the residues contributing to specificity for histamine located to several discrete regions within the transporter that contributes to an amine-binding domain (226). The essential proton-exchange function of the VMATs is found even in bacterial proton antiporters, leading to the inclusion of the VMATs in an evolutionarily distinguishable superfamily called the toxin-extruding antiporters (TEXANS). This suggests that amine uptake into vesicles is a coopted function of toxin extrusion from single-celled organisms (560), and presaging the function of VMAT2 as a "vesicle-sequestering" protein protecting CA-containing CCs, as well as dopaminergic neurons, from oxidative damage caused by CA metabolites resulting from CA degradation by MAO and other oxidative enzymes (253).

The parallel process of biosynthesis and storage in SGs for the protein and peptide exportable products of the CC is discussed later. Synthesized as the so-called preprohormones in the rough ER, proteins of the SG are imported into the granule through the trans-Golgi network (TGN) and condensed into nascent CGs at that location through a process that is still ill-defined (312). The CC has been a model system for understanding the role(s) of proteolytic converting enzymes (PCs) as well as "trimming" and amidating enzymes in the production of bioactive peptides in neuroendocrine cells. For an overall historical perspective, see Ref. 672; for prohormone convertases, see Ref. 459; for carboxypeptidase processing enzyme (CPE), see Refs. 307 and 231; and for peptidylglycine α-amidating monooxygenase (PAM), see Ref. 192.

An important factor that largely contributes to the accumulation of amines is the vesicular ATP. ATP is present, and highly concentrated, in almost all secretory vesicles from all animal species (67). Although its physiological role in the concentration of CA was suggested since the 1960s and demonstrated *in vitro* (361, 603), it was not easy to test its role *in vivo*, as the removal of cellular ATP would mean the death of the cell. However, the cloning of the vesicular nucleotide transporter (VNUT, Figure 9) by the group of Moriyama (554) allowed the study of the contribution of vesicular ATP in the accumulation of CA. Thus, using siRNA against VNUT, Estevez-Herrera and collaborators demonstrated that the reduction of vesicular ATP largely reduced the accumulation of CA (214).

The result of amine uptake and storage, protein processing, and the collaboration of granins, ATP and Ca^{2+} in the production of the SG core is the "secretory cocktail" of the adrenal medulla: proteins, bioactive peptides, and small molecules, including CAs, GABA, and ATP that are released upon depolarization of the CC. The remainder of this article describes the molecular, cellular, and system biology of how the release of this secretory cocktail, via hormonal, neuronal, and inflammatory first messengers, affects mammalian organisms in both normal and pathophysiological states.

Metabolism of CAs

Two different mechanisms terminate CA activity: active cell membrane transporters and metabolizing enzymes. The final disposal of CAs and their metabolites is achieved by renal excretion. In neurons, 90% of released NE is taken back up into the cell by the NE transporter at the presynaptic membrane (193), while EPI released from the adrenal medulla is removed from the circulation by nonneuronal monoamine transporters (198). CAs taken up into neurons or CCs can either be metabolized by intracellular enzymes or sequestered into storage vesicles (194). Sympathetic neurons and CCs, contain monoamine oxidase (MAO), whose

principal substrates are, as the name implies, the CAs DA, NE, and EPI. The major deaminated metabolite of NE is 3,4-dihydroxyphenylglycol (DHPG) (194). The adrenal medulla contains catechol-*O*-methyltransferase (COMT). This enzyme is responsible for the intramedullary conversion of EPI into metanephrine and NE into normetanephrine (198). Over 90% of metanephrine and about 23% of normetanephrine in circulation are formed within the adrenal medulla from CA leaking from granules into the cytosol (198). The remainder of the circulating metanephrine derives from extra-adrenal metabolism by COMT after adrenal release of EPI.

Chromaffin Cell Excitability

What makes CCs excitable? The ion channels controlling the resting potential and action potential generation in CCs

As explained above, adrenal CCs release CA into blood circulation in response to sympathetic stressful stimuli. Typically, CA secretion is regulated neurogenically by the activity of the splanchnic nerve whose nerve terminals in the adrenal medulla release ACh sufficiently close to CCs to induce postsynaptic responses well above the threshold to activate *all-or-none* APs (303). This excitatory response is associated with the activation of nicotinic excitatory postsynaptic currents (48, 304, 334) that cause cell depolarization and AP trains of the same frequency as splanchnic nerve stimulation, with no evidence of fatigue or desensitization when stimulated at low rates (0.3–3 Hz) (303). In this way, CCs act as "relay elements." They couple splanchnic nerve activity to CA secretion by generating AP trains that trigger Ca^{2+} entry through voltage-gated Ca^{2+} channels and regulate the Ca^{2+}-dependent cascade of events leading to neurotransmitter release.

To accomplish this task, CCs contain a "palette" of ion channels that is as rich as that found in neurons (389), appearing even excessive for a "relay element" that should passively follow the neural input. Despite the central importance of neurogenically evoked release of CAs (155), it is possible that the ion channel "palette" can also sustain an *intrinsic electrical activity* of CCs that, under humoral conditions, may contribute to characteristic forms of CA secretion. Consistent with this, recent work on mouse models reveals that mouse CCs exhibit several voltage patterns of intrinsic excitability, including a slow-wave burst activity that may be significant for CA secretion (12, 260, 263, 426, 434, 633).

In this section, we will describe all the ion channels expressed by CCs that contribute to the resting membrane potential and generate the patterns of APs and slow-wave burst activity under physiological and pathological conditions. Table 1 summarizes all the ion channels that will be described and that are proposed to play specific roles in the function of CCs.

Na^+ channels (Nav1.3 and Nav1.7)

CCs of most mammalian species exhibit robust voltage-dependent Na^+ (Nav) currents (222, 407, 633) that are entirely tetrodotoxin (TTX)-sensitive. Na^+ currents in CCs start activating at around −35 mV and reaches maximal amplitude at −10 mV in 2 mM extracellular Ca^{2+}. They activate in less than 1 ms and inactivate quickly within 5 ms at 0 mV. At this potential, the AP reaches its maximal rate of rise (dV/dt_{max}) and decay ($-dV/dt_{max}$) before and after reaching its peak amplitude between +20 and +40 mV in mouse CCs (51, 633). Recovery from full inactivation of Nav channels is relatively fast and complete at −90 mV (90% in 100 ms) and at resting potentials of −50 mV (70% in 100 ms) (633). In this way, the Nav channels of CCs are designed to sustain the fast response to sympathetic nerve stimulation repeated at its maximal rate of 20 Hz with no decay of AP amplitude (303).

Na^+ currents in CCs have been reported to be carried by Nav1.7 channels (358, 654, 655), but evidence on this point is incomplete. Recent work, using quantitative PCR and western blots, suggests a contribution of both Nav1.3 and Nav1.7 in MCCs, with a preponderance of the Nav1.3 isoform (633). However, the voltage dependence of both activation and steady-state inactivation of mouse Nav currents is consistent with one type of sodium channel (633). Based on the studies of heterologously expressed Nav1.3 and Nav1.7, both channels share similar functional properties, but the voltage at which Nav1.3 is half inactivated is shifted to the right compared to that for Nav1.7 (146, 297, 638). Thus, if both isoforms are expressed in mouse CCs in similar proportions, the Nav currents should exhibit double rather than single Boltzmann inactivation functions. However, this is not the case in either rat or mouse CCs (633). Thus, Nav currents seem most consistent with a dominant Nav1.3 channel in MCCs. Indeed, preliminary results using Nav1.3 KO mice confirm that Nav current is completely lost in MCCs (Chris Lingle, personal communication) (389). Interestingly, Nav1.3 channels have strong structural homologies to Nav1.1 and Nav1.2, which are the Nav channels of brain neurons implicated in AP initiation, AP conduction, and repetitive firing (117).

Ca^{2+} channels (Cav1, Cav2, and Cav3)

Following the early patch-clamp recordings of voltage-dependent Ca^{2+} currents (32, 222, 309), the gating properties, pharmacological diversity, and modulation of these currents in CCs have been the subjects of extensive investigation using different CC species (7, 30, 31, 35, 36, 74, 98, 103, 121, 136, 236, 404, 406, 411, 417, 419, 427, 484, 495, 509, 510, 542, 575, 634). Nearly all known subtypes of high-voltage-activated Ca^{2+} current, including Cav1 (L-type) and Cav2 (P/Q-, N-, and R-type) channels have been described in rat (523), mouse (11, 417), cat (7), cow (30, 411) and human (510) CCs. Cav3.2 T-type Ca^{2+} channels are also expressed, particularly during immature stages and stressful conditions preserving their particular low-voltage range of activation

and fast-inactivating kinetics in rat and mouse CCs (74, 103, 107, 108, 298, 379, 479).

The expression density of Cav channels varies remarkably among animal species studied (human, bovine, pig, cat, mouse, and rat; 224), cell conditions, and development. In adult animals, L-, N-, P/Q-, and R-type channels are the dominant species (240, 262, 426, 427). They shape AP waveforms and regulate CA secretion and vesicle retrieval. Despite the many reports, there is still no convincing proof of a specific colocalization of any of the expressed Cav channel subtypes with the secretory apparatus. Each channel type controls CA release with the same linear Ca^{2+}-dependence (30, 100, 103, 206, 248, 352, 417, 543, 609, 627). Why do simple relay elements like CCs need so many different Ca^{2+} channel types for their task? A possibility is that depending on their different sensitivity to voltage, external signaling, and intracellular second messengers, the many expressed Cav channel types (see Table 1) permit compensatory paracrine and autocrine responses to regulate the proper CA release during different CC functioning conditions. The large number of Cav channels also derives from the dual role (chemical and electrical) that Ca^{2+} channels exert in the control of both CA release and AP firing (426, 631, 632).

Cav channel contributions to AP firing are conditioned by the voltage dependence of activation of the different Cav channel isoforms. Cav2.1 (P/Q-), Cav2.2 (N-), and Cav2.3 (R-type) channels activate at significantly more positive voltages than Cav1.2 and Cav1.3 (L-type) channels and therefore contribute mostly to the fast AP upstroke. Cav1.2 and Cav1.3 channels activate at more negative potentials and thus contribute to the slow phase of depolarization between consecutive APs (interspike potential). It is also important to notice that L-type channels are expressed at high densities in CCs. They inactivate slowly and carry about half of the total current in mouse, rat, and cat CCs (46, 121, 240, 428) and, most likely, also in human CCs (see Ref. 631). For this reason, they pass sufficient currents at very negative potentials and are thus favored to set the "pacemaking" current regulating the repetitive AP firing of CCs near resting potential. Looking more closely at the biophysical properties of these two L-type

Table 1 Na^+, Ca^{2+}, and K^+ Channel Types Expressed in CCs

Current	Channel type	Function	Animal	References
INa	Nav1.3	AP upstroke	Mouse	(389, 633)
	Nav1.7		Rat	(358, 654, 655, 681)
ICa	Cav1.2 (L)	AP upstroke, pacemaking, neurosecretion	Mouse, rat, bovine, human	(46, 429)
	Cav1.3 (L)			(429, 632, 635)
	Cav2.1 (P/Q)	AP upstroke, neurosecretion	Mouse, rat, bovine, human	(12, 30, 35, 240, 418)
	Cav2.2 (N)			(12, 30, 35, 240, 418)
	Cav2.3 (R)			(11, 121, 418)
	Cav3.2 (T)	AP threshold, neurosecretion	Mouse, rat	(104, 107, 427)
IKCa	BK (Slo1)	V_{rest}, burst generation, AP repolarization	Mouse, rat	(429, 434, 436, 468, 632)
	SK1-3	V_{rest}, burst duration, burst repolarization	Mouse, rat	(436, 468, 504, 635)
IKv	Kv1-3	V_{rest}, AP repolarization	Bovine, rat, mouse	(564)
	Kv4 (IA)	V_{rest}, AP repolarization, AP trains	Bovine, rat, mouse	(564)
	Kv7 (KCNQ)	V_{rest}, AP repolarization, burst generation	Bovine, mouse, rat	(660)
	Kv11 (ERG)	V_{rest}, AP repolarization	Mouse, rat	(263)
IK_{ATP}	Kir6.1 Kir6.2	V_{rest} repolarization during hypoxia	Rat	(73, 550)
IK	K2P (TASK)	V_{rest}, pH sensitive	Mouse, rat	(260, 316)

channels, it appears evident that Cav1.3 possesses the proper parameters to act as a pacemaker channel. Data derived from heterologously expressed channels indicate that (i) Cav1.3 activates with steeper voltage dependence and at more negative voltages than Cav1.2 (362, 679) and (ii) Cav1.3 has slower and less-complete voltage-dependent inactivation as compared to Cav1.2 (362).

Given the lack of specific blockers for either Cav1.2 or Cav1.3 (both are nifedipine-sensitive) in CCs, the dominant role of Cav1.3 as a pacemaker channel could only be revealed using the Cav1.3 KO mouse ($Cav1.3^{-/-}$) (516). Previous studies on the role of L-type channels in CC pacemaking showed that these channels could pass 10 to 20 pA of current in WT MCCs at rest, and nifedipine could either decrease the firing frequency or block the spontaneous firing (426, 428). A subthreshold inward Ca^{2+} current of this size passing through the high input resistance of CCs (3–5 GΩ) (222, 428, 429) is sufficient to generate pacemaker potentials of 10 to 20 mV in amplitude to drive the cell from resting (−50 mV) to the threshold of the AP upstroke (∼−30 mV). Deletion of Cav1.3 drastically reduces the amplitude of this pacemaker current and the fraction of MCCs firing spontaneously (429). Loss of Cav1.3 (i) decreases markedly the level of the nifedipine-sensitive currents driving spike generation, (ii) raises the rheobase (the minimal amount of current required to elicit a train of APs) from 4 to 6.6 pA, and (iii) decreases the extent of spike frequency adaptation during sustained current injections (635). In conclusion, Cav1.3 in CCs plays a key role in the control of AP firing either spontaneous or evoked during step depolarization. Compared with other neuronal pacemaker channels that carry mostly Na^+ currents, like the hyperpolarization-activated cation channels (HCN) (502), the persistent (143) and resurgent (527) Na^+ channels, and background channels (409), Cav1.3 appears unique and worthy of being further studied in cardiac cells and brain neurons.

Ca^{2+}-dependent SK and BK potassium channels

CCs were among the first cells from both rat and cow in which Ca^{2+}-dependent small- and big-conductance (SK and BK) potassium currents were recorded (29, 408, 434, 436, 467, 468). SK currents are voltage independent and blocked by the bee-venom toxin apamin (319). They activate robust outward currents following the release of cytosolic Ca^{2+} stores (467). Single SK channels have small K^+ conductance and are half activated at ∼0.7 μM cytosolic Ca^{2+} (503). Mouse CCs express all the identified SK channel subtypes: SK1, SK2, and SK3 (635). SK are open at any potential if sufficient cytosolic Ca^{2+} is available, and therefore they contribute to setting the CC resting potential. Their current builds up between successive APs and sets the basal frequency of spontaneous repetitive firing. In fact, block of SK channels by apamin usually increases the spontaneous firing rate of CCs, suggesting that there is sufficient SK activation between spontaneous APs to prolong the interspike interval (ISI) (635). SK channels respond to small and persistent increases in cytosolic Ca^{2+} (218). In this way, they play a negative feedback role in response to Ca^{2+} influx and set the firing frequency in resting CCs. SK currents build up markedly during high-frequency firing or prolonged bursts and help terminate the firing or the burst (635). More specifically, SK channels are the main K^+ channel types that activate near rest, which is with mild depolarization, or during the ISI, with a degree of activation proportional to the amount of Ca^{2+} entering the cell during the repeated APs or a burst. In this way, SK channels act as an effective "brake" on firing cells, to promote more regular firing and adapting the AP for constant maximal amplitude during sustained cell activity.

BK channels are voltage dependent. They activate following cytosolic Ca^{2+} elevation, but their probability of opening depends on the degree of cell depolarization. BK channels are also less sensitive to Ca^{2+} with respect to SK, requiring ≥10 μM cytosolic Ca^{2+} to be activated (218). Given the diversity and multiplicity of intracellular Ca^{2+} buffering systems, and their tight regulation, such high concentrations occur only within Ca^{2+} "nanodomains" near Ca^{2+} sources, that is in the vicinity of Cav channels (218). This implies that BK channels are localized near a Cav channel, and usually they are colocalized with more than one Cav channel (522, 632). In CCs, upon sufficient Ca^{2+} elevation and depolarization, the BK current is typically the most prominent outward current in CCs of all mammalian species (408, 429, 434, 468). This greatly conditions the shape of single APs that exhibit a marked afterhyperpolarization (AHP) during the falling phase of the action potential, depending on the number of BK channels open upon cell repolarization. In CCs, BK currents can be either inactivating (BK_i) or noninactivating (BK_s) (429, 434, 468, 522) in varying degrees, from completely inactivating to completely noninactivating.

BK channel inactivation rate in CCs depends on the presence of a regulatory β2 subunit (661, 676) whose cytosolic N-terminus mediates a pore occlusion type of inactivation (677). Genetic ablation of β2 subunit expression results in complete loss of inactivation of BK current in mouse CCs (434). The functional role of BK channel inactivation *per se* remains unclear (598). BK channel inactivation is very slow (>35 ms, when 2 β2 subunits are bound to the channel); thus, during trains of APs at 10 Hz, there is little cumulative BK channel inactivation (167, 434). However, besides producing inactivation, the regulatory β2 subunit also shifts the range of channel activation to more negative voltages (676). With a full set of four β2 subunits bound, the activation of BK channels shifts by about 60 mV to more negative voltages with 10 μM cytosolic Ca^{2+}. This shift is critical for the effective range of channel gating and results in different AP firing patterns between CCs with BK_i or BK_s currents (434, 581, 598). In the absence of any β2 subunit bound, BK channels will activate less at a given Ca^{2+} (434), causing an increased AP amplitude, slowed AP repolarization, and reduced AHP. This, in turn, slows the rate of recovery of Nav channels from inactivation and reduces the contribution of Nav channels to the next AP. This, eventually, results in AP

block after sufficient cumulative Nav inactivation (390). How these properties of BK channels affect burst firing will be considered in the following sections.

Voltage-dependent Kv (Kv1–3, Kv4, Kv7, and Kv11), K_{2P} TASK, and K_{ATP} potassium channels

CCs express a variety of voltage-gated Kv channels that mainly control the resting potential, the AP repolarization phase, and the mode of AP firing (tonic vs. burst) of the cells. Bovine rat, and mouse CCs express Kv currents that are either slowly (Kv1–3) or rapidly inactivating (Kv4; I_A current) (429, 436, 468, 564). Kv4 currents are prominent in bovine CCs and possess activation/inactivation characteristics that suggest that they may play a key role in the regulation of repetitive AP firing frequency during sustained stimulation (564).

CCs also express Kv7 (KCNQ; M-currents) and the Kv11 (ERG) channels. Kv7 is highly expressed in cardiac cells, postganglionic sympathetic neurons, and brain and blocked by mAChR activation through a membrane-delimited PIP_2 depletion mechanism mediated by Gq/PLC-β [for review, see (161)]. Kv7 are "low-threshold" K^+ channels that are already open at rest, activate very slowly with membrane depolarization, and do not inactivate during prolonged depolarization. In bovine CCs, there is evidence of an M-current inhibited by histamine (660). The existence of Kv7 in CCs, along with mAChRs, suggests a role for Kv7 channels in cell depolarizations induced by mAChR activation. However, data on this issue do not lead to a firm conclusion on this point (489). This derives most likely from the existence of different isoforms of mAChRs and different coupling to Kv7. In MCCs, muscarine causes either hyperpolarization plus a depolarization (31%), a depolarization alone (30%), hyperpolarization alone (21%), or no effects (18%). In contrast, blockade of Kv7 channels by XE991 causes a sizeable depolarization in 100% of the cells, followed by an increased firing frequency or a switch of spontaneous firing from tonic to bursts (402) (I. Méndez-López, A.G. García, and E. Carbone, unpublished observations). ERG or Kv11 channels have been identified in rat CCs (263). Block of Kv11 by the selective blocker WAY-123,398 causes marked cell depolarization, increased spontaneous firing frequency, and even burst firing in some cases, indicating that Kv11 channels may have an important role in regulating CC excitability (263).

Rat and mouse CCs also express the pH-sensitive K^+ channels TASK-1 and TASK-3 that are two-pore "leak" channels (K_{2P}) contributing to the membrane potential and firing activity of cells at rest (260, 316). In rat CCs, TASK-1 channels are blocked by the activation of mAChRs, and thus are postulated to be targets of the mAChR stimulatory action that leads to increased CA secretion (316). In mouse CCs, blockade of TASK-1 channels by the selective blocker A1899 and BK channels by paxilline mimics the effect of lowering the extracellular pHo causing cell depolarization, burst firing, and increased CA release (260). Given the expression of TASK channels, MCCs act as "pH sensors" which trigger an effective physiological response that compensates for the acute acidosis and hyperkalemia generated after vigorous exercise and muscle fatigue (441).

CCs also express K_{ATP} (Kir6.1 and Kir6.2) channels (73, 375, 550, 615). As in other cells, these channels are expressed to protect the cell during low O_2 pressure (PO_2) conditions that lower cytoplasmic ATP. In rat CCs, K_{ATP} channels are effectively activated during acute hypoxia, most likely due to the transient reduction of ATP at low PO_2. The involvement and upregulation of these channels is evident in rat CCs when acute hypoxia is tested in the presence of the K_{ATP} channel blocker glibenclamide. The addition of glibenclamide has no direct effect on K^+ current but enhances the inhibitory action of acute hypoxia on K^+ currents (550), suggesting that K_{ATP} channels are recruited only in response to low O_2.

The neuron-like spontaneous action potentials: tonic versus burst firing

In CCs, like in neurons, spontaneous firing takes place when sufficient inward current is driven by small voltage perturbations near resting potential. Critical for triggering spontaneous AP oscillations is the net balance between inward (leak, Na^+, and Ca^{2+}) and outward K^+ currents near resting potential. When the inward depolarizing current exceeds the outward current, a more positive unstable potential is reached at the start of the "negative conductance" region of the IV characteristics (680). At this unstable potential, any small depolarization causes a small inward current that depolarizes the cell further and triggers a train of APs. Subthreshold spontaneous membrane potential oscillations occur easily if the cell possesses high input resistance (3–5 GΩ) and is equipped with sufficiently large densities of Nav and Cav channels that activate readily at low voltages as in CCs (222, 418, 428). An additional critical requirement for driving spontaneous APs in slowly firing cells (~1 Hz) is the presence of a weakly inactivating inward current that sustains the slow pacemaker potential. Spontaneous firing is rather variable in CCs regardless of whether the cells are isolated and plated in culture or in adrenal gland slices (for a review, see Refs. 389 and 631). In many CCs, the repetitive firing is "irregular," that is spikes occur at a variable frequency (Figure 1A). In mouse CCs, the degree of regularity is somehow correlated with spike frequencies. Fast spiking cells are typically more tonic and display "regular" repetitive firings (Figure 1B).

As in neurons, a small fraction of CCs can also undergo spontaneous bursts at normal physiological conditions (434, 633). Burst firing occurs as trains of APs on top of a sustained plateau potential of variable duration (Figure 1C). The burst terminates with a robust AHP whose amplitude sets the duration of the ISI. Deeper AHPs generate longer ISI. The origin of this "neuron-like" bursting mode in CCs is largely unknown, but one can speculate that it derives from a combination of accumulated BK- and Kv-channel inactivation and sizeable slowly inactivating Na^+ and Ca^{2+} currents that sustain the plateau potential and part of the spike amplitude.

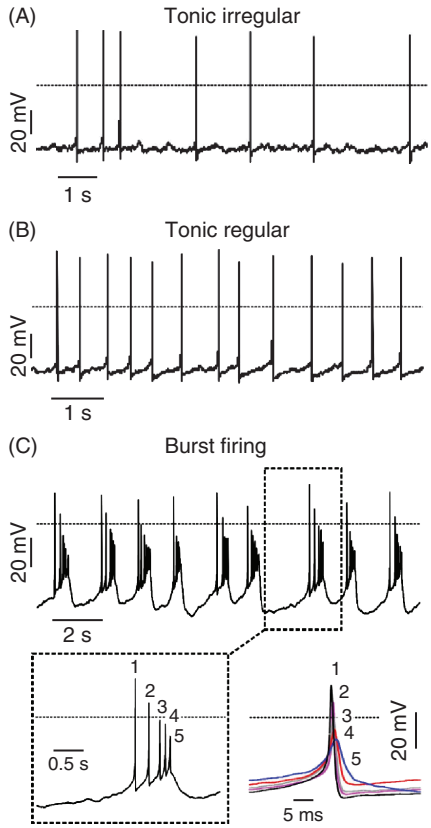

Figure 1 Different firing modes of spontaneously active chromaffin cells. (A, B) Spontaneous AP trains recorded from two different rat CCs displaying "irregular" and "regular" tonic firings, respectively. (C) Spontaneous AP trains exhibiting slow-wave bursting recorded from a mouse CC. Below is shown a single burst at an expanded time scale (dashed rectangle) and the overlap of consecutive APs within a burst. Numbers indicate the sequential position in the burst. Adapted, with permission, from Vandael DH, et al., 2015 (633).

Any reduction of the K^+ currents activated during the first AP attenuates the AHP to less negative values (plateau potential) where Cav and Nav channels can sustain the series of AP during bursts. Several ion channels contribute to set the plateau potentials and spike amplitudes. The key issue is to uncover how the degree of expression, gating properties, and sensitivity to endogenous and exogenous modulators of these channels may alter the equilibrium between repetitive and burst-firing modes in CCs.

Tonic firing in CCs

The first intracellular and extracellular recordings of spontaneous firing activity in rat CCs were reported almost 50 years ago (61, 76). In these studies, the emphasis was on the identification of the current components that participate in nerve-evoked depolarization and could influence the Ca^{2+} influx required for CA secretion. Subsequent reports confirmed that CCs can generate APs when stimulated and that they fire spontaneously both *in situ* or when isolated from the glands (47, 263, 467). In some cases, they exhibited considerable rhythmic complexity suggestive of bursting (263). The existence of a spontaneous (nonneurogenic) firing activity was compelling and received strong experimental attention. The leading idea was to identify the resting currents that control the timing of repetitive AP firing, after having observed that the block of L-type channels by 3 μM nifedipine reduced or fully blocked the spontaneous activity of mouse CCs (426, 428). Given this, the primary issue was to determine whether the slowly inactivating Cav1.3 channel was the main L-type channel responsible for the spontaneous firing activity. This could be done using the Cav1.3 KO mouse generated by the Striessnig laboratory (516). By comparing the ionic conductances and firing properties of WT and Cav1.3 KO mice, it was possible to show that both a Cav1.3-mediated inward current and a BK-mediated outward current are active during the interspike interval and the initial rising phase of an individual AP (429). The two currents balance each other with a slight predominance of Cav1.3 that drives the spontaneous firing. The time course of the subthreshold inward Cav1.3 current became evident in WT cells after blocking BK channels with 1 μM paxilline or 5 mM TEA, while this current was clearly absent in Cav1.3 KO cells.

A dominance of subthreshold Cav1.3 currents in regulating AP firing is also evident when studying their functional coupling with SK currents and how the coupling effectively adapts sustained AP trains during prolonged cell depolarizations. The Cav1.3-dependent SK currents that build up during the interspike intervals in WT cells lead to spike frequency adaptation that is strongly attenuated in Cav1.3 KO cells (635). Low adaptation ratios due to reduced SK channels activation associated with Cav1.3 channel deficiency prevent the effective recovery of Nav1.3 channels from inactivation. This promotes a rapid decline in AP amplitudes and facilitates early onset of depolarization block following prolonged stimulation. Thus, in Cav1.3 KO mice, both the spontaneous and evoked AP firing are significantly altered.

The involvement of Cav1.3 in regulating AP firing in mouse CCs is also supported by the altered firing properties observed in mutated CCs in which the C-terminal automodulatory (CTM) domain is interrupted with a hemagglutinin tag (556). The CTM domain regulates the Ca^{2+}-dependent inactivation (CDI) of the channel and the replacement of the "distal C-terminal regulatory domain" (DCRD) with an HA-epitope generates a mouse ($Cav1.3DCRD^{HA/HA}$) that displays faster CDI. Indeed, the mutated CCs exhibited L-type currents with faster inactivation and reduced CDI. Spontaneous cell firing was significantly altered. An increased percentage of cells lost their spontaneous activity due to their more negative resting potential (silent cells), and evoked AP bursts had increased firing frequency due most likely to the reduced number of SK channels activated by the accelerated CDI of $Cav1.3DCRD^{HA/HA}$ channels (556). In conclusion, łoss or mutations of Cav1.3 channel gating induce significant changes in the spontaneous and evoked CC activity, suggesting a key role of these L-type channels in the generation and maintenance of AP firing.

Slow-wave bursting in CCs

Slow-wave bursting activity is an intrinsic firing pattern that can be recorded in 15% of WT MCCs either grown in culture (633) or in adrenal medulla slices (434). Burst firing may become dominant when CCs need to release CAs to sustain several physiological responses such as acute acidosis and hyperkalemia during heavy exercise and muscle fatigue (441) (see below).

AP bursts have variable shapes. In general, an initial AP of usual amplitude exhibits a slow and reduced repolarization. The slow repolarization leads to a depolarized plateau potential upon which a series of truncated, presumably Ca^{2+}-dependent secondary, APs develops before terminating with a strong repolarization. The number of secondary APs varies from burst to burst, but it is likely the amplitude and duration of the plateau potential that determines the amount of Ca^{2+} entry during the burst. Given that a slow-wave bursting lasts more than 300 ms and the plateau potential varies between −30 and −20 mV, the quantity of Ca^{2+} entering a cell is likely to be more than an order of magnitude larger than the Ca^{2+} entering during a single AP. This is far more than the Ca^{2+} entering by simply increasing the frequency of firing from 1 Hz to its maximal value (20–30 Hz). This implies that any cell manipulation, ion channel blocker, or channel agonist able to switch the spontaneous firing of a CC from tonic to bursting can induce a significant Ca^{2+} overload that boosts massive CA release during stress or perhaps some ongoing pathological condition (i.e. hypertension). In this view, an increased percentage of burst-firing CCs could be a marker of an increased functionality or a pathological state for the adrenal CCs.

It is of key importance to understand how Na^+, K^+, and Ca^{2+} channels contribute to the generation of slow-wave bursting. Figure 2 shows the time course of Nav, Cav, Kv, BK, and SK currents during burst firing using as voltage-clamp commands three different AP bursts previously recorded in current clamp (see Ref. 633). It is evident that Nav, BK, and Kv currents are maximal during the first and second AP, while they decrease rapidly during the remaining APs (Figures 2A, 2B, and 2D). At the end of the burst, their contribution falls drastically. Quite different is the time course of inward Cav currents that decreases slowly during the burst but keep contributing significantly to Ca^{2+} entry almost up to the end of the burst (Figures 2C and 2D). Conversely, the SK currents build up progressively during the burst to reach a critical size that terminates it (Figures 2C and 2D).

Recent studies have highlighted cell manipulations that enhance or unmask this slow-wave burst (260, 434, 633). In one case, reducing Nav1.3 channel availability by either slow depolarizations that induce partial Nav channel inactivation or application of low concentrations of TTX to reduce the number of active Nav1.3 channels unmasked bursting activity (633). In the second case, when the β2 auxiliary subunit of BK channels is genetically deleted, almost all CCs exhibit repetitive slow-wave bursting which occurs at a frequency (~1 Hz) similar to the spontaneous AP frequency of WT cells (434). In the third case, lowering of extracellular pH (pHo) from 7.4 to 6.6 causes a marked cell depolarization that induces sustained slow-wave bursts (Figure 3) and a nearly sevenfold increase in CA secretion. Cell depolarization is attributed to the blockade of pH-sensitive TASK and BK channels (260). As summarized in Table 2, burst firing is also induced when Kv7 channels are blocked by histamine (660), Kv11 channels are blocked by their specific blocker WAY-123,398 (263), and Kv, SK, and BK channels are blocked by acute hypoxia (550). Burst firing is also induced in mouse CCs when Kv and BK channels are blocked by TEA (464) and when Cav1 channels are potentiated with the selective agonist Bay K 8644 (260). There is also evidence that the pronounced depolarization

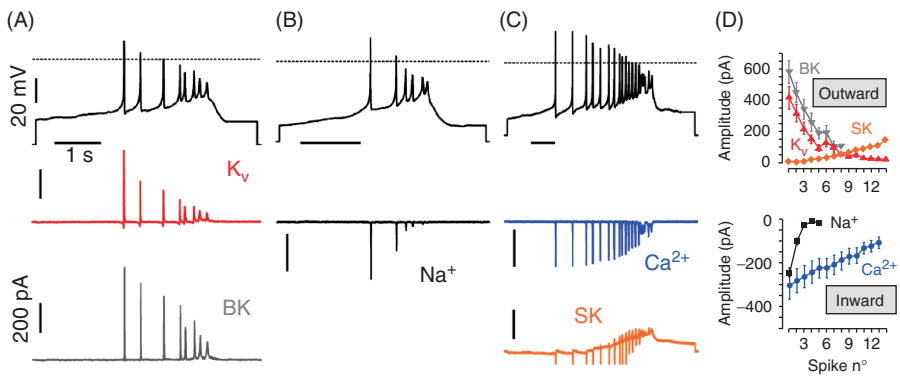

Figure 2 Time course of Na^+, Ca^{2+}, and K^+ currents during slow-wave bursts in mouse CCs. (A) AP-clamp experiment measuring Kv and BK currents. Top: AP bursts elicited by current steps were recorded at current-clamp mode and used as voltage command (black trace) in voltage-clamp experiments. Bottom: Kv currents are shown in red and BK currents in gray. (B, C) As in (A), but the currents isolated were Ca^{2+} currents (blue), SK (orange), and Na^+ (black). (D) Top: BK, SK, and Kv outward current amplitudes versus the spike number of the burst. Bottom: Same, but for the Na^+ and Ca^{2+} inward currents. Adapted, with permission, from Vandael DH, et al., 2015 (633).

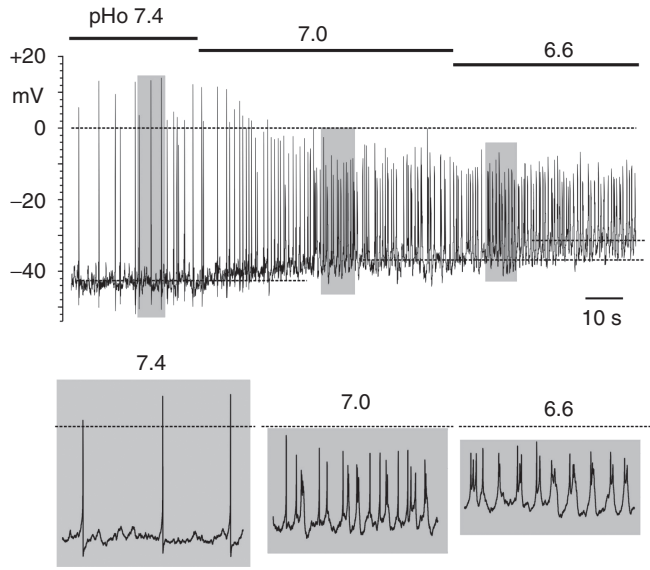

Figure 3 Low pHo induces burst firing in mouse CCs. Spontaneous firing (no current injection) recorded in mouse CCs at pHo 7.4, 7.0, and 6.6. Bottom: AP recordings on an expanded time scale corresponding to the gray window above. A decrease in pHo results in resting membrane depolarization and the switch of firing modes from tonic (pHo 7.4) to mildly bursting (pHo 7.0), to sustained bursting (pHo 6.6). Intermittent and sustained burst firing are accompanied by a net decrease of AP peak amplitude associated with the slow inactivation of Nav1.3 channels at depolarized potentials. The dotted line indicates the 0-mV level. Adapted, with permission, from Guarina L, et al., 2017 (260).

induced by 10 μM muscarine drives the spontaneous firing of a large percentage of mouse CCs into a slow-wave bursting mode (I. Méndez-López, A.G. García, and E. Carbone, unpublished results).

Burst firing occurs typically when CCs are either depolarized by blockade of Kv, SK, TASK, or BK channels or when Cav1 channels are potentiated. In the first case, Nav1.3 channels are steadily inactivated by the sustained cell depolarization and are thus unable to activate a sufficient number of BK and Kv channels to produce marked AHP that warrants the fast recovery of Nav1.3 and Cav channels from their inactivation and the generation of the next AP. In the second case, the increase of slowly inactivating Cav1 currents is sufficient to produce enough inward current to sustain the plateau potential of the burst. It seems likely that other cases of channel modulation or cell manipulation that induce bursting will be discovered, to join those listed in Table 2.

Action potentials elicited by current injection

The ability of CCs to generate different patterns of spontaneous firing is helpful in understanding the causes of altered CC excitability and identifying the molecular targets that are altered. In some cases, CCs do not fire spontaneously (for a review, see Ref. 155), and when it does occur, spontaneous firing never reaches the high-frequency levels obtained during intense sympathetic stimulation (20–30 Hz). In addition, detailed analysis of CC firing during sustained high-frequency sympathetic stimulation is often required to obtain a more physiological view of stimulation-secretion coupling (153). In these cases, an alternative to recording spontaneous firing is to elicit trains of APs by applying step depolarizations of increasing current amplitude (2–20 pA) and variable length (0.1–10 s) to induce AP firing of increasing frequencies. Using current steps above 2 to 4 pA, CCs from all animal species examined undergo AP firing and CA secretion, confirming

Table 2 Cell Conditions, Hormones, Neurotransmitters, Blockers, and Agonists That Induce Slow-Wave Bursting in CCs

Stimuli, compound	Action on channels	Intensity of depolarization	Animal	References
Cell depolarization	Slowly inactivated Nav1.3	+++	Mouse	(633)
TTX	Block of Na1.3	+	Mouse	(633)
Bay K 8644	Potentiation of Cav1.2 & Cav1.3	++	Mouse	(260)
Low pHo	Block of TASK and BK	+++	Mouse	(260)
Paxilline	Block of BK	+	Mouse	(260)
A1899	Block of TASK-1	+	Mouse	(260)
Deletion of BK-β2	Removal of fast inactivation; leftward shift of BK activation	+	Mouse (slices)	(434)
TEA	Block of Kv and BK	++	Mouse (slices)	(464)
Acute hypoxia	Block of Kv, SK and BK	+++	Rat	(550)
Histamine	Block of Kv7 (IM)	+++	Bovine	(660)
Muscarine	Block of Kv7 (IM)	+++	Mouse	I. Méndez-López, A.G. García, and E. Carbone (unpublished results)
WAY-123,398	Block of Kv11 (ERG)	++	Rat	(263)

that CCs, like neurons, are highly excitable cells whose firing frequency controls Ca^{2+}-dependent CA secretion (303).

Evoked AP firing with step depolarization was first recorded 40 years ago (61, 76) using sharp glass microelectrodes. With the advent of the patch-clamp technique, evoked AP firing was detected in CCs of many animal species with variable results (153). AP recordings were used to identify the ion channel types involved in CC excitability (389). As in neurons, the analysis of AP trains with increasing step depolarizations furnishes a detailed view of the ion channels involved and their role in sustaining the firing. Well-resolved recordings of current-evoked AP firing have been reported in cow (153), rat (178, 581, 689), and mouse CCs (244, 434, 556, 635).

Figure 4 shows an example of AP recordings evoked with increasing current steps (from 5 to 15 pA) illustrating how CCs respond to constant depolarizations. Current injections induce trains of APs that gradually decrease in frequency (Figure 4A). The instantaneous firing frequency toward the end of the pulse (f_{ss}) is always smaller than one measured at the onset (f_o), giving rise to an adaptation ratio f_o/f_{ss} always >1 (Figure 4B). Increasing the injected current (from 5 to 15 pA), the CC responds with a marked increase of f_o (from 8 to 16 Hz) and f_{ss} that rises with the same proportion (from 2 to 4 Hz), indicative of the adapting behavior of these cells. The maximal f_o at very large depolarizations (15–20 pA) reaches values (16–18 Hz) that are comparable with those recorded in adrenal medulla slices (434) and with those induced by maximal sympathetic stimulation. The frequency of the adapted APs is significantly lower in mouse CCs (4–6 Hz) than in rat CCs (10–12 Hz) (178, 303, 689), most likely due to the different expression of BK channels in the two cell preparations (434). In all cases, the AP peak adapts steadily to a lower value, due to the incomplete recovery of Nav channels and BK channel activation induced by the constant (20 mV) cell depolarization.

AP firing adaptation is the result of an ionic equilibrium between ion channels carrying inward and outward currents. Figure 4C shows clearly that when SK channels are blocked with 100 nM apamin, the AP firing increases remarkably. The cell adapts more slowly to higher f_{ss}, and the AP amplitude falls even below overshoot (Figure 4B). Thus, the current-evoked AP firing is a potent tool to identify the key molecular components of CC excitability. Current-evoked AP firing has been successfully used to highlight the key roles of some ion channel and modulatory pathways in regulating CC excitability. Specifically, they have revealed: (i) the role of SK channels in setting firing frequency of mouse CCs (635), (ii) the dual action of leptin-mediated upregulation of BK channels through the activation of an IP3K pathway regulating spontaneous and evoked AP firing in mouse CCs (244), (iii) the BK channel inactivation regulating AP firing in rat CCs (581), (iv) the Cav1.3 channel regulation of AP firing in KO (635) and KI Cav1.3 in mouse CCs (556) and, (v) the existence of burst firing during sustained cell depolarization (434) and its block by nifedipine in mouse CCs (260).

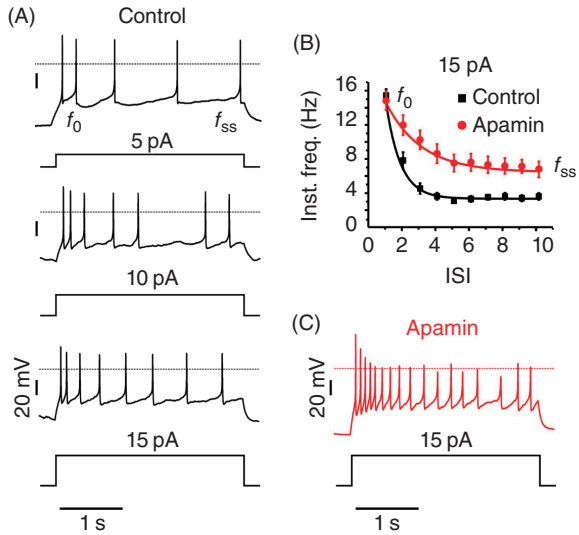

Figure 4 Spike frequency adaptation during current injections in mouse CCs. (A) Representative current-clamp recordings from WT mouse CCs in response to 5, 10, or 15 pA current injection from Vh = –70 mV (from top to bottom). (B) Evolution of the instantaneous firing frequency in WT mouse CCs at 15 pA in control (black squares) and in the presence of the SK channel blocker apamin (200 nM; red circles). (C) AP recordings in the presence of 200 nM apamin during 15 pA current injection, to be compared with the control trace to the left. Adapted, with permission, from Vandael DHF, et al., 2012 (635).

Action potentials elicited by splanchnic nerve stimulation and pulses of ACh

APs in CCs are generated by sympathetic splanchnic nerve stimulation that releases ACh and activates the nAChRs of the cells. Opening of nAChRs triggers excitatory postsynaptic potentials that initiate synchronous APs following splanchnic nerve stimulation. Alternatively, APs can be activated directly by brief pulses of ACh on cultured cells or adrenal gland slices (240). These two forms of stimulation are used sometimes for studying the modulatory effects of hormones, neurotransmitters, and drugs, which affect the stimulus-secretion coupling machinery in a cell system near physiological conditions (for a review, see Ref. 153). In the first case, recordings of APs are performed on the CCs of isolated bisected adrenal glands, which preserve their splanchnic innervation intact. CCs respond to low-frequency sympathetic stimulation (0.3, 1, and 3 Hz) with single APs that follow each stimulus synchronously, with no evidence of fatigue or desensitization (303). At higher frequencies (10 Hz), some stimuli fail to evoke a response. Failures of synaptic transmission are also observed in the superior cervical ganglion neurons during continuous stimulation at 10 and 20 Hz (58), suggesting strong similarities between the responses to splanchnic nerve stimulation of CCs *in situ* and sympathetic ganglion cells, and a weak linear correspondence between splanchnic stimulation and CC responses at a higher frequency. This approach has been used in the past to establish a correlation between the frequency of neuronal stimulation and the amount of released CA, with contrasting results on whether the two parameters

are linearly related or not in the range of physiological frequencies (0.3–30 Hz) (303, 658). Presently, this approach is not often used due to the complexity of the preparation and the technical difficulties to record stable APs, but it remains nevertheless a valuable tool to study stimulus-secretion coupling in intact innervated glands. A recent work, using a modified version of this technique, has shown remarkable spatial and activity-dependent differences in stimulus-secretion coupling between CCs releasing EPI and cells releasing NE (674).

APs induced by short puffs of ACh have been used recently for the purpose of mimicking the quantal release of ACh during single splanchnic nerve stimulation. de Diego et al. were first to design a protocol made of a train of brief pulses of 30 μM ACh lasting 25 ms of variable frequency (154) that mimicked the CC responses to trains of splanchnic nerve stimulations (153). In bovine CCs, the protocol could elicit trains of APs with similar waveforms to those recorded using either brief step depolarizations or single splanchnic nerve stimulations. All-or-none APs had a proper amplitude (65 mV from V_{rest}), half-width (2.7 ms), and well-resolved AHP (−17 mV from V_{rest}) indicative of the excellent state of excitability of the cells. Tonic firings with no sign of adaptation and failures could be achieved with frequencies ≤1 Hz. At higher frequencies (≥3 Hz), APs occurred irregularly and resting potential became unstable, most likely due to the incomplete fast washout of ACh after each brief application (153). Accumulation of ACh during repeated stimulation could induce nAChRs desensitization, activation of mAChRs, cell depolarization, and Nav channel inactivation. Thus, ACh-evoked APs appear to be a physiologically interesting way to induce cell firing in CCs, although the method requires technical improvements to study stimulus-secretion coupling mechanisms using AP frequencies above 3 Hz.

Nicotinic acetylcholine receptors of chromaffin cells

Stimulation of the sympathetic splanchnic nerve elicits the release of ACh from their nerve endings. ACh binds to nAChRs and mAChRs present in the CCs membrane. Membrane depolarization and AP firing were initially recorded in rat CCs upon activation of nAChRs (349). nAChRs play a central role in triggering fast CA secretion from adrenal CCs in response to ACh (175, 220). Pharmacological and immunological studies support the view that nAChRs expressed by CCs are of the neuronal type (142). nAChRs are formed by five subunits that span the membrane four times with the agonist-binding site located at the extracellular region, which is also structurally and functionally linked to the ion channel (152). Crystal structures of complete receptors reveal a cylinder-like structure made of five subunits symmetrically located around the ion channel. Single-channel nAChR conductance is ∼44 pS (221). The channel is permeable to Na^+, K^+, and Ca^{2+}, the latter contributing only 2% to 4% of the current (691).

The most conspicuous nAChR subtype involved in CA secretion is the heteromeric receptor formed by α3 and β4 subunits. In bovine CCs, two nAChR subtypes have been described. The main subtype is a heteromeric assembly of α3, β4, and possibly α5 subunits insensitive to α-bungarotoxin. The other subtype is α-bungarotoxin-sensitive homomeric α7 nAChRs (142). The transcription factor Sp1 (specificity protein 1) coordinates the synthesis of α3, β4, and α5 subunits that form the α-bungarotoxin-resistant nAChRs (57), while the transcription factor Egr-1 (early growth response protein 1) controls the differential expression of both α7 nAChR (114) and PNMT (185) in adrenergic CCs.

In addition to the typical nicotinic ligands, several unrelated substances of clinical relevance bound to nAChRs in CCs act as agonists, antagonists, or allosteric modulators. *Agonists* include ACh, carbachol, dimethylphenylpiperazinium, cytosine, and (+)-anatoxin-a that act on α3β4 receptors (142). Choline elicits $[Ca^{2+}]_c$ signals and secretion by acting on α7 receptors in bovine CCs (159, 232). A study on human CCs showed that the α7 nAChR-selective agonist PNU-282987 elicited whole-cell inward currents that were potentiated by allosteric α7 modulators 5-hydroxyindole and PNU-120596. Since α7 nAChR agonists choline and ONU-282987 augment secretion, it was concluded that human CCs express functional α7 nAChRs (508). In a similar study in bovine CCs, the authors conclude that CA release might be regulated by α7 and α3β47 nAChRs, depending on agonist concentrations and the presence of allosteric modulators of α7 nAChRs (159). The *antagonist* pharmacology of CCs nAChRs shows a classical "ganglionic" profile, with some additional blockers, like the spasmolytic otilonium (237), nanomolar concentrations of atropine (256), or the diuretic cyclothiazide (478). The involvement of α7 nAChRs in human CCs is supported by its partial blockade of CA secretion with α-bungarotoxin (405, 508). Some neuropeptides, that is catestatin, a fragment of CgA (294), substance P (132), and calcitonin gene-related peptide (CGRP) (164) also modulate the nAChRs of CCs.

In conclusion, although various subunits of nAChRs have been cloned and are expressed by CCs, the α3β4 is the predominant nAChR subtype in mediating the whole-cell nicotinic currents, $[Ca^{2+}]_c$ signaling, and the release of CA triggered by ACh in CCs. The α7 nAChR is also expressed by CCs, although its function is not yet fully characterized. A rich pharmacology is at hand either for activation or blockade of CC nAChRs, or to modulate them allosterically.

Ca^{2+} and Stimulus-Secretion Coupling

Ca^{2+} channel-secretion coupling

Ca^{2+} channel regulation by neurotransmitters: a historical background

Douglas and Rubin (172, 176) working in CCs and Katz and Miledi (337, 338) in presynaptic nerve terminals were the first to identify the role of Ca^{2+} influx as the key regulator of hormone and neurotransmitter release. Later studies uncovered the steep dependence of neurotransmitter release on Ca^{2+}

influx (169) and demonstrated the link between presynaptic Ca^{2+} currents and postsynaptic responses using the squid giant synapse (398). These results focused interest on Cav channels and their physiological role, with special emphasis on neurotransmitter and hormone release. It soon became evident that Cav channels are plastic and can be modulated by neurotransmitters, hormones, second messengers, and other intracellular chemicals, as well as by pharmacological agents. The first hormone-mediated pathway was described in cardiac cells where EPI or elevation of intracellular cyclic adenosine monophosphate (cAMP) facilitated Ca^{2+} channel activity (531, 621). The second neurotransmitter-mediated pathway was reported in chick sensory neurons where the addition of NE inhibited Ca^{2+} channel activity, the amplitude of Ca^{2+} currents, and the duration of APs (182, 183). In this section, we will describe a variety of mechanisms for the regulation of Cav channels of CCs by autocrine/paracrine neurotransmitters, hormones, and other modulators.

The role of Cav2.1 and Cav2.2 on excitation-secretion coupling in CCs

Cav2 channels (N, P/Q, and R) are highly expressed in the nervous system, where they carry the presynaptic Ca^{2+} currents that trigger neurotransmitter release and synaptic transmission (216, 530). Neurotransmitter release controlled by these voltage-gated channels is steeply Ca^{2+} dependent (third to fourth power) (169), making them an important locus of synaptic regulation. Among Cav2 channels, Cav2.1 and Cav2.2 are the main sources of the Ca^{2+} that initiates the rapid release of glutamate, ACh, and GABA from neurons. In addition, Cav2.1 and Cav2.2 contain a synprint region that binds to syntaxin 1A and SNAP-25 (115), suggesting that they colocalize to regions of the membrane where the SNARE complex is formed and synaptic vesicle fusion occurs to initiate neurotransmitter release. Cav2 channel activation is also critically regulated by several G-protein subunits (686), which form the basis of Ca^{2+}- and voltage-dependent signal transduction at the synaptic terminal (116).

In CCs, Cav2.1 and Cav2.2 are highly expressed, but at different densities, in all animal species (240). The two channels are effectively coupled to secretion, with Ca^{2+} dependence of secretion not as steep as in presynaptic terminals. The dependence on Ca^{2+} is nearly linear and comparable to that seen with the other Ca^{2+} channels expressed in CCs (L, R, and T types) (100, 103, 248, 308, 352, 543, 609). This means that Cav2 channel opening is loosely coupled to readily releasable secretory vesicles, which is consistent with the idea that Ca^{2+} channels and release sites in CCs are not closely colocalized as in presynaptic neuronal terminals (130). Ca^{2+} channels and release sites are distributed within nanometric distances (200–300 nm) to form specialized membrane regions with dimensions of several micrometers (357, 469). In this way, the dominant Ca^{2+} signal regulating vesicle fusion and neurotransmitter release derives from the activation of several Cav channel subtypes distributed over areas covering tens of square micrometers, rather than from dense Ca^{2+} channel clusters localized near some readily releasable vesicles at the presynaptic secretion sites similar to the active zones (469, 470).

Regarding the regulatory role of Cav2.1 and Cav2.2 channels in secretion, most electrophysiological studies support the idea that both channels are equally accessible to the secretory apparatus, without any preferential coupling (30, 100, 206, 230, 352). Nonetheless, using Cav2.1 KO mice, a predominant role of P/Q-type channels in regulating the fast release of vesicles from the immediately releasable pool (IRP; see below for an accurate definition) has been proposed (20). Using short square pulses (10 ms) to 0 mV from −80 mV to specifically recruit vesicles from the IRP, it was shown that Cav2.1 channels control most of the IRP. Finally, it is worth noticing that depending on the activation gating of Cav2 channels, it is likely than Cav2.1 channels exert a key role in secretion when using trains of triangular APs rather than square pulses (122). This is likely associated with the fast activation of Cav2.1 with respect to the other Cav channels, which is more evident with short stimuli since less affected by the fast Ca^{2+}-dependent inactivation (CDI) of the channel. This suggests that channel activation and the type of stimuli applied, rather than the specific colocalization of the secretory apparatus with Ca^{2+} channels, critically regulate exocytosis in CCs.

The voltage-dependent modulation of Cav2.1 and Cav2.2 by neurotransmitters: physiological significance

As shown in the previous section, due to their higher threshold of activation, Cav2 channels contribute mainly to the upstroke and falling phase of APs, while Cav1 channels (Cav1.2 and Cav1.3) control the subthreshold current regulating AP firing in rat and mouse CCs (426, 429). Cav2 channels are also uniquely modulated in a voltage-dependent manner by G-protein-coupled receptor (GPCR) pathways (314). In CCs, Cav2.1 and Cav2.2 are autocrinally inhibited by ATP and opioids that are released together with CAs during cell activity (8, 10, 149, 235). The inhibition occurs in membrane microdomains, without the involvement of diffusible second messengers, and is manifested by a delay of channel openings (slow activation) at low potentials (Figures 5A and 5B) (98, 102). This effect, however, can be reversed by depolarizing voltage steps. The normal fast activation is recovered during strong depolarizations to +100 mV (52, 430) or when a strong depolarizing prepulse anticipates a test depolarization to 0 mV (Figures 5A-5C) (201). This phenomenon is called "voltage-dependent facilitation" and is attributed to a protein-protein interaction between the activated GPCR βγ subunit and several sites on both the Cav2 α1 subunits: the N-terminus, the I–II linker domain, and probably the C-terminus (see Ref. 686 for a recent review). Remarkably, this inhibition of Cav2.1 and Cav2.2 can be partially recovered during brief depolarizations repeated at high frequency (150) as it occurs during high-frequency AP

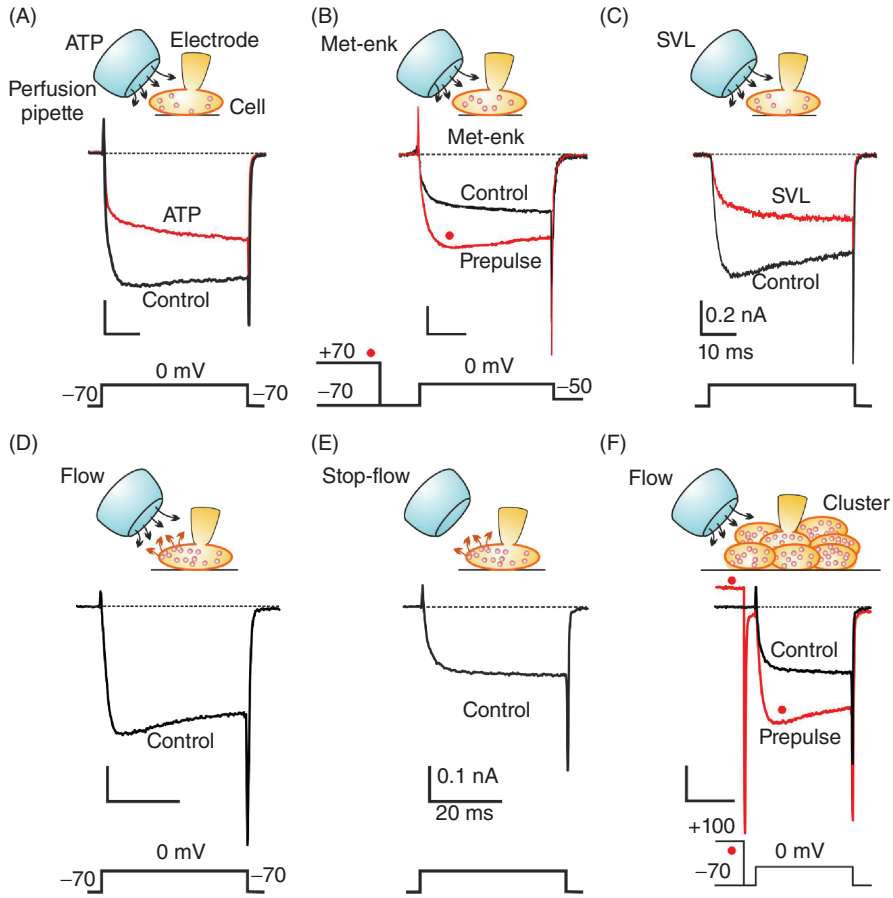

Figure 5 Voltage-dependent modulation of $Ca_V2.1$ and $Ca_V2.2$ in CCs. (A) Acute application of ATP (50 μM) slows down the activation of N- and P/Q-type Ba^{2+} currents recorded at 0 mV in a bovine CC (for details, see Ref. 98). (B) The delayed activation of N- and P/Q-type Ba^{2+} currents induced by the acute application of met-enkephalin (10 μM; black trace; control) is recovered by a 50-ms prepulse step depolarization to +70 mV (red dot; red trace) (for details, see Ref. 573). (C) Acute application of the soluble vesicle lysate (SVL) containing (EPI, NE, opioids, and ATP) causes N- and P/Q-type current delayed activation in bovine CCs (for details, see Ref. 10). (D, E) Autocrine inhibition of N- and P/Q-type channels revealed by changing cell superfusion from "stop-flow" to "flow" condition, in bovine CCs. In the "flow" condition, there is no autocrine inhibition. Ba^{2+} currents at 0 mV are fast activating. In the "stop-flow" condition, the autocrine-released material inhibits through GPCRs the opening of Ca_V2 (N- and P/Q-type) channels, inducing marked activation delay. Adapted, with permission, from Carabelli V, et al., 1998 (98). (F) Ba^{2+} currents recorded from a cell that is part of a cluster undergo robust autocrine modulation (black trace, control). A prepulse to +100 mV is able to rescue the fast channel activation by removing the autocrine inhibition induced by the neurotransmitters released by the surrounding cells (for details, see Ref. 290).

trains occurring during stress-mimicking conditions. The result is that the reduced Ca^{2+} entry through $Ca_V2.1$ and $Ca_V2.2$ at rest, due to the downregulation induced by the released opioids and ATP, can be partially recovered during sustained cell activity. This "autocrine inhibition" of Ca_V2 channels was first discovered by applying the purified low molecular weight components of secretory vesicles (soluble vesicle lysate, SVL), containing EPI, NE, opioids, and ATP, directly onto bovine CCs (Figure 5C) (10). Application of SVL caused the typical slowdown of Ca_V currents induced by applied neurotransmitters that could be removed by strong pre-step depolarizations. This report also showed that the same autocrine inhibition occurs by stopping the flow of external solution perfusing the cell (*stop-flow*), allowing the unwashed released neurotransmitters (ATP and opioids) to activate the inhibitory GPCR pathway that slows down Ca_V2 currents (Figure 5E). This resting autocrine inhibition of Ca_V2 channels is particularly evident in clusters of bovine CCs (290) (Figure 5F) and in mouse adrenal gland slices (292). In these tissue preparations, the packed organization of the cells is well preserved and the released neurotransmitters can accumulate in the extracellular space to exert a basal tonic inhibition on cell firing and CA secretion that is facilitated by prepulse depolarizations. The application of opioids or ATP

directly on bovine CCs mimics the effects of SLV application (Figures 5C and 5F).

The effective action of this autocrine modulation derives from the high concentration of neurotransmitters stored in the CC granules (0.8–1 M CA, ~200 mM ATP, and 1–2 mM met-enk; see below) and the high density of adrenergic (α_2-AR, β_1-AR, and β_2-AR) (78, 121, 355), opioidergic (μ and δ) (8, 355, 624), and purinergic (P_{2y}) receptors (98, 149, 519, 627) expressed in bovine, rat, and mouse CCs. It is noteworthy that rat CCs also possess ligand-gated purinergic receptors (P_{2X3} and P_{2X7}), which are upregulated during pain-induced stress in chronic pain rat models (28). This suggests a key role of P_{2Y} and P_{2X} receptors in regulating cell depolarization, AP firing, and CA release. The role of secreted ATP modulating the quantum characteristics of CA exocytosis through purinergic receptor activation has also been recently demonstrated (421).

In conclusion, it seems that the autocrine inhibition of the Ca_V2 channel acts primarily as negative feedback to regulate the amount of secretion. With increased firing rates, the autocrine inhibition may still act to prevent excessive CA release, but when under intense APs stimulation, a large surge of CAs is required. CCs are able to overcome the inhibition by facilitating Ca_V2 currents in an activity-dependent manner.

The voltage-independent modulation of $Ca_V2.1$ and $Ca_V2.2$ by neurotransmitters

Voltage-independent inhibition of Ca_V2 channels also exists and likely includes several distinct modulatory mechanisms. In CCs, the neuronal Ca^{2+} sensor-1 (NCS-1) exerts a tonic voltage-independent inhibition of $Ca_V2.1$ channels (665). NCS-1 acts through an autocrine, purinergic, and opioidergic receptor-mediated pathway and the activation of PTX-insensitive G-proteins. To complete the $Ca_V2.1$ channel inhibition in CCs, it is also necessary that the $Ca_V2.1$ α1-subunit be phosphorylated at the tyrosine residues by a Src-like kinase, strictly controlling Ca^{2+}-dependent exocytosis through a series of biochemical steps (665). Specifically, NCS-1 is shown to regulate CC secretion by favoring PIP_2 production, leading to $InsP_3$ increases and Ca^{2+} release from intracellular stores (500). A similar voltage-independent inhibition of $Ca_V2.1$ and $Ca_V2.2$ channels mediated by PIP_2 and PKC has been reported in the sympathetic superior cervical ganglion and transfected TsA201 cells (596). Voltage-independent inhibition of Ca_V2 channels is also produced by Gq-coupled H1-histamine receptors in bovine CCs (151).

Modulation of $Ca_V2.1$ and $Ca_V1.3$ channels in CCs: a role in stimulus-secretion coupling

The important participation of L-type channels in the Ca^{2+}-dependent release of CAs from the adrenal medulla was first reported in a pioneering set of experiments (243). Subsequent reports confirmed this observation and showed that due to their slow inactivation, Ca_V1 are the Ca^{2+} channels mostly contributing to CA secretion when long-lasting stimuli are applied. Sustained depolarization with high KCl solutions (404), prolonged applications of ACh (325, 461), or repeated splanchnic nerve stimulation (6) in isolated cells or intact adrenal glands of various animal species revealed a predominant L-type channel regulation of CA release (see Refs. 70, 240, 417, 463, 634 for recent reviews). This slow action occurs independently of the expression densities of Ca_V1 channels and it is associated with the unique gating properties of these channels. Two features, in particular, are the slow Ca^{2+}-dependent inactivation that allows sustained Ca^{2+} fluxes during prolonged (or repeated) depolarizations and the weak steady-state inactivation of both channels at −50 and −60 mV, the latter favoring channel availability near the resting membrane potentials. When exocytosis is evaluated using capacitance changes on single isolated cells, the L-type Ca^{2+} current possesses the same Ca^{2+} sensitivity of Ca_V2 and Ca_V3 channels and contributes proportionally to their density of expression (100, 103, 206).

The voltage-independent direct and remote autocrine modulation of L-type channels

The Ca_V1 (L-type) channels, like the Ca_V2 type, experience basal and stimulus-induced autocrine modulation, which affects the amount of Ca^{2+} entry that regulates CA secretion. This autocrine effect is mediated by a variety of GPCRs, including adrenergic, opioidergic, and purinergic, expressed in CCs. It occurs in isolated CCs (Figures 5A-5C) (10, 121, 288, 419) but is more prominent in cell clusters (236) and in adrenal gland slices (292), in which, as *in vivo*, released neurotransmitters accumulate between closely packed CCs (Figure 5F).

The autocrine modulation of L-type channels in CCs is mainly voltage independent and occurs through two opposing distinct pathways (see Refs. 109 and 417 for a review): (i) rapid inhibition of Ca_V1 channel gating via PTX-sensitive G-proteins (*direct inhibition*) or (ii) slow upregulation of Ca_V1 channel activity through a cAMP/PKA-mediated phosphorylation (*remote upregulation*), similar to the upregulation of cardiac $Ca_V1.2$ channels under β1-AR-mediated sympathetic stimulation. The *direct inhibition* is fully apparent in membrane microdomains where Ca_V1 channels and GPCRs interact to reduce the open channel probability and Ca^{2+} entry (98, 101, 288, 292). The *remote upregulation* requires the presence of β-adrenoceptors (β-ARs), functional adenylate cyclases, PKA, and Ca_V1 channels and occurs in nearly all the CCs of all animal species including humans (101, 121, 138, 229, 428); for a review, see Refs. 46, 109, 417, 426, 427, and 634.

L-type channels are also regulated by a remote mechanism, in addition to neurotransmitter-mediated modulation, involving the NO/cGMP/PKG pathway which tonically inhibits Ca_V1 channels and CA release in bovine (99, 561) and mouse CCs (419). The NO/cGMP/PKG-mediated downregulation of L-type channels and cAMP/PKA-mediated upregulation act oppositely, just as they act on cardiac $Ca_V1.2$ channels. An interesting question is whether $Ca_V1.2$ and $Ca_V1.3$,

which are equally expressed in both mouse and rat CCs, are both modulated by NO/cGMP/PKG- and cAMP/PKA-driven mechanisms. Using $Ca_V1.3^{-/-}$ KO mice, it was demonstrated that both channels are equally up- or downregulated by the two opposing pathways (419). The two pathways act independently on the two channels and may induce almost an order of magnitude variation of L-type current amplitudes when concomitantly regulated. Figure 6 illustrates the cascade of events of the two opposing modulatory pathways. The upper part shows cAMP/PKA-mediated upregulation driven by the autocrine activation of β-ARs. The lower part shows the NO/cGMP/PKG-mediated downregulation driven by different stimuli. Figure 7 summarizes the two main experiments that showed how Ca_V1 currents in WT mouse CCs can be upregulated by inhibiting the NO/cGMP/PKG cascade and activating the cAMP/PKA pathway (bottom part) or drastically downmodulated by inhibiting cAMP/PKA pathway and stimulating the NO/cGMP/PKG cascade (top part) (see Figure 7 legend for further details).

Concerning the functional relevance of this dual modulation of $Ca_V1.2$ and $Ca_V1.3$ L-type channels, we point out that cAMP/PKA and cGMP/PKC are likely active even at rest due to the basal activity of the two kinases. PKA can be stimulated by PACAP (526), Ca^{2+} entry, and G-protein subunits that are activated by the basal activity of first messengers released from sympathetic neurons (24), surrounding capillaries (431, 670), and by the autocrine activity of CCs (10, 121). The soluble GC is activated by the resting NO levels generated by the Ca^{2+}-calmodulin-mediated activation of NO synthase (NOS) expressed in most CCs (494, 561). Under these conditions, cGMP/PKG appears to work as a "brake" to limit the potentiating effects of cAMP/PKA and helps set the resting levels of $Ca_V1.2$ and $Ca_V1.3$ currents, which regulate the resting potential and basal repetitive firing.

A *synergistic potentiation* of Ca_V1 channels could occur during sustained splanchnic nerve stimulation that releases PACAP (*vide infra*) and induces a massive release of EPI from CCs, which would further elevate the levels of cAMP/PKA

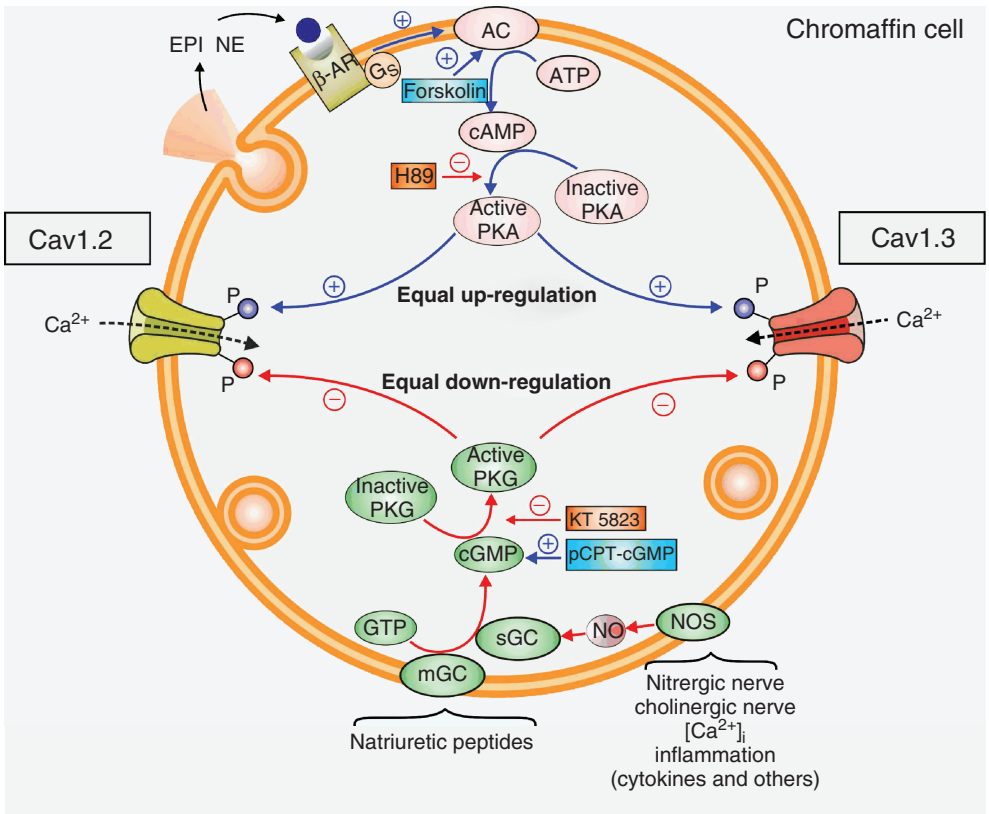

Figure 6 Up- and downmodulation of $Ca_V1.2$ and $Ca_V1.3$ channels by the cAMP/PKA and NO/cGMP/PKG pathways in mouse CCs. The top half illustrates the molecular components and the sequential steps of the β-AR-mediated upregulation of $Ca_V1.2$ and $Ca_V1.3$ L-type channels through the activation of adenylate cyclase (AC), leading to cAMP production, activation of PKA, and Ca_V1 channel phosphorylation, causing an increase in open channel probability. The bottom part shows the molecular components and the sequential steps of the NO-mediated downregulation of $Ca_V1.2$ and $Ca_V1.3$ L-type channels through the activation of a soluble guanylate cyclase (sGC), leading to cGMP production, activation of PKG, and Ca_V1 channels phosphorylation, causing a decrease in open channel probability. NO is produced by membrane NO-synthases. cGMP is also produced by a membrane guanylate cyclase (mGC). Adapted, with permission, from Mahapatra S, et al., 2012 (417).

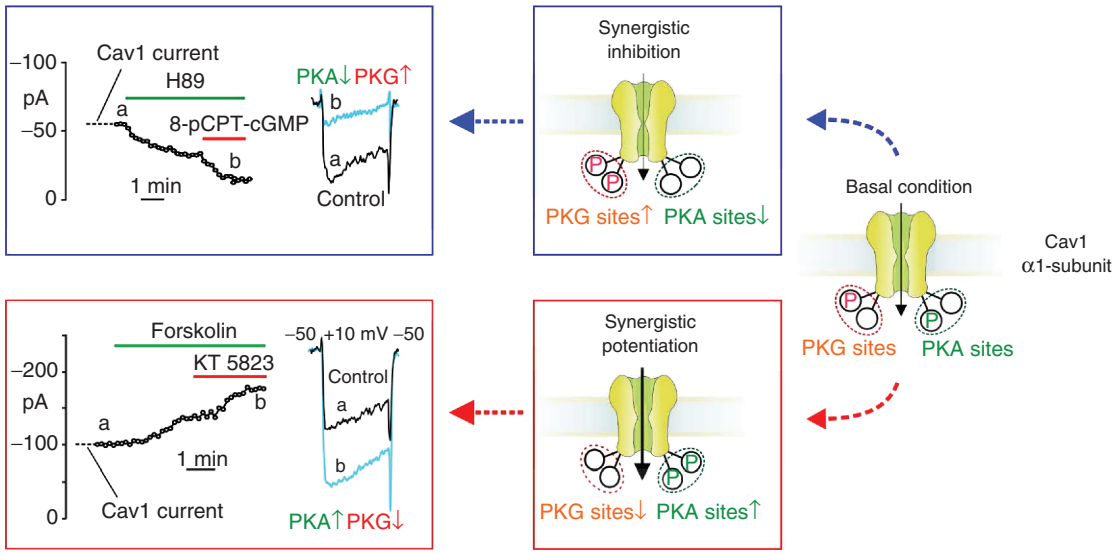

Figure 7 Synergistic effects of cAMP/PKA and cGMP/PKG pathways on Cav1 currents of WT mouse CCs. The right side is a schematic representation of Ca_V1 channel α1 subunit with two hypothetical PKA and PKG phosphorylation sites (P). The schemes represent the situation under basal conditions (middle), a synergistic potentiation (top), and a synergistic inhibition (bottom) of Ca_V1 channel. PKA and PKG P-sites are partially phosphorylated (P) or unphosphorylated under resting conditions. Phosphorylation and dephosphorylation drove by up- and downregulation of PKA and PKG proceed independently of each other and can reach two extreme conditions. In one case, the two PKA P-sites are dephosphorylated and the two PKG P-sites are phosphorylated (minimal Ca_V1 current; cyan trace, on the top left panel). Alternatively, the PKA sites are phosphorylated and PKG P-sites are dephosphorylated (maximal Ca_V1 current; cyan trace, on the bottom left panel). The two panels to the left show the time course of synergistic downregulation of Ca_V1 channels induced by the sequential application of the PKA inhibitor H-89 and the PKG activator 8-pCPT-cGMP (top) and the synergistic upregulation of Ca_V1 channels by sequential application of the PKA activator forskolin and PKG inhibitor KT 5823 (bottom). Adapted, with permission, from Mahapatra S, et al., 2012 (417); Adapted, with permission, from Vandael DHF, et al., 2013 (634) (drawing and experimental data).

through the autocrine activation of the β_1-ARs (121, 428). The increased Ca^{2+} entry could, in turn, upregulate PDE1, the cGMP-hydrolyzing and Ca^{2+}-calmodulin-activated PDE that controls the resting levels of cGMP (561, 637). Activation of a cGMP-specific PDE that lowers cGMP, inhibiting PKG, and the parallel activation of PKA during PACAP release and β_1-AR stimulation could markedly boost the L-type Ca_V1 currents. This regulation would overall serve to sustain the rapid increase of cell firing activity and CA release during the "fight-or-flight" response to stressors in CCs. On the other hand, a *synergistic inhibition* could occur if, as in mammalian ventricular myocytes, CCs possess cGMP-activated PDE2 isoforms that hydrolyze cAMP (410). Any robust upregulation of the NO/cGMP/PKG pathway under these conditions would enhance cGMP and downregulate cAMP, rapidly depressing $Ca_V1.2$ and $Ca_V1.3$ channel gating. The existence of various PDEs acting on cAMP in CCs is supported by the observation, in mouse CCs, that the nonspecific PDE blocker IBMX increases basal cAMP levels more potently than the PDE-4-specific blocker rolipram (428).

Regulation of endocytosis by L-type Ca^{2+} channels

To ensure that the size of nerve terminals and neuroendocrine cells is preserved during cell activity, excess vesicle membrane incorporation into the cytosolic membrane during exocytosis must be compensated by an equivalent membrane retrieval during subsequent endocytosis (see the following section). This mechanism ensures that a given number of vesicles are available to replenish the secretory vesicle pool and participate in subsequent rounds of exocytosis during repetitive cell activation (33, 118, 284). Like exocytosis, endocytosis is a Ca^{2+}-dependent process in CCs (33, 481). The question is whether some Ca_V channels (Ca_V1 and Ca_V2) are preferentially coupled to endocytosis or not.

In bovine CCs stimulated with long depolarizing pulses, there is a preferential coupling of L-type Ca_V1 channels to endocytosis (541). In spite of the small contribution of Ca_V1 channels to the whole-cell Ca^{2+} current, this shows that nifedipine abolishes the endocytotic response, without significantly affecting exocytosis that is also sustained by $Ca_V2.1$ and $Ca_V2.2$ channels. In the presence of FPLA64176 (an L-type Ca_V channel agonist), the increased Ca^{2+} entry doubles the endo/exocytosis ratio, indicating a selective augmentation of endocytosis associated with the selective Ca^{2+} entry increase through Ca_V1 channels (543). In mouse CCs, the membrane excess retrieval following strong Ca^{2+} entry is suppressed by nitrendipine, suggesting the direct involvement of Ca_V1 channels (511).

Immunofluorescence experiments on bovine CCs have shown negligible colocalization of clathrin and dynamin to

L-type Ca^{2+}-channels (543). This, and the additional studies in CCs (136), support the idea that a low-rate, non-inactivating Ca^{2+}-entry through Ca_V1 channels ($Ca_V1.3$) might be more critical to trigger compensatory as well as excess exocytosis. These data also support the idea that not all Ca^{2+} that enters CCs during depolarization through slow-inactivating Ca_V1 channels or through fast-inactivating $Ca_V2.1$ and $Ca_V2.2$ channels has the same physiological function (463).

Ca^{2+} handling by CCs: impact on exocytosis

That Ca^{2+} is the coupling agent in the stimulation-secretion process was established in pioneering experiments in perfused cat adrenal glands: ACh stimulation triggered a Ca^{2+}-dependent CA release (176) that was associated with enhanced Ca^{2+} entry via Ca_V channels (174). Since then, we have learned much more about the complexities of enhanced Ca^{2+} entry and secretion. The details of the stimulus-secretion coupling process (172) have been amply clarified using Ca^{2+}-sensitive probes, particularly aequorins targeted to different organelles (537) as well as patch-clamp (271) and amperometric techniques (668) to measure the exocytotic release of CAs with high temporal resolution.

Several ion channels and transporters tightly regulate Ca^{2+} handling in excitable cells. This exquisite control of the cycling of Ca^{2+} is vital to cell function and survival. Concerning CCs, we will review the cycle of Ca^{2+} that comprises four processes: Ca^{2+} entry, Ca^{2+} efflux, Ca^{2+} redistribution into organelles, and Ca^{2+} release from those organelles.

Ca^{2+} entry

Ca^{2+} entry through the various Ca_V channels is the primary determinant in shaping the $[Ca^{2+}]_c$ elevations occurring during CC activation. Although Ca^{2+} entry through the plasmalemma is mainly associated with Ca_V channels, Ca^{2+} can reach the cytosol through the nAChRs as well as the store-operated Ca^{2+} channels (SOCCs). The characteristics and functions of Ca_V channel subtypes and their role in the regulation of CA release have been described earlier (*Ca^{2+} channel-secretion coupling*). Those of nAChR and SOCC channels in Ca^{2+}-handling and transport have been discussed in detail elsewhere (242).

Ca^{2+} efflux

There are two plasmalemma transporters for pumping Ca^{2+} out of the CCs. These are the plasma membrane Ca^{2+}-ATPase or Ca^{2+} pump (PMCA) and the Na^+/Ca^{2+} exchanger (NCX). The Ca^{2+} pump, inhibited by vanadate and lanthanum, has a high affinity for Ca^{2+} (submicromolar K_D) and transports one Ca^{2+} ion for each ATP hydrolyzed (79). The H^+/Ca^{2+} transport ratio for PMCA in reconstituted *in vitro* systems has been estimated at 1:1 (165), and therefore PMCA pumping would be electrogenic (551) and sensitive to membrane potential. Nevertheless, in neurons, PMCA exchanges two H^+ for each Ca^{2+} ion extruded, that is it is electroneutral (612). PMCA is modulated by numerous factors, but mainly calmodulin. When intracellular Ca^{2+} increases, calmodulin binds to PMCAs, increasing their Ca^{2+} affinity. The PMCAs are encoded by four different genes: PMCA1 and PMCA4, which are ubiquitously expressed, and PMCA2 and PMCA3, expressed predominantly in the CNS (106). The Na^+/Ca^{2+} exchanger uses the energy of the Na^+ gradient, to exchange three external Na^+ ions for one internal Ca^{2+} ion (42); its function is, therefore, electrogenic. The exchanger can also work in the "reverse mode," moving Na^+ out of the cell and Ca^{2+} into the cell; this occurs during membrane depolarization and/or when intracellular Na^+ concentration increases, for instance after intense AP firing (63).

Using plasma membrane purified from bovine adrenal medullae, a first functional characterization of the two transporters was achieved (335). Later, it was found that bovine CCs express NCX1, the major isoform of the exchanger (497). NCX1 can adopt two functional modes: the forward mode that favors Ca^{2+} efflux (520) and the reverse mode that favors Na^+-dependent Ca^{2+} influx (391). Changes in the Na^+ gradient, either directly through manipulation of ion concentrations (499, 501, 536) or indirectly through inhibition of the Na^+ pump with ouabain (213, 241), augment Ca^{2+} entry and the release of CAs. This has suggested a role of the NCX in the regulation of Ca^{2+} signaling and exocytosis in CCs. However, an alternative explanation in the case of experiments with ouabain involves its ability to release Ca^{2+} from the ER (443).

Ca^{2+} sequestration

The membrane of the ER and sarcoplasmic reticulum (SR) contains a Ca^{2+} pump, the sarcoendoplasmic reticulum Ca^{2+}-dependent ATPase (SERCA), of which there are three major isoforms (SERCA1–3). SERCA1a-b and SERCA2a are present in skeletal and cardiac muscles, while SERCA2b is expressed in all nonmuscle tissues including nerve cells. SERCA keeps intracellular Ca^{2+} concentration low and Ca^{2+} concentration high inside the ER/SR, creating the conditions for cytosolic Ca^{2+} transients and oscillations in response to a variety of external stimuli that allow ER Ca^{2+} release. Phospholamban (PLB), a small protein expressed in muscle and integral to the ER/SR membrane, when nonphosphorylated, binds to SERCA and decreases its affinity for Ca^{2+}, and when phosphorylated, it detaches from the pump, relieving the inhibition. The activity of all SERCA isoforms is specifically inhibited by thapsigargin with half-maximal inhibitory potency of 10 to 20 nM (106).

Redistribution of Ca^{2+} into, and release of Ca^{2+} from, the endoplasmic reticulum

Several laboratories have approached the study of Ca^{2+} circulation in and out of the cytoplasm and ER Ca^{2+} store of CCs. Upon cell activation, increased cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) activates Ca^{2+} uptake into the ER lumen

through the SERCA. For instance, upon stimulation of bovine CCs (17, 640, 678) or rat CCs (295), Ca^{2+} is effectively taken up by the ER, to reach concentrations as high as half millimolar within the ER lumen (17).

Various stimuli are known to release Ca^{2+} from the ER through two channels, namely, the inositol tris-phosphate receptor ($InsP_3R$) and the ryanodine receptor (RyR). Carbachol, histamine, bradykinin, and angiotensin II augment the synthesis of $InsP_3$ in bovine CCs (477, 518, 553) in parallel with $[Ca^{2+}]_c$ increase (589). These ER Ca^{2+}-mobilizing actions are mimicked by direct stimulation with $InsP_3$, suggesting that they are mediated by the plasmalemmal receptors for these first messengers to generate $InsP_3$ that will finally elicit Ca^{2+} release via $InsP_3Rs$ (589, 590).

The functional correlation of the $InsP_3$ ER Ca^{2+} release pathway has been studied in more detail with agonists for metabotropic receptors. Thus, stimulation of bovine CCs with histamine (29) or guinea-pig CCs with muscarine (487) augments $[Ca^{2+}]_c$ to elicit the activation of Ca^{2+}-dependent SK and BK channels that gives rise to cell hyperpolarization. In line with this is the observation that SK and BK channels regulate the nicotinic and muscarinic secretory responses of cat and bovine CCs (371, 625, 626). A short application of histamine causes a transient mild secretory response (83), while a longer application causes a greater effect (83, 397, 476). Most likely, the histamine-elicited $[Ca^{2+}]_c$ elevation has two components: an initial transient phase linked to ER Ca^{2+} release and a late, sustained phase due to Ca^{2+} entry (485, 589, 590, 687); but see (64). The second component has been linked to the inhibition of a K_V7 (M-current) potassium channel with an associated depolarization and augmented Ca^{2+} entry through open Ca_V channels (660). Angiotensin II also increases cytosolic Ca^{2+} and stimulates exocytosis in bovine CCs, but to a lesser extent than histamine (126, 590).

The second pathway for Ca^{2+} release from the ER is the RyR Ca^{2+}-release channel. An activator of this channel, caffeine, causes rapid Ca^{2+} release in bovine CCs (126). Caffeine effects displayed a "quantal" pattern as if Ca^{2+} release from the ER occurred in steps; thus, it was concluded that the caffeine-sensitive Ca^{2+} pool is composed of different functionally discrete stores (124, 126, 127). The presence of separate or overlapping Ca^{2+} stores sensitive to histamine or caffeine and their functional significance have been under debate since the 1990s (123, 392, 538, 589).

How the $InsP_3$-sensitive and the caffeine-sensitive Ca^{2+} stores interact was partly clarified in bovine CCs transfected with ER-targeted aequorin (17, 640). These experiments suggest that in CCs, the ER behaves as a single thapsigargin-sensitive Ca^{2+} pool that releases Ca^{2+} through a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism mediated by RyR or $InsP_3Rs$. Later reports suggested that in mouse CCs in the intact adrenal gland, CICR is either small or nonexistent (535), but in another study in cultured mouse CCs, a functional CICR mechanism was found (675). The contribution of the ER Ca^{2+} store to the regulation of exocytosis has been studied by several laboratories. In voltage-clamped bovine CCs, depletion of the ER Ca^{2+} store with a SERCA blocker either did not affect exocytosis (451) or depressed it (498). In a third study also in bovine CCs, ER Ca^{2+} depletion depressed the ACh secretory responses but not that triggered by depolarizing high-K^+ solution. This difference was explained assuming that ACh produces discrete and more localized $[Ca^{2+}]_c$ transients at sub-plasmalemmal regions, while high K^+ elicits greater $[Ca^{2+}]_c$ transients that spread quickly throughout the cytosol (145). Nevertheless, sustained stimulation of bovine CCs with caffeine caused ER Ca^{2+} depletion and a decrease of the K^+-elicited secretory responses. It seems, therefore, that depending on its grade of filling, the ER may behave as a Ca^{2+} sink when empty, or to augment $[Ca^{2+}]_c$ signals and exocytosis by a CICR mechanism when full (370).

The ER Ca^{2+} store could also have a regulatory function on Ca^{2+}-dependent pre-exocytotic steps, that is promoting the flow of secretory vesicles from a reserve pool (RP) toward the sub-plasmalemmal readily release vesicle pool. This is supported by experiments in voltage-clamped bovine CCs that use histamine to elicit mild $[Ca^{2+}]_c$ elevations elicited by ER Ca^{2+} release; although these $[Ca^{2+}]_c$ elevations were subthreshold for exocytosis, they caused a clear augmentation of the exocytotic response elicited by depolarizing pulses (652).

Redistribution of Ca^{2+} into, and release of Ca^{2+} from, mitochondria

Mitochondria use the driving force of the large electrical potential across their membrane (nearly −180 mV) to import vast amounts of Ca^{2+} through their Ca^{2+} uniporter (532). Mitochondria behave as a temporary Ca^{2+} store and export Ca^{2+} back into the cytosol by exchanging one Ca^{2+} for 2 Na^+ ions via the mitochondrial electroneutral Na^+/Ca^{2+} exchanger (mNCX). A less-active H^+/Ca^{2+} exchanger has also been described (105, 264). As the uniporter has a low affinity for Ca^{2+}, mitochondria take up large quantities of this cation efficiently only when overall intracellular Ca^{2+} concentrations are high, or if mitochondria are close to cytosolic high Ca^{2+} microdomains (HCMDs) that build-up upon Ca^{2+} entry through Ca_V channels at sub-plasmalemmal sites (640). This also occurs with HCMDs close to $InsP_3$ receptors that are sensed by neighboring mitochondria (537).

During stimulation, mitochondria of rat CCs behave as rapid and reversible Ca^{2+} buffers (40, 504). However, the use of Ca^{2+} probes in these experiments to monitor Ca^{2+} concentrations in the mitochondrial matrix ($[Ca^{2+}]_m$) revealed increases only in the low micromolar range (40). This is likely due to an underestimation due to the saturation of the Ca^{2+} probe. In bovine CCs, mitochondria also contribute to the clearance of large cytosolic Ca^{2+} loads (640, 678). Mitochondria-targeted aequorins with different Ca^{2+} affinities in bovine CCs revealed surprisingly rapid $[Ca^{2+}]_m$ transients, nearly in the millimolar range upon stimulation with ACh, caffeine, or high K^+ (453).

The impact on exocytosis of such avid mitochondrial Ca^{2+} uptake has been explored in several laboratories. For instance, dissipation of the proton gradient by protonophores, which drastically decreases the Ca^{2+} buffering capacity of mitochondria (453, 682), results in a pronounced augmentation of the exocytotic responses (452, 453). Such potentiated secretion has also been observed in fast-perifused bovine CCs stimulated with ACh, caffeine, or K^+ (112, 144, 145, 453), as well as in embryonic and adult rat CCs (636) and in mouse CCs after pharmacological interfering with the mitochondrial Ca^{2+} uniporter (MICU) and the mNCX (402). The inhibition of the Ca^{2+} uniporter also augments the secretory response of bovine CCs (682). However, in mouse CCs, the protonophores depressed the K^+-evoked $[Ca^{2+}]_c$ signals and the secretory responses (112). This could be due to a different expression of Ca_V channel subtypes and different inactivation rates during the blockade of mitochondrial Ca^{2+} sequestration by the protonophores (291).

An integrative view of Ca^{2+} handling by CCs

The rates of Ca^{2+} entry, its redistribution into CC organelles, its release back to the cytosol, and its extrusion to the extracellular space have been calculated with both direct and indirect approaches (640). Ca^{2+} entry into CCs through the various Ca_V channel subtypes is the primary determinant of the initial $[Ca^{2+}]_c$ transient. Thus, for a 15-µm diameter bovine CC, the rate of Ca^{2+} entry can be calculated from the measured Ca^{2+} inward current (692) or from the rate of $^{45}Ca^{2+}$ uptake into K^+ depolarized cells (32). The mean rate of Ca^{2+} entry from the two sets of experiments is around 500 µmol L cell^{-1} s^{-1}. After generating the sub-plasmalemmal HCMDs, which can reach concentrations as high as 10 to 100 µM (38, 471), Ca^{2+} redistributes into various intracellular compartments, particularly at the ER and mitochondria. This $[Ca^{2+}]_c$ elevation activates the SERCA and the ER avidly takes up Ca^{2+}; thus, during stimulation of bovine CCs (17, 640, 678) and rat CCs (295), the rate of uptake by the ER is about 60 µmol L cell^{-1} s^{-1}.

Mitochondria are highly effective in clearing $[Ca^{2+}]_c$ transients. Using mitochondria-targeted aequorin in bovine CCs maximally stimulated with K^+, it was found that mitochondria took up Ca^{2+} through their uniporter at about a rate of 1100 µmol L cells^{-1} s^{-1} (452, 453, 640). This value is close to that found for Ca^{2+} entry through Ca_V channels, meaning that most Ca^{2+} entering the bovine CC during stimulation is initially taken up by mitochondria and then released back into the cytosol through the mNCX. The rate of Ca^{2+} efflux through this transporter at 37 °C in bovine CCs is about 800 µmol L cells^{-1} s^{-1}; its kinetics are exponential and K_{50} approaches 20 µM Ca^{2+} (640). Finally, Ca^{2+} extrusion from the cytosol to the extracellular space depends on the activity of both SERCA and NCX. The joint action of both transporters decreases $[Ca^{2+}]_c$ at a maximal rate of 20 µmol L cells^{-1} s^{-1} in rat CCs at 27 °C (295, 504). In bovine CCs at 37 °C, this rate is close to 100 µmol L cells^{-1} s^{-1}.

After a brief opening of Ca_V channels in bovine CCs, an HCMD is formed near sub-plasmalemmal exocytotic sites (Figure 8). This HCMD has been estimated to have about 0.3-µM diameter and reach 10 µM or higher $[Ca^{2+}]_c$ (38, 129, 357, 442, 471–473). The formation of HCMDs is favored by colocalization of Ca_V channel clusters and secretory vesicles (490, 539, 690) to trigger fast exocytosis (54).

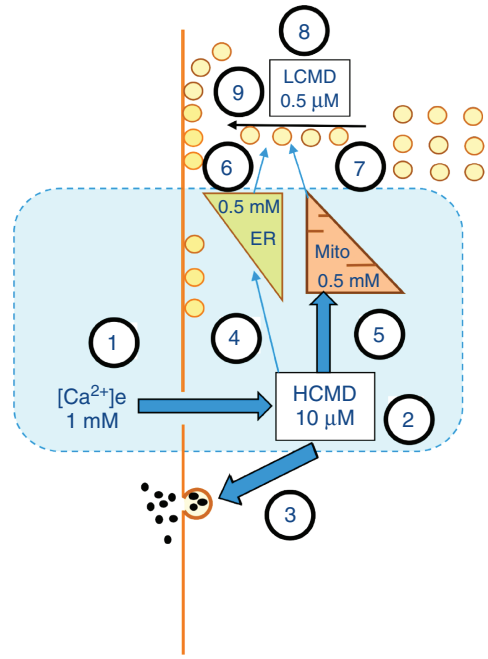

Figure 8 The concept of functional triads that regulate the generation of local $[Ca^{2+}]c$ transients and exocytosis responses upon stimulation of CCs. (1) After cell depolarization with the physiological neurotransmitter ACh, the voltage-dependent Ca^{2+} channels open. (2) Ca^{2+} enters the cell through a huge electrochemical gradient, giving rise to the formation of a high-Ca^{2+} microdomain (HCMD) of about 10 µM or higher near the sub-plasmalemmal exocytotic sites. (3) High Ca^{2+} is required to trigger the fast exocytotic release of CAs. (4) The HCMD quickly dissipates initially by mobile Ca^{2+} buffers (not drawn) and more slowly by Ca^{2+} uptake by the ER Ca^{2+}-ATPase (SERCA). (5) The second more relevant pathway for the clearance of the $[Ca^{2+}]c$ transient is the mitochondrial Ca^{2+} uniporter, which has a low-affinity high-capacity for Ca^{2+} sequestration into the mitochondrial matrix. (6, 7) In both ER and mitochondria (Mito), the matrix $[Ca^{2+}]c$ can reach near half a millimolar. ER Ca^{2+} can be released back into the cytosol through a CICR mechanism via ryanodine receptors, $InsP_3$ receptors (6), or through the mitochondrial Na^+/Ca^{2+} exchanger (7). (8) Ca^{2+} diffusion serves to redistribute Ca^{2+} at inner areas of the cell core to generate low-Ca^{2+} microdomains (LCMD) of around half micromolar that are required for the cytoskeleton-mediated Ca^{2+}-dependent vesicle traffic. (9) The Ca^{2+} levels of the LCMD refills with new vesicles the RRP at sub-plasmalemmal exocytotic sites, securing that new rounds of exocytosis take place, thus completing the exocytotic process.

The dissipation of HCMDs has two components. The fast one occurs in the time range of milliseconds and is associated to nearby cytosolic mobile Ca^{2+} buffers that accelerate Ca^{2+} diffusion and oppose the development of HCMDs (287, 480, 692). Consistent with this is the observation that added external Ca^{2+} buffers such as fura-2 increase the apparent rate of Ca^{2+} diffusion about fourfold (692).

The slower component of the $[Ca^{2+}]_c$ transient clearance is linked to the redistribution of Ca^{2+} into the ER and

mitochondria. This occurs particularly under conditions of intense stimulation of CCs. Using mitochondria-targeted aequorins with different Ca^{2+} affinities, two pools of mitochondria, M1 and M2, were characterized in bovine CCs (453). Mitochondrial pool M1, located near sub-plasmalemmal sites can sense the HCMDs occurring during physiological stimulation (295, 453, 504, 639, 640) (a local $[Ca^{2+}]_c$ rise of about 20 μM) and tune the mitochondrial generation of ATP to match the local energy needed for ATP-dependent vesicle docking at the plasmalemma and later exocytosis. Pool M2 exhibits a much lower rate of Ca^{2+} redistribution, which moves to inner regions to serve other functions, for instance, the intracellular transport of secretory vesicles from a RP to the readily releasable pool of vesicles (RRP) at sub-plasmalemmal sites (453, 640).

Concerning the functional role of ER Ca^{2+}, it is noteworthy that using ER-targeted aequorins, a reduction of 60 to 100 μM ER Ca^{2+} was observed in bovine CCs stimulated with high K^+, suggesting that CICR takes place during cell depolarization (17). RyR channels mediating CICR seem to colocalize with Ca_V channels and the mitochondrial uniporter of the M1 pool. Finally, after stimulation of CCs, the residual $[Ca^{2+}]_c$ released from the M1 pool of mitochondria and through the CICR mechanism from the ER is extruded out of the cytosol through the SERCA and the NCX.

We summarize our simplified view of the global Ca^{2+} handling by CCs in terms of what we refer to as a "functional triad" to shape the $[Ca^{2+}]_c$ transients. It has been proposed that functional triads formed by clusters of Ca_V channels, the ER RyR, and the mitochondrial Ca^{2+} uniporter are responsible for the generation and shaping of local sub-plasmalemmal $[Ca^{2+}]_c$ transients at the HCMDs that control the rate of exocytosis (17, 453, 640). Ca_V channels will act as the initiating signal, the RyR as the signal amplifier through CICR, and mitochondria as a barrier to limit diffusion further into the cell's core, where such HCMDs are not required. Ca^{2+} uptake into mitochondria has at least three functions, namely, to serve as a Ca^{2+} buffer, thus dissipating the large local Ca^{2+} transient; to redistribute the matrix Ca^{2+} into other mitochondria and the ER compartment into the cytosol; and to stimulate mitochondrial respiration and couple increased energy production to finely regulate the exocytotic machinery of the CC. As mitochondria release their matrix Ca^{2+} into the cytosol slowly, respiratory stimulation lags behind the cessation of cell stimulation, until the mitochondria Ca^{2+} load is completely cleared. Most Ca^{2+} taken up by mitochondria at sub-plasmalemmal sites may diffuse to other cell locations through the mitochondrial network, and eventually will be released near the cell core. Thus, mitochondrial Ca^{2+} release likely contributes, in a spatially specific way, to maintaining elevation of $[Ca^{2+}]_c$ after cell stimulation ends, to facilitate vesicle transport from a RP to a RRP (652). Ca^{2+} diffusion from sub-plasmalemmal sites to inner cytosolic sites is achieved by the propagation of a Ca^{2+} wave assisted by diffusible Ca^{2+} buffers and maintained by the CICR mechanism of the ER (17) (Figure 8).

From a physiological point of view, the concept of a functional triad suggests that plasma membrane Ca_V channels, together with the uptake and release of Ca^{2+} by ER and mitochondria, regulate Ca^{2+} handling in CCs. Ca^{2+} fluxes through these channels, and reservoirs are strongly activated during cell depolarization, but they also operate during inter-stimulation periods contributing to the Ca^{2+} homeostasis. Dysregulation of Ca^{2+} fluxes under pathological conditions at, for instance, the mitochondria will alter energy production and the exocytotic release of CAs. Recent experiments have demonstrated that Ca^{2+} handling and exocytosis of the sympathoadrenal axis and adrenal medullary CCs are altered in hypertension and various transgenic mouse models of neurodegenerative diseases (see below).

Exocytosis and endocytosis in CCs

Chromaffin granules

CCs are secretory cells and their most characteristic organelles are the CGs, which are very similar to the large dense-core vesicles (LDCVs) found in most neuroendocrine cells and neurons. In contrast to the synaptic vesicles found in neurons, CGs contain an intravesicular matrix that is visualized by electron microscopy as a dark structure within the CG. Granules are also of a larger size (≈200 nm) than synaptic vesicles (≈50 nm). Not only the size and matrix define CG, also the nature of their releasable cargo as they, beside amines and ATP, also release GABA, peptides, and granins. CGs contain astonishing concentrations of solutes whose resulted osmolarity would be expected to cause the swelling of CGs and their lysis. The ways used by nature to overcome the theoretical osmotic gradient from granule toward the cytosol have fascinated scientists for over five decades. A typical CG contains 0.8 to 1 M CAs, an estimated concentration of ATP of ≈200 mM, 40 mM Ca^{2+}, and significant amounts of peptides, nucleotides, and chromogranins. This inner cocktail, whose interaction enables the accumulation of neurotransmitters, is crucial for neurotransmission and for the successful secretion of hormones; processes that require the packaging of highly concentrated releasable cargo. Two components, chromogranins (166) and ATP (214), are essential for the accumulation of solutes. A concise description of the role of ATP and chromogranins contributing to the accumulation of adrenal CAs has been recently published (215).

The general view is that proteins that will form the granule matrix are synthesized in the ER, and protein assembly continues through the Golgi. CGs are produced in the trans-Golgi network by a budding process encapsulating the organized proteins that will form the matrix. The trans-Golgi network is also the sorting place for newly formed organelles. CGs are differentially sorted toward the regulated exocytosis pathway. A detailed description of the biogenesis and sorting processes occurring in neurosecretory cells is out of the scope of this article. However, excellent reviews have been published elsewhere (37, 604).

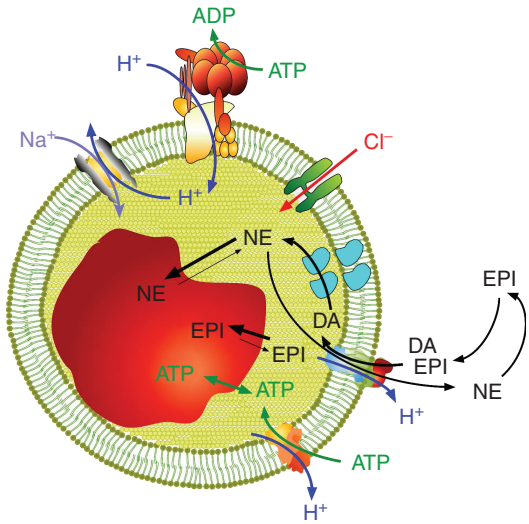

Figure 9 Main mechanisms involved in the accumulation of amines and ATP into the CG. A specific vesicular carrier (V-ATPase) pumps H^+ against a concentration and voltage gradient. To reduce the Ψ gradient, Cl^- channels open, allowing the pH to drop. The pH gradient is also regulated by Na^+ (and probably K^+) channels. Protons are used for exchange with CAs (dopamine, DA, or NE) or ATP. The synthesis of NE must occur inside the CG by the enzyme dopamine-β-hydroxylase (DBH, light blue) using DA as a substrate. In adrenergic cells, NE must leave the granule to be transformed into EPI in the cytosol (by an enzyme called phenylethanolamine-*N*-methyltransferase, PNMT, not shown) Most solutes bind the granule matrix, thus permitting their accumulation by maintaining the isotonicity of CG versus cytosol. Ca^{2+} is accumulated and released by a combination of H^+/Ca^{2+} exchange, Ca^{2+} pump, $InsP_3$-receptors (683), and ryanodine receptors (552). For clarity, Ca^{2+} turnover is not shown.

The inner milieu of CG is then further acidified from the Golgi value (pH ≈ 6.5) (555), and the matrix condenses to allow the uptake of amines, ATP, and Ca^{2+}. It is generally accepted that acidification will occur rapidly and that the uptake of cargo species starts immediately. This process is driven by an ATP-dependent specific H^+ pump, the vesicular proton pump (V-ATPase), which accumulates H^+ until the Ψ gradient reaches around +80 mV. To obtain the requisite lowering of pH, Cl^- enters CG acting as a counterion, and finally, the interior milieu reaches pH ≈ 5.5. Protons are then used as a counter-carrier to concentrate CAs, ATP, and Ca^{2+}. Thus, any alteration in the pH gradient will result in a loss of accumulation of these three species (94, 552). Most solutes will bind chromogranins, thus forming the granule matrix (Figure 9). It has been estimated that more than 90% of amines (669) and Ca^{2+} (552) are tightly bound to the matrix, thus forming a bicompartmental store, which will become important to determine the kinetics of exocytosis (558) and the participation of CGs in cellular Ca^{2+} dynamics (93). It should be noted that although CGs constitute approximately 20% of CC volume and contain Ca^{2+} at 40 mM, they do not appear to be a functionally important part of Ca^{2+}dynamics/regulation as are the ER, mitochondria, and plasma membrane (683) (Figure 9).

The complex process of secretion

As CCs are cellular machines evolutionarily optimized for secretion, they contain all the elements necessary to readily release solutes to the bloodstream. Each CC possesses around 12,000 CGs (671). The total amount of CAs present in both adrenal glands, if released instantly, would be a lethal dose for the organism. To prevent CA poisoning, CCs have multiple mechanisms to regulate secretion. An important initial limiting factor is that only a small fraction of CGs are available (the "readily releasable pool") for immediate release, with 70% to 80% of granules requiring further priming before they are available for secretion.

CG release occurs by regulated exocytosis, a Ca^{2+}-dependent process that entails the fusion of a CG with the plasma membrane. Roughly, exocytosis starts with the approach of a CG to the membrane, docking it and the formation of a fusion pore, and the expansion of the fusion pore allowing the release of CG products (cargo), followed by CG membrane recapture from the plasma membrane by a process called endocytosis. Exocytosis is common to most secretory cells, including neurons, and most of the molecular steps leading to vesicle exocytosis are shared by neurons and CCs. This has been one of the historical reasons for the use of CCs to unveil the mechanisms underlying secretion (470). Probably, the first evidence indicating that the secretion of neurotransmitters occurs by exocytosis was the finding of intravesicular proteins in the extracellular media after the stimulation of adrenal medulla. No other mechanism could easily explain how CgA (a soluble protein of ~70 kDa discovered in CGs) could cross the plasma membrane together with CAs (62). Once the mandatory role of Ca^{2+} for secretion was established (176, 360), the main question was to determine how Ca^{2+} entry could trigger a very energy-unfavorable reaction like the fusion of two membranes. The elucidation, mainly in CCs, of the main steps of protein-protein, lipid, and lipid-protein interactions involved in triggering and regulating exocytosis has taken more than 40 years of extensive research.

The sites of exocytosis, either in CCs and in synapses, are not widely distributed along the plasmalemma but restricted to specialized areas, active zone-like release sites. These regions, characterized by cytoskeletal elements arranged to direct SGs toward docking and fusion sites, are specifically enriched in lipids like cholesterol and PIP_2 (306, 363, 662), contain the acceptor proteins for fusion, and are relatively close to Ca_V channels, hence the fusion machinery is in close proximity to the Ca^{2+} source. Contrary to active zones in neurons, where the probability of exocytosis is high, the role or even the presence of the piccolo/bassoon anchoring proteins has not been described in CCs so far. Consequently, the transport of CGs to active zone-like release sites likely performed sequentially by tubulin and actin. F-actin was initially considered to fulfill a role as a cortical barrier that prevents the access of granules to the plasmalemma (39, 125, 642). This concept has evolved, and the cytoskeleton is currently considered a dynamic element, where a close interaction of actin filaments

with granules occurs (326, 369, 486, 592). Myosins II, V, and VI also seem essential for the delivery of granules from the Golgi to the periphery of the cell (545, 618, 641). Figure 10A illustrates our current view of the SG cycle in CCs. The F-actin cytoskeleton and myosin II could also be important for providing the "extrusion, squeezing" force needed for the fast release of neurotransmitters through the open fusion pore (466, 666).

The secretory machinery

The discovery that exocytosis can be selectively blocked by proteolytic neurotoxins like tetanus and botulinum toxins (359) indicated the existence of specific exocytotic proteins, as these toxins do not affect endocytosis (465). In addition, the discovery of the *N*-ethylmaleimide-sensitive factor (NSF) and the protein complexes involved in its action (the soluble NSF-attachment receptor complex or SNARE) (594, 595) started a stimulating scientific race to elucidate the molecular steps of regulated exocytosis. Although not all of the exocytotic steps have been defined, there is a general consensus about the main actors and steps involved in the process, which are summarized in Figure 10B (685). The so-called SNARE complex is formed by the interaction of one specific protein in the membrane of the granule (synaptobrevin or VAMP)

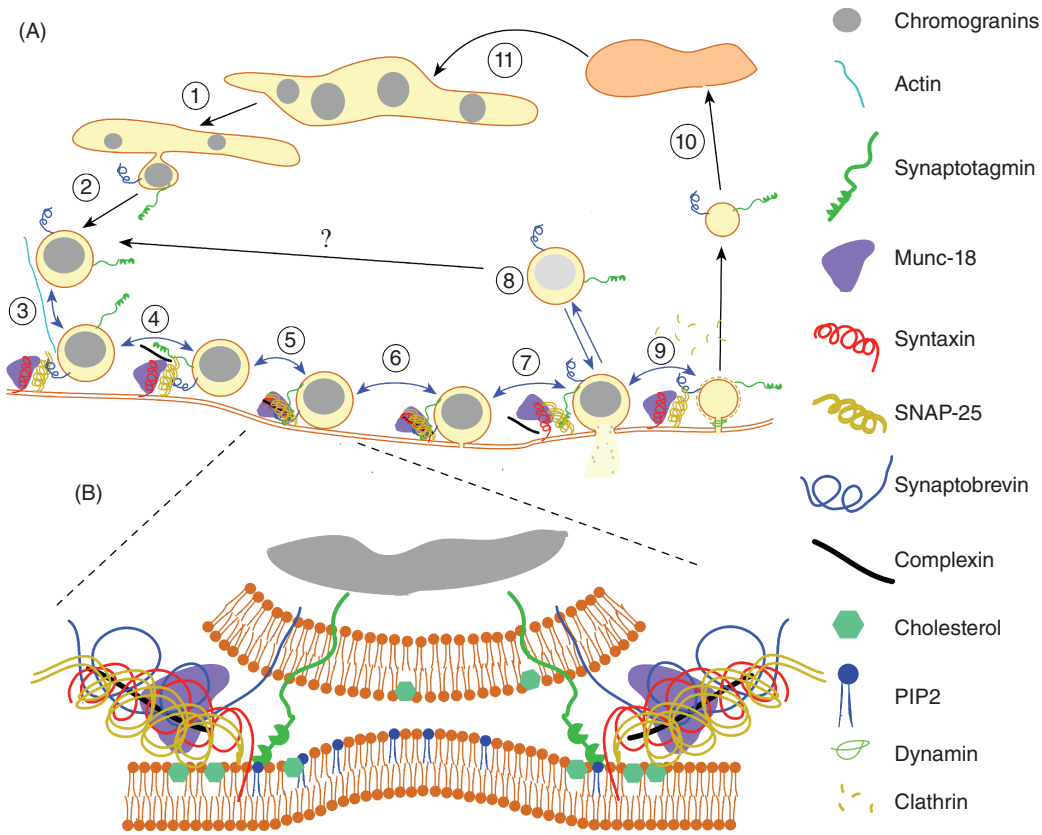

Figure 10 Exocytosis and endocytosis of CGs. (A) For clarity, only one SNARE complex is shown, while some of the accessory proteins are omitted: (1) granule proteins are sorted and packaged into the Golgi apparatus; (2) granules are transported to the release sites by tubulin (not shown); (3) actin filaments drive granules to specific tethering points; (4) "granule docking," the SNARE complex starts to organize and the granule docks to the plasmalemma; (5) priming, mediated by Munc-18 and complexin, thus allowing a tighter interaction of SNARE complexes. Munc13 acts on syntaxin changing its conformation, thereby leading to SNARE proteins zippering and the formation of fusion pore; (6) the initial fusion pore allows a limited exchange of water and solutes; (7) fusion pore dilatation allowing the partial or complete release of the granule content; depending on the extent of dilatation and duration of the Ω state, more or fewer solutes will be released. The SNARE complex is disassembled; (8) cavicapture. This partial exocytosis occurs when a dilated, but reversible, fusion pore allows the partial release of small molecules like peptides. Whether these granules can go to the SRP or get exocytosed again is unknown; (9) membrane recovery by endocytosis. Two major mechanisms might be involved: depending on clathrin and dynamin 2 or clathrin-independent that uses dynamin 1 to promote granule fission. The inner content of the granule rapidly acidifies; (10) clathrin is disassembled and the granule can travel deeper inside the cell toward either endosomes or lysosomes from where they can reenter in the secretory cycle after sorting in the Golgi (11). Note that sizes of the resulted endocytotic granules are now smaller, and granule matrices are clearer as a result of protein loss during exocytosis. Note also that some of the steps are reversible (blue double head arrows). (B) Organization of the SNARE complex in the priming state. The presence of cholesterol and lysophospholipids allows the curvature of the cell membrane. Note the lateral disposition of coiled-coil of proteins and the proposed situation of Munc-18 and complexin.

and two proteins at the plasma membrane (syntaxin and SNAP-25) (see Ref. 163 for a recent review). The exocytosis of CGs is much slower than that of synaptic vesicles, probably because despite using similar Ca^{2+}-triggering mechanisms and exocytosis machinery, they differ in the degree of preparation (docking) for fusion and in the composition of their vesicular matrix. Only six proteins, syntaxin, synaptobrevin, SNAP-25, Munc18-1, synaptotagmin, and complexin, form the core of the exocytosis machinery. Once the energetic barrier opposing membrane fusion is overcome, the interior of the granule is connected to the external media, thus starting the release of its contents.

The following are the currently most accepted steps involved in exocytosis (Figure 10B). The remodeling of the actin cytoskeleton allows SGs to get in contact with the plasma membrane (docking). At this stage, SNARE proteins are not yet fully associated. Ca^{2+} and the interaction of Munc13 and CAPS are crucial mechanisms involved in the priming process (423). Then, complexin binds to the SNARE complex, hence leaving it ready for Ca^{2+} action (super priming). Ca^{2+} will subsequently bring synaptotagmin to bind syntaxin, thereby promoting fusion pore opening (partial fusion). If Ca^{2+} concentration is sufficiently large or maintained long enough, the process evolves to "full fusion." Probably, there are intermediate steps between partial and full fusion. CCs possess two types of synaptotagmin (1 and 7), which have been implicated in the fast and slow components of the secretory response, respectively (528, 557). Ca^{2+} binds synaptotagmin at two binding sites (C1 and C2) to trigger granule fusion.

Pools of secretory granules

Since the first rudimentary recording of the time course of CA release (Figure 11A), it became clear that adrenal response decreased progressively regardless of stimulus persistence. This signaling "fatigue" is probably a physiological mechanism of defense to avoid the deleterious effect of oversecretion of CAs into the bloodstream, as occurs in some forms of adrenal pheochromocytoma (see below). This phenomenon is partially dependent on desensitization of nAChRs as well as Na_V and Ca_V channels (45). However, the "fatigue of secretory response" was also observed in the so-called leaky cell using either electroporation (43) or detergents like digitonin (184), indicating that the secretory machinery cannot maintain secretion for long periods of time, especially during strong stimuli (Figure 11B).

With the arrival of electrophysiological techniques, especially whole-cell capacitance, the phenomenon of "fatigue" was localized to the existence of distinct pools of granules (Figure 11C) with different probabilities of fusion (277, 387, 472). The nomenclature used to classify the differences in the release rate of CG populations varies from one research group to another, with sometimes confusing results. Hence, the granules that were ready to enter exocytosis in the first seconds after depolarization, as they are close to the "open mouths of Ca^{2+} channels" were considered to be in the immediately releasable pool (IRP). This pool would comprise only about 17 granules per cell in mouse CCs (582), although its exact size is difficult to determine as most studies were carried out in cultured cells whose channel distribution is significantly affected by denervation and adhesion to the cell culture surface.

Large Ca^{2+} entry, or sudden elevation of Ca^{2+} experimentally caused by uncaging of trapped Ca^{2+} using light pulses, will promote the release of about 200 granules from a CC within 20 to 40 ms when intracellular Ca^{2+} levels are around 20 μM (644). This is the rapidly releasable pool (RRP), which is observed by whole-cell capacitance as a rapid increase in cell surface resulting from the addition of granular membranes to the plasmalemma (Figure 11C). The analysis of the kinetics of release observed either by whole-cell capacitance or by integrating amperometric recordings shows that the rapid secretion from the RRP is followed by a slow phase of release from an RP or slowly releasable pool (SRP) with a time constant of ≈200 ms (644).

Finally, it should be noted that most CGs are either nonreleasable or are exocytosed only very slowly. The physiological role of this unreleasable pool has received little attention from scientists. Historically, it was considered as formed by immature granules that progressively move toward the releasable pools. However, novel experimental approaches have concluded that young granules will "jump the queue" and be the first released. This was shown using either total internal reflection fluorescent microscopy (TIRFM) with fluorescent dyes that changed color with age (180) or by analyzing the secretion of EGFP from CCs expressing EGFP-tagged neuropeptide Y (214) (Figure 11D). The classical theory uses a geographical distribution of granules to explain the kinetics of release, assigning to the RRP those granules situated near the membrane and to the RP those that are behind the actin barrier. TIRFM analysis suggests that only a small fraction of the granules that are already within the evanescent plane (<200 nm from membrane) do not get exocytosed and remain immobile for minutes regardless of the presence of stimuli. The unreleasable pool in CCs is about 70% to 80% of the whole population (≈10,000 granules per cell), and its physiological role is still unknown. Furthermore, it is the RRP that requires repletion, after its reduction by stimulation. Theoretically, refilling the RRP could be accomplished by (i) refilling of granules only partially exocytosed (kiss-and-run or cavicapture), (ii) formation of new granules after a short travel through endosomes, or (iii) the arrival of new granules to the cortical domain of the CCs. The precise contribution of each alternative is still under debate.

The exact contribution of the different pools to secretion is difficult to establish because of the Ca^{2+} dependence of both exocytosis and granule motion processes. However, the latter requires lower intracellular Ca^{2+} levels (≈1 μM) compared with the higher concentration required for exocytosis (>2 μM). These features have been used to determine the

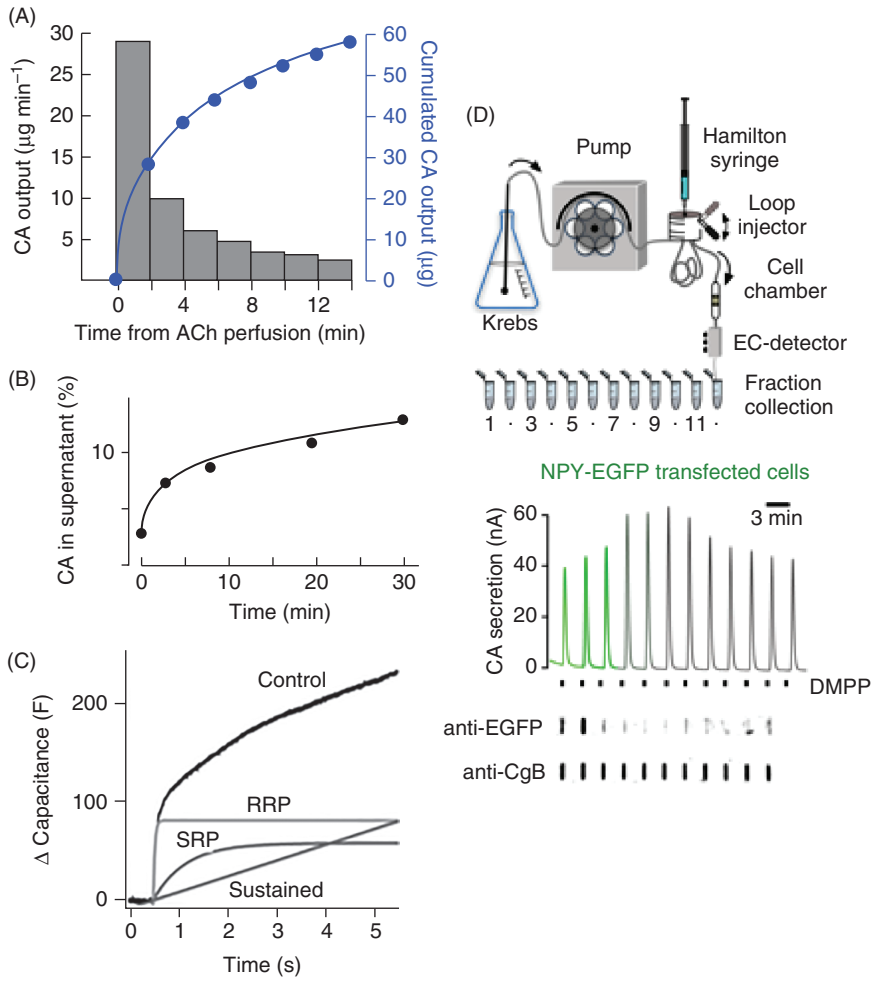

Figure 11 Different pools of granules are involved in the secretory responses. (A) Even in the continuous presence of Ach, the secretion of CA from perfused cat adrenals progressively decays (gray bars). Cumulative CA secretion is superimposed (blue trace). Modified, with permission, from Douglas WW, and Rubin RP, 1961 (176). (B) Although the time courses are different, a similar situation occurs when secretion is elicited by rising intracellular Ca^{2+} in permeabilized CCs (leaky cell). Modified, with permission, from Baker PF, and Knight DE, 1981 (44). (C) Recordings of whole-cell capacitance from mouse CCs evidence the presence of at least two exocytotic kinetics: rapid (RRP) and slow (SRP). Authors tested the effect of rising intracellular Ca^{2+} by flash photolysis of NP-EGTA (taken from Figure 2A in Ref. 591). Notice the different time course compared with panels A and B. (D) Newly produced granules are the first to be released. Perfused CCs are stimulated with a nicotinic agonist and CA secretion continuously recording by an electrochemical detector. The analysis of the perfusate shows that EGFP (labeling neuropeptide Y) is released only during the first pulses. Modified, with permission, from Estevez-Herrera J, et al., 2016 (214).

size and contribution to the secretion of RRP and SRP, that is by low versus high levels of intracellular Ca^{2+} released dose-dependently by controlled flash photolysis of Ca^{2+}-caged compounds (168, 643). An important limitation of the capacitance measurements to indicate the extent of CG release is the difficulty in determining, and subtracting, the contribution of endocytosis to the recordings. Capacitance change recordings might overestimate secretion as it also detects the fusion of granules that are devoid of CAs, a phenomenon observed when capacitance and amperometry measurements of exocytosis are compared (599).

Measuring exocytosis

Exocytosis from CCs has been measured by several methods including the chemical analysis of secreted products. However, the latter occurs too slowly for assessing a phenomenon that occurs in tens of milliseconds. For this reason, only modern live imaging, electrophysiological or electrochemical techniques, and the combination of them get a reliable time resolution. TIRFM uses the evanescent wave principle, by which excitation is restricted to ~150 nm beneath the cell surface situated in direct contact with the bottom coverslip. This noninvasive technique also allows examining the movement

of a granule prior to exocytosis and also membrane recapture. However, it requires the use of fluorescent dyes (170).

Cell capacitance change (ΔC) measurements with the patch-clamp are based on the notion that the cell membrane is a capacitor and its capacitance increases when the granule membrane fuses with the plasmalemma (or decreases when a portion of the membrane is retrieved during endocytosis). Modern equipment incorporates lock-in amplifiers to resolve exocytosis occurring at the whole-cell level. This is an invasive technique that permits control of the intracellular media but in general, is unable to resolve single-granule fusion events and is also blind to all phenomena occurring prior to fusion. The advantage of ΔC measurements is the monitoring of the whole-cell secretion and not just that restricted to the area covered by an amperometric electrode or the attached surface of a glass cover slide as in TIRFM measurements. The capacitance changes observed from a whole cell are the sum of two phenomena (exocytosis and endocytosis) that usually overlap. The RRP can be measured by applying strong stimuli to promote the fusion of the entire pool, whereas the refilling of this pool could be measured by the slower kinetics that follows the initial rise in capacitance (Figure 11C). One variation of the technique uses the cell-attached patch-clamp configuration to resolve single exocytotic events, allowing the study of the initial stages of fusion pore formation. These observations, however, are restricted to the patched area (386).

Electrochemical methods comprise amperometry and cyclic voltammetry. They detect the CAs released with a precise time resolution but do not detect other secreted species like ATP or peptides. This technique is noninvasive (the cell integrity is preserved), but it does not provide information about what happens to granules prior to exocytosis nor after their recapture (endocytosis). Voltammetry has less time resolution but allows the quantification of the released CAs. Single-cell electrochemistry also allows the analyses of the kinetics of exocytosis, including the pre-spike phenomenon—the so-called foot—that is associated with the escape of CAs through the fusion pore. However, some of the published data should be taken with caution unless proper calibration and correction for artifacts (e.g. use of the same calibrated electrode throughout the experiment, reduction of bias caused by changes in electrode responsiveness, and appropriate statistical analyses) are employed (69, 412). The kinetics of the exocytotic fusion pore can be resolved with high-resolution amperometric techniques (668). Thus, with a carbon-fiber microelectrode placed on the surface of a CC, several modes of exocytosis can be distinguished: (i) CA release occurs through a transient fusion pore that opens and closes without giving rise to full fusion and pore expansion, the so-called kiss-and-run; (ii) formation of a more stable fusion pore that, however, does not give rise to full fusion, the so-called kiss-and-stay exocytosis; (iii) formation of a fusion pore with subsequent expansion to release all vesicle content, followed by the collapse of the vesicle membrane and its integration in the plasmalemma, referred to as "full fusion" (630). Another interesting feature of the individual amperometric spikes is that many of them are preceded by a small "foot" (fusion pore formation) before the abrupt upstroke occurs (pore expansion and full fusion, see below) (130).

Finally, amperometry and cell-attached capacitance measurements have been successfully combined for simultaneous measurements (9). This technique, called patch amperometry, allows the direct characterization of fusion pore and the direct measurement of granule content of CAs (254, 454). The main disadvantage of this procedure is its technical difficulty.

Partial versus full exocytosis

Classically, exocytosis has been considered as an all-or-none process. The concept of partial exocytosis, that is when only one part of the granule content is released upon fusion, was first suggested by Bruno Ceccarelli in frog neuromuscular junction (119). This phenomenon, however, received real attention only after it was observed in mast cells and CCs (9, 18) and has finally gained acceptance as a technology for measuring secretion evolved. The stable and reversible formation of a fusion pore allows the release of cargo species through its limited diameter and lasting duration (170). There is not a general consensus about the definition of the fusion pore: the concept ranges from a channel-like structure (with a diameter of 1–2 nm), measurable by capacitance, to a wider Ω-shaped vesicle as observed by electron microscopy (of about 20–30 nm in size). The former probably is governed by a mixture of lipids and proteins, whereas the expanded pore seems to be composed only of lipids (574). Nevertheless, it is now accepted that most of the exocytotic events are partial, and the granule usually retains a significant fraction of its content after fusion. This comprises both low molecular weight species such as CAs and high molecular weight proteins such as chromogranins and their fragments.

The comparison of the quantum size measured in the same cell cultures by amperometry and patch amperometry (455) or by "electrochemical cytometry" (181) indicates that only about 30% of CAs are released in most of the exocytotic events. In addition, the monitoring of fluorescent-labeled CgA (chromogranin coupled with EGFP) by TIRFM shows that most granules retain some fluorescence after fusion (170). An emerging novel concept about the regulation of secretion of CAs comes from the amount released during partial exocytosis (491). It seems that strong stimuli tend to favor full release, whereas mild stimulation triggers partial release. The main regulators of this process seem to be the intracellular Ca^{2+} concentration (199) and cAMP (413). The nature and physiology of the fusion pore have been recently reviewed (19).

Endocytosis

The increase of membrane resulting from secretion or constitutive exocytosis has to be compensated by membrane retrieval. This fission process is called endocytosis, and it is necessary not only to maintain constant the membrane surface

but also for renewal of proteins and lipids from the membrane. This latter type of endocytosis, named "constitutive," is common to all cells and it is not dependent on Ca^{2+}. Constitutive endocytosis is used to retrieve receptors, carriers, and channels, and it is essential for downregulation processes common to transporter activity, receptor desensitization, and, more generally, cellular responsivity (285, 649). This is a slow process, and does not necessarily occur at the site of exocytosis even for proteins involved in the exocytotic process itself.

Triggered endocytosis occurs in CCs rapidly after exocytosis (Figure 10). Plasma membrane internalization does not occur randomly to maintain the total surface stable. Rather, despite full fusion, exocytosed granules and plasmalemma maintain their specific protein and lipid composition. This exo-endocytosis coupling was first observed by morphological studies showing that granule membrane-bound components are retrieved after exocytosis (507). Also, a large increase of *coated pit* structures containing granule components immediately after stimulation of CCs was observed by electron microscopy (245). The endocytosis process seems to occur at the same lipid-raft structures where exocytosis took place. These areas are enriched in both cholesterol and phospholipids like PIP_2 (322). Taken together, these data indicate that exocytosis and endocytosis are tightly coupled phenomena intended not only to maintain the cell surface constant but also to recapture most of the granule membrane components and part of the granule matrix.

In CCs, two endocytic pathways exist: a rapid endocytosis, which takes place in tens of milliseconds to a few seconds, and it is probably associated with cavicapture and kiss-and-run secretory modes (605), and a slow clathrin-mediated exocytosis (200). Rapid endocytosis does not require clathrin and seems to involve GTP, Ca^{2+} (85, 541), and dynamin-1 (34). The clathrin-mediated endocytosis is the best-studied and involves clathrin and dynamin-2, but not Ca^{2+}; this slow endocytosis occurs in tenths of seconds and can last for minutes (200). Dynamins are envisaged to function in constricting the fusion pore and promoting the formation of an endocytic vesicle capable of traveling toward the cell interior. It is, therefore, possible that these proteins can also regulate the amount of cargo released during each exocytotic event. Data derived from dynamin manipulation in CCs are still contradictory (128, 233). Even the expression of dynamin-1 in CCs has been questioned (255). Likewise, the functional role of amphiphysin, another protein involved in fusion pore constriction in other tissues, has not been conclusively established in CCs.

Endocytosis often results in retrieving more membrane than that previously incorporated during exocytosis. This excess retrieval is faster than rapid endocytosis and seems to be triggered by different mechanisms (207, 579) unlinked to exocytosis and at a rate that could internalize approximately 10% of the total cell surface within a few seconds (49). Excess retrieval is frequently accompanied by the formation of large nonreleasable endosomes (611). This formation of large endosomes during excess retrieval is called "bulk" endocytosis (111, 533) (for clarity, this is not shown in Figure 10).

Clathrin-dependent membrane retrieval produces vesicles of 50 to 100 nm, notably smaller than CGs (180–250 nm). The discrepancy in size suggests that the membrane from CGs can be recaptured as small pieces rather than as a whole (311). Both recycling from partial exocytosis and a degradation pathway from full granule collapse coexist, and their fraction may depend on secretory activity, as mentioned above. Vesicles resulting from the endocytotic clathrin-mediated pathway might go to early endosomes to produce new granules or fuse with lysosomes for degradation. The degree of contribution of both pathways and their regulation is not known. Since retrieved vesicles are composed of pieces from CGs and not as a whole, the early endosomes could be a transient organelle where endocytic vesicles are repackaged to reconstitute functional granules before going to the retrograde transport pathway to the trans-Golgi network (311). Using specific antibodies against membrane-bound granule proteins, it was shown that they were taken up through clathrin-mediated vesicles and internalized toward lysosomes (56). These proteins were then found in the trans-Golgi, and a few hours later were localized in newly formed granules (507, 649) (Figure 10A).

From *kiss-and-run* to full granule collapse, there are different degrees of exocytotic modes. The same can be said for endocytosis where several mechanisms for membrane retrieval coexist. Capacitance changes and patch-amperometry studies conclude that the fusion pore can open and close (or "flicker"); this suggests that this transient structure can be reversible at least up to certain pore size. Therefore, kiss-and-run retains membrane and most of the matrix proteins of CGs. The limit for reversibility for CGs is around 3.4 nm, as larger sizes convert fusion to an irreversible process (9, 15). However, observations using TIRFM (512) and fluid-phase capture (650) suggest that even larger pores are reversible. Many CGs reseal in a few seconds after exocytosis. These granules are rapidly acidified (606) and might be partially or completely empty of CAs and other soluble components (599). The physiological contribution and importance of the cell mechanisms operating, for the various modes of exocytosis/endocytosis, opens exciting research prospects for the coming years.

The Sympathoadrenal Medulla Axis in Stress Responding: Roles of PACAP, Other Transmitters and Peptides, and Organ Remodeling

PACAP as a stress transmitter at the splanchnicoadrenomedullary synapse

The ability to generate an AP via nAChR-dependent Na^+ channel activation on CCs explains how ACh acts as a secretagogue for EPI release from the adrenal medulla (see the

preceding sections). In the late 1970s, however, the possibility of adrenomedullary CA secretion by first messengers other than ACh began to emerge. The Wakade laboratory in particular, explored the long-term changes in CC content driven by high stimulation rates thought to be typical of the frequency of firing of the splanchnic nerve during stress. It was discovered that the adrenal medulla releases an amount of CAs equivalent to its entire initial content during a bout of secretion lasting several hours. Yet even after such prolonged secretion, CCs remain able to continue CA secretion, due to constant CA repletion via biosynthesis during the secretory episode (see (576, 659) for similar observations in the calf adrenal gland). How the adrenal gland resynthesizes its entire store of accumulated EPI, as well as its complement of releasable proteins and peptides, following prolonged and even acute but intense secretion, has been a subject of considerable investigation, subsumed under the rubric of "stimulus-secretion-synthesis coupling" (3, 190). One mechanism for enhanced repletion of CAs linked to increased depletion by secretion was discovered in the 1970s by Costa and Chuang. They learned that exposure to cold, which is associated with massive sympathoadrenal activation, causes an increase in the total capacity to convert tyrosine to L-Dopa within the adrenal medulla (126), due to induction of tyrosine hydroxylase (TH), the rate-limiting step in CA biosynthesis (131). Since nicotinic cholinergic stimulation has a limited ability to induce TH as well as peptide synthesis in the gland, it was concluded that this "stimulus-secretion-synthesis coupling" might be afforded by activation of mAChRs on CCs following enhanced splanchnic nerve stimulation during episodes of stress (217). It was noted subsequently, however, that reflex stimulation of the splanchnic nerve *in vivo*, by pharmacological treatment with reserpine, caused an induction of TH that could not be reversed by blockade of either nicotinic or muscarinic receptors, and these authors concluded that either a novel type of cholinergic receptor was expressed on CCs, or that a noncholinergic substance might be released along with ACh from the splanchnic nerve, to allow TH induction under conditions of stress (610). Similarly, stimulation of the sympathetic trunk at high frequency also causes TH induction in the postganglionic sympathetic neurons of the superior cervical ganglion, and this also occurs by a mechanism only partially accounted for by action of ACh released from preganglionic terminals (318).

The discovery of the neuropeptide PACAP (447) by Miyata, Arimuraa, and colleagues in 1989 finally offered a clue to the identity of this additional sympathetic neurotransmitter. PACAP, as its name implies, was initially discovered as a hypothalamic peptide able to stimulate cAMP production in cells of the anterior pituitary. However, it was subsequently learned that the physiological role of PACAP extends far beyond its role as a hypothalamic releasing factor, to that of neurotransmitter in both brain and autonomic nervous system (26). In particular, and consistent with a potential role as a splanchnicoadrenomedullary transmitter along with ACh, PACAP potently stimulates EPI secretion from the adrenal gland *in vivo*, from the perfused adrenal gland, and from CCs in primary culture (155, 265, 270, 302, 600, 663; and references therein).

The localization of PACAP to the splanchnic nerve innervating the adrenal medulla required the creation of mice genetically deficient in PACAP expression. This in turn allowed the certification of antibodies to PACAP that could be shown clearly to detect PACAP specifically, especially compared to the very closely related neuropeptide VIP, in the cholinergic nerve terminals of the splanchnic nerve (269). The role of PACAP in stress-induced CA release and biosynthesis in the adrenal medulla was further examined with the aid of PACAP-deficient mice. Injection of insulin lowers blood glucose levels and stimulates maximal reflex firing of the splanchnic nerve, causing greatly elevated blood EPI levels and enhanced TH activity in the adrenal gland: these effects were blunted or abolished in PACAP-deficient mice. PACAP-deficient mice could be rescued from death after prolonged hypoglycemia by treatment with either PACAP or glucose (269). In adrenal slices taken from the mouse, EPI secretion upon splanchnic nerve stimulation at high frequency (2 Hz), but not at low stimulation rates (0.2 Hz), is blocked by the PACAP antagonist $PACAP_{(6\text{-}38)}$ and is abrogated by PACAP deficiency (i.e. is absent in slices from PACAP-deficient mice) (368, 593).

In summary, PACAP is colocalized with ACh (i.e. the cholinergic vesicular marker VAChT) at nerve terminals of the splanchnic nerve that innervate CCs in the mouse. Stress-induced CA secretion and TH regulation require the expression of PACAP. Experiments with adrenal slices *ex vivo* show that this PACAP dependence occurs at the level of the splanchnic nerve itself, and is probably due directly to the release of PACAP there, since the morphology and chemical neuroanatomy of the splanchnicoadrenomedullary synapse in PACAP-deficient mice are otherwise normal. These experiments suggest that PACAP released at the adrenomedullary synapse mediates CA secretion from the adrenal medulla during stress (see Figure 12) (189, 580). Residual CA release provoked by prolonged hypoglycemia even in the PACAP-deficient mouse suggests that ACh also contributes to stress-induced CC CA secretion *in vivo* (269), consistent with the many previous reports of high-frequency CA secretion from the adrenal glands of various mammalian blocked by nicotinic and muscarinic antagonists (342, 356, 364, 374, 623, 658, 684). Of relevance to this issue are the observations of Arun Wakade's lab on isolated perfused adrenal glands from rats (657). The group reports that prolonged high-frequency splanchnic nerve stimulations (10 Hz for >3 h) produce the massive release of CA that is almost completely abolished (>80%) by cholinergic receptor antagonists, while sustained low-frequency stimulations (1 Hz for >6 h) are effectively controlled (>60%) by noncholinergic receptors activation (PACAP and VIP) (422, 656).

It is evident from the above arguments that the relative contributions of ACh and PACAP at the splanchnicoadrenomedullary synapse during high levels of splanchnic

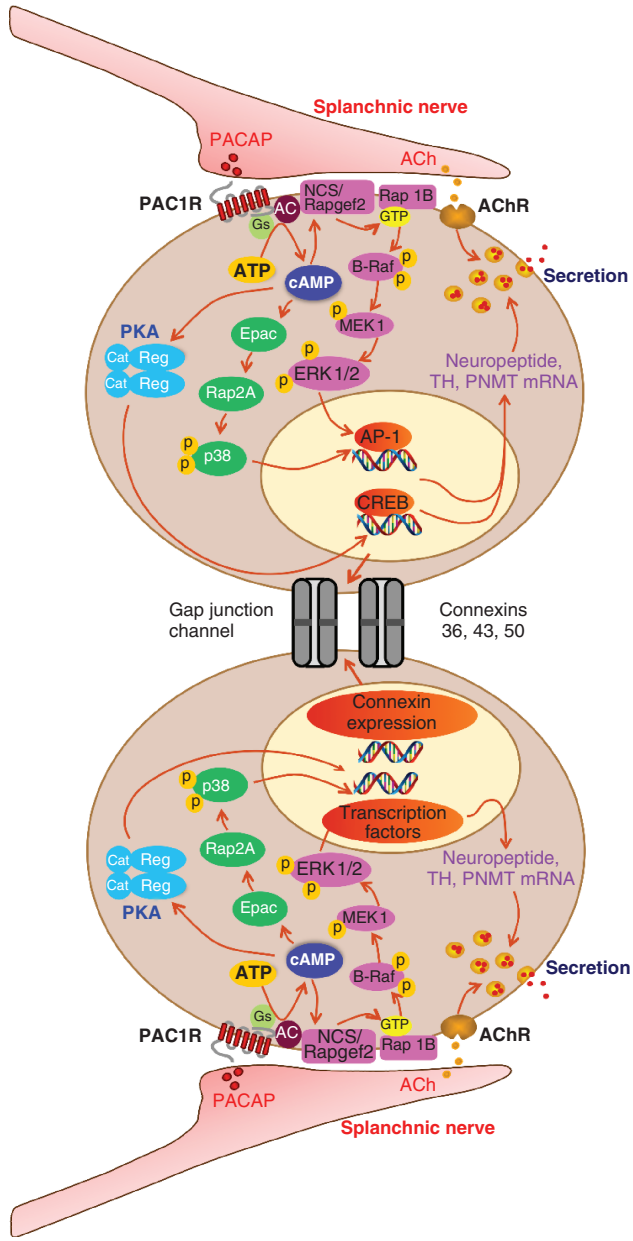

Figure 12 Intracellular signaling pathways and physiology of the chromaffin cell. Acetylcholine and PACAP (sympathetic preganglionic) release from splanchnic nerve activates the nicotinic (AChR) and PACAPergic (PAC1R) receptors, triggering catecholamine and neuropeptide secretion from CG, and increased transcription of genes encoding biosynthetic enzymes for catecholamines (TH, PNMA), and prohormones from which processing to mature neuropeptides occurs, and stimulus-secretion-synthesis coupling in the chromaffin cell. PAC1R activation promotes Gs coupling to adenylate cyclase (AC), the elevation of cyclic AMP (cAMP), and the activation of three separate cAMP effectors. Protein kinase A (PKA) mediates CREB-dependent gene transcription; Epac mediates Rap-dependent activation of the MAP kinase p38, leading to activation of transcription factors including AP1; NCS-Rapgef2 mediates Rap-dependent activation of the mixed-function kinase B-Raf, allowing MEK upregulation of the MAP kinase ERK (extracellularly regulated kinase) and gene activation through a combination of transcription factors. Stimulus-secretion-synthesis coupling also involves on the intercellular level increased expression of connexins that form gap junctions, which help to amplify chromaffin cell secretion from the adrenal gland as a whole. There is as yet no direct proof for PACAP or acetylcholine as the principal mediator of what might be termed the "gap-junction response." IFN, TNF, IL-1, and IL-6 effects on cognate receptors on chromaffin cells and cellular sequelae in the chromaffin cell are not shown, but synergize with PACAP stimulation to modulate both catecholamine and peptide secretion and chromaffin cell gene transcription. Splanchnic nerve input affords synergistic as well as antagonistic interactions between cytokines and PACAP under physiological conditions in which both stress and inflammation may play a role. Finally, secretory products of the chromaffin cell itself, including substance P, other neuropeptides, and chromogranin-derived peptides such as catestatin, modulate chromaffin cell secretion via paracrine actions, in part through modulation of AChR function. See text, and references in text, for further details.

nerve stimulation that occurs during stress may vary significantly across mammalian species. Like ACh, PACAP stimulates secretion of CAs from the adrenal *in vivo* or from CCs in primary culture, in mice, rats, cows, dogs, humans, and other mammals (reviewed in Ref. 270). In terms of PACAP action at other sympathetic ganglia/synapses, there are well-documented PACAP actions at sympathetic postganglionic neurons, at least in culture, that include secretion and changes in gene expression, suggestive of a role for PACAP in stimulus-secretion-synthesis coupling at sympathetic as well as sympathoadrenal synapses (53, 440). Postganglionic parasympathetic neurons of intrinsic ganglia of the heart are also PACAP-responsive (620). These findings support the concept that PACAP acts as an effective neurotransmitter, along with ACh and NE, in the autonomic nervous system. It is also evident that understanding the details of the specific roles of this stress-inducing neurotransmitter in supporting ACh-driven secretion and gene expression in the adrenal medulla deserves further investigation.

Implications of PACAP transmission for GPCR-mediated stimulus-secretion-synthesis coupling in the CC during stress

Endocrine cells, in general, the must maintain their stores of secretable material, even during intense episodes of stimulation and secretion, to preserve their efficacy for future homeostatic responding. Secretion and synthesis need not be coupled processes if they occur at about the same rate constitutively. This is rarely the case, however, as hormone secretion is commonly episodic, occurring in response to varying homeostatic demands. Repletion of hormone content lost by secretion from endocrine cells could occur by several mechanisms, but two mechanisms seem to dominate for the CCs of the adrenal medulla. The first is a chronic overproduction of prohormone protein relative to the storage capacity. In this case, the excess hormone is degraded at times of low secretory activity, and diverted to the secretory pathway at times of high secretory activity and demand. CgA, the major protein constituent of the CG, appears to be regulated in this way. Since CgA is a component in the process of SG biogenesis itself, the CC can package no more CgA than there are available vesicular membranes to contain it. At rest, and when the CG complement is maximal, excess chromogranin is apparently degraded in the trans-Golgi network, and when the CG complement is depleted by an episode of secretion, this excess is redirected toward granule production. This mechanism allows CgA levels (and CGs) to be maintained at a sufficient level across even prolonged episodes of secretion without the need for compensatory increases in chromogranin mRNA abundance and protein synthesis (191, 227). For (pro)hormones that represent a smaller fraction of total SG protein content, and are therefore not "rate-limiting" for CG biosynthesis, and also for CAs, however, a regulatory mechanism must exist to compensate hormone lost from the cell by secretion, via enhanced hormone biosynthesis.

The hypothesis of stimulus-secretion-synthesis coupling arose during the search for this second mechanism of the hormone loss and repletion cycle, which centered on whether or not secretagogues, while stimulating the release of stored hormones, signal at the same time to the nucleus to activate transcription of the specific genes responsible for hormone biosynthesis (190). For bioactive peptides produced by prohormone processing within the CG, this is accomplished by an increased rate of transcription of the prohormone gene and subsequent increase in translation of its mRNA into the prohormone, which is then processed into mature peptide(s). In the adrenal medulla, the "secretory cocktail" is a mixture of protein (chromogranins and processed neuropeptides) and small molecules (CAs, ATP, and GABA), and stimulus-secretion synthesis coupling must occur for both categories of secreted material. Thus, binding of secretagogues to their receptors stimulates Ca^{2+} influx, activation of CG exocytosis, and release of CG contents. Simultaneously, secretagogue signaling stimulates transcription of prohormone protein-encoding genes as well as biosynthetic enzymes responsible for CA and another small-molecule biosynthesis. In the case of CA production, this means activation of TH through phosphorylation at key serine/threonine residues, and increased production of the TH protein itself, through enhanced transcription of the TH gene (276).

The cellular mechanisms for stimulus-secretion-synthesis coupling in the adrenal medulla have been well investigated throughout the 20th century and have functioned as a model for understanding this process in other types of endocrine cells (Figure 12). The second messenger most prominent in regulation of stimulus-synthesis coupling is Ca^{2+}, consistent with its role in stimulating secretion in most endocrine cells. In the adrenal medulla, cAMP was implicated early in both the transcriptional and posttranslational phases of TH activation. Study of stimulus-secretion-synthesis coupling related to co-stored peptides such as substance P, NPY, enkephalin, and galanin revealed that while cAMP appears to be the main second messenger for activation of gene transcription of the genes associated with these peptides, cAMP-dependent gene activation by the classical third messenger protein kinase A (PKA) was not always clearly identified as a regulated step in the process.

Kuo and Greengard (367) initially proposed that PKA was the sole effector for cAMP in mammalian cells. It was later discovered that cAMP gates Ca^{2+} channels on some mammalian cells (81), and that in addition, many non-PKA and non-Ca^{2+}-dependent cAMP-initiated signaling events exist in mammalian cells, especially neuroendocrine cells. In the 1990s, two proteins within the family of Rapgefs (rap-activating guanine-nucleotide-exchange factors) were identified as cAMP-regulated enzymes. These two proteins are most commonly called EPAC1 and EPAC2 (other names include Rapgef 3 and 4, or cAMP—GEF1 and 2 (72, 158, 341). The EPACs extended the range of cAMP effectors—from protein kinases to guanine-nucleotide-exchange factors—and also brought into the

stimulus-secretion-synthesis coupling pathway downstream signaling molecules activated preferentially by Rapgefs, such as the MAP kinase ERK (Figure 12) (202, 204, 205).

For example, galanin biosynthesis in the CC was found to be regulated by PACAP via a non-PKA-dependent cAMP signaling pathway acting through ERK (203). Experiments in cellula allowed the identification of a Rapgef cAMP effector molecule, Rapgef2, in this pathway, by both loss-of-function after gene ablation and gain-of-function after expression in nonendocrine cells (202). The Rapgef2 gene emits two classes of transcripts by alternative splicing, now called NN (for nonneuronal)- and NCS (for neuritogenic cAMP sensor)-Rapgef2. NCS-Rapgef2 is expressed mainly in neuronal and endocrine cell types/tissues in adult rodents and other mammals, and links cAMP elevation to ERK activation in CCs, in neurons, and in pancreatic beta cells (324). The initial discovery of NCS-Rapgef2 in the CC has led to the understanding that cAMP signaling in these and other endocrine cells and neurons is parcellated among at least three effectors, NCS-Rapgef2, EPAC, and PKA, that independently signal through distinct Rap isoforms (NCS-Rapgef2 and EPAC), and through CREB and other threonine/serine kinase acceptor proteins, to mediate distinct and separate cellular functions (204, 305, 324, 347, 403, 544).

A relevant emerging question is how PACAP receptor activation, since it does not involve the opening of sodium channels, engages the machinery for Ca^{2+} influx in the CC. A plethora of reports on this subject convey the general impression that Ca^{2+} channel opening via activation of the PAC1 receptor (PAC1R) is likely to involve initial cAMP-dependent phosphorylation of Ca^{2+} channels themselves, albeit the mechanisms ultimately responsible for secretion and regulation of gene expression for CA and secretory protein biosynthesis by PACAP in CCs will require additional investigation (*vide supra*).

The role of peptides secreted from the adrenal medulla

The discovery that bioactive neuropeptides, in particular opioid peptides are released along with CAs from the CCs of the adrenal medulla provided a source tissue for the isolation of the prohormones for the enkephalins, and later the mRNAs encoding them (135, 380, 381, 395). These developments were in turn made possible by the earlier identification of chromogranins as secreted proteins of the adrenal medulla (*vide infra*) and the discovery that enkephalin peptides are a major constituent of CGs (380, 395). Progress in understanding the generation of small opioid peptides from larger precursors led in turn to the realization that CgA is itself a prohormone, generating the bioactive peptides pancreastatin, vasostatin, catestatin, and others (188, 281, 313, 608). One of the proenkephalin-derived peptides found to exist in CGs BAM-22P was structurally identified through a systematic peptidomics analysis of the CG (448). Likewise, the novel bioactive peptide adrenomedullin was identified, in extracts of CC-derived human pheochromocytoma tissue, similar to PACAP extracted from the hypothalamus, via a proteomics-based search for novel cAMP-elevating neuropeptides (354). The finding of a highly variegated secretory cocktail, including CgA and enkephalin-derived peptides, as well as substance P, galanin, and NPY, also later shown to be prohormone derived, increased appreciation that the CC might participate in organismic endocrine regulation through these peptides as well as via the release of CAs. The ability to determine where, and how, these peptides exerted their physiological roles was initially made difficult by ignorance of the location of their receptors, which might suggest their autocrine, paracrine, or hormonal actions *in vivo* (281). Indeed, the molecular nature of the receptor for catestatin, one of the CgA-derived peptides of the adrenal medulla, is still uncertain. Catestatin, for example, inhibits ACh-induced CA secretion from CCs, but not that caused by potassium depolarization, by the Ca^{2+} "agonist" barium, or by ionophores that mobilize Ca^{2+}, suggesting that catestatin acts through a receptor that either is or acts immediately downstream of the nAChR of CCs (420). In PC12 cells, catestatin affects not only ACh-induced but also PACAP-induced CA secretion, suggesting that it may act to modulate both resting and stress-induced adrenomedullary secretion (549). Thus, catestatin may function as an autocrine regulator of CA secretion and biosynthesis, based on its negative modulation of nAChR activation in CCs, as has been suggested for substance P produced in CCs (84, 310, 396).

Catestatin has multiple effects on distant organs, suggesting a hormonal role, in addition to its autocrine functions within the adrenal medulla itself. It is noteworthy that, given the presence of several other bioactive peptides within the CgA primary sequence, administration of catestatin alone rescues the major aspects of the CgA-deficiency phenotype in CgA-knockout mice (416). Catestatin and other chromogranin-derived peptides affect virtually all stages of the secretory function of the CC (416), including CG biogenesis (353), and this function appears to be shared with other granin proteins in CCs (455). Modulation of CA release is a major function of catestatin that is increasingly well defined pharmacologically (622). Physiologically, catestatin not only modulates CA release, but, as a hormone in its own right, acts in synergy with CAs on cardiovascular function and metabolism, and regulation of other endocrine cells and immunocytes, and their metabolism and secretory morphology (348, 588). Other CgA-derived peptides with potent effects on heart and vasculature are reported, including vasostatin, and these potential hormones create multiple regulatory linkages among adrenomedullary secretion, metabolic function, and muscle performance, and therefore between stress transduction and cardiovascular and metabolic physiology (279).

Another adrenal medulla-derived neuropeptide worthy of consideration is adrenomedullin, which was discovered as a constituent of human pheochromocytoma (354). Adrenomedullin activates a GPCR linked to Gs, and its receptors are found throughout the cardiovascular system.

It has profound hypotensive properties and thus could be envisaged as a counter-regulator or modulator of CA actions. Unlike peptides present at only low levels in CCs, for which autocrine and paracrine are more likely than hormonal ones (*vide supra*), adrenomedullin, like catestatin, may function as a bona fide hormone, like the CAs, in mammalian physiology, as well as a paracrine/autocrine factor in the adrenal medulla itself (25) in addition to actions at sites of local synthesis outside of the adrenal medulla. The possible functional role of adrenomedullin in the regulation of the heart, vasculature, and kidney has been studied, and this peptide shows some promise for clinical utility (186).

The role of peptide hormones of the adrenal medulla, including CgA, CgA-derived peptides, opioid peptides, NPY, substance P, adrenomedullin, and others, has opened a new and important avenue of adrenal medulla physiology. These peptides and their receptors represent adaptive components of the endocrine network and their fine-tuning of homeostasis, throughout mammalian evolution, including the tissue-specific production of specific hormones and the tissue-specific expression of their receptors. BAM22P, for example is a 22-amino acid peptide that is proteolytically processed from proenkephalin almost exclusively in the adrenal medulla, perhaps because the uniquely slow or incomplete processing of this opioid prohormone in the adrenal CC, unlike in neurons, generates a multitude of peptide "processing intermediates," which are characteristic of the adrenal medulla. BAM22P appears to be one of these which is uniquely bioactive upon release, because its receptor is expressed mainly within sensory neurons. This receptor prefers BAM22P to met-enkephalin with a potency ratio of greater than 50-fold. Consistent with this preference, the BAM22 receptor is only distantly related to the μ-opioid receptor, for which enkephalin pentapeptides are thought to be the main endogenous ligands (377). The expression of the BAM22P receptor in sensory neurons, together with high concentrations of BAM22P contained in and therefore secreted from the adrenal medulla, invites the speculation that BAM22P may act as a transmitter or paracrine factor for cross-communication between CCs and the sensory nerves that innervate the adrenal gland (505) (see Figures 12 and 13).

The concept of organ plasticity in stress responding of the adrenal medulla

During development, endocrine organs arise as cell colonies with a very specific secretory mission. Some are spatially quite compact, as the adrenal medulla, and others dispersed throughout other organs, such as the cells of the endocrine pancreas and the entero CCs of the gut. Regardless of their physical coherence, it is generally considered that each cell within an endocrine organ acts in parallel with, but independently of, its neighbors in hormone secretion in response to the presence of secretagogues. The detailed study of vasopressin secretion from the posterior lobe of the pituitary, however, caused a reappraisal of this point of view. Several investigators began to advance the idea of a concerted function of the secretory cells as a cohort, through cell-cell communication about the secretory status of individual cells, with integration to the function of the entire gland (80). This perspective allows endocrine organs to be viewed not only as a "massively parallel" system, but takes into account plasticity in the secretory status and responsivity of individual cells in response to secretory demand and even organismic

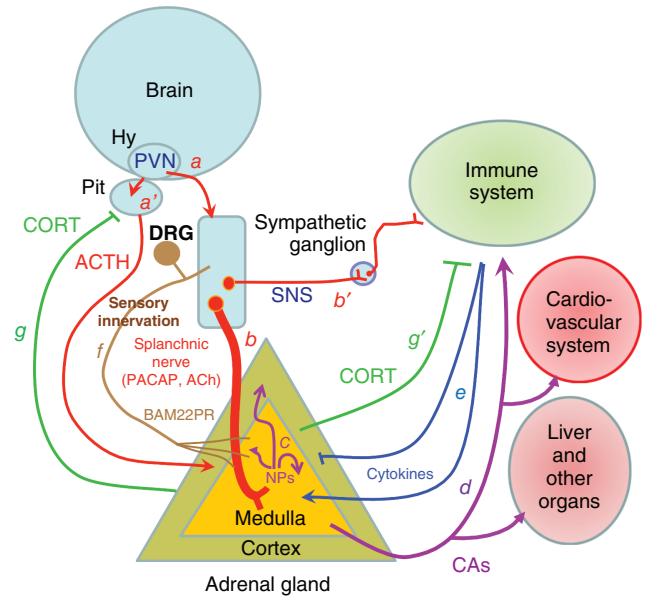

Figure 13 The adrenal medulla as a stress transducer and neuroimmunoinflammatory and cardiovascular regulator. Lower figure. a. The "final common pathway" for stress responding in the CNS is the activation of neurons in the paraventricular nucleus (PVN) of the hypothalamus (Hy), which projects both to the cell bodies of sympathetic preganglionic neurons in the intermediolateral column of the spinal cord and to the median eminence: a′. for ACTH release from the pituitary (Pit) to stimulate corticosterone/cortisol (CORT) release from adrenal cortex; b′. cell bodies in the intermediolateral column of the spinal cord innervate the CCs of the adrenal medulla via the splanchnic nerve, and sympathetic postganglionic nerve targets via para- and prevertebral postganglionic sympathetic neurons b. via the splanchnic nerve, releasing ACh basally, and ACh and PACAP during stress, with activation of both secretion by ACh and PACAP, and of CC signaling pathways by PACAP increasing expression of genes encoding neuropeptides (NPs), additional mediators, catecholamine biosynthetic enzymes, and adhesion factors and connexins that increase cell-cell communication among CCs and amplify CA, neuropeptide, and chromogranin output in response to stress; c. NP (neuropeptide) release from CCs has autocrine effects on CA secretion from CCs themselves (e.g. catestatin, substance P, and others), activation of sensory neurons (e.g. BAM22P, acting on specific receptors expressed in sensory nerves), and modulation of CORT secretion from the adrenal cortex (galanin, VIP, and other peptides), as well as hormonal effects on distant organs; d. CA release from CCs into the general circulation, and affecting metabolism, heart rate, blood pressure, and immune cell mobilization; e. *Cytokines* released as blood-borne molecules or locally from circulating monocyte macrophages act as inhibitors of CORT secretion in the adrenal cortex, and as modulators of peptide secretion in adrenal cortex via receptors on CCs themselves; f. Sensory inputs to adrenal medulla sense CC secretory activity via release of BAM-22P, for which sensory neurons express specific receptors. Also depicted are the targets of glucocorticoid (CORT) release from the adrenal cortex at the pituitary g and immune system g′, the latter decreasing in turn cytokine secretion, which affects adrenomedullary function during stress. For a further explication of the figure, see the text.

conditions. Paracrine/autocrine regulation, discussed above, is one way in which the activity of individual cells can be aggregated and modulated. This autocrine/paracrine regulatory concept may explain the roles of several of the peptide hormones produced by and released from the adrenal medulla (see previous section), in particular, those that are released at concentrations too low, relative to their potencies, to be active at distant receptors.

A second mode of regulating the collective secretory response of cells within an endocrine organ involves altered cellular adhesion, through the formation of gap junctions among secretory cells themselves (Figure 12). Gap junctions lower the resistance between adjacent cells through physical connections between them. This occurs, at the molecular level, through the dynamic expression of connexins, proteins that form hexameric hemichannels to promote the spread of electrical excitability. Connexins are expressed by rat adrenal CCs (433) and are regulated *in vivo* by changes in splanchnic nerve activity in response to stress (see Ref. 133 for a comprehensive review). It was initially observed that cellular resistances of isolated CCs in primary culture are greater than those of CCs in intact adrenal glands, and this difference inferred to exist due to loss of gap junctions in dissociated cells (120, 433). A series of *in vivo* experiments assessed changes in gap-junction formation in the adrenal medulla *in vivo* as a function of cold stress (summarized in Ref. 261). Rat adrenomedullary slices examined before and after five days of cold stress (134) showed morphological remodeling of both synapses onto CCs and CC cell-cell borders; increased dye permeation between CCs; and increased electrical coupling between CCs after depolarization of single cells. Finally, application of a several-day stress paradigm *in vivo* caused a noticeable increase in CA secretion after an applied electrical stimulation that was attenuated by pharmacological inhibition of gap-junction formation. Further investigation of the detailed biochemical constitution of gap junctions in the adrenal medulla, and the modulation of gap-junction formation in concert with altered secretory function after stress, has been reported (133, 162). Thus, in addition to shifts between the balance of cholinergic and PACAPergic neurotransmission from rest to stress, physical reorganization of the gland itself anticipates further episodes of stress. The actual first messengers mediating this adaptive response, whether the neurotransmitters ACh and/or PACAP, or autocrine factors released from the CCs in response to stress transduction, are as yet unidentified.

The role of gap junctions in adrenomedullary function may well apply to other endocrine organs (219). It is worth noting that the stress stimuli so far selected for the study of gap-junction-driven plasticity of the adrenal medulla are not among the most "selective" for that organ (252). Cold stress, for example, activates NE secretion rather more than EP secretion, suggesting that its major effect is on postganglionic sympathetic neuronal activity rather than CC activation. It remains to be seen if principal ganglion cells of the SNS respond to stress as the adrenal medulla does. An additional unresolved issue is whether PACAP, or ACh, or both are the principal regulator(s) of either type of adrenomedullary stress-induced plasticity. Stress also regulates CC responsiveness through alteration of other components of the CC. Two important examples are the recruitment of T-type ($Ca_V3.2$) Ca^{2+} channels during chronic hypoxia and β-AR stimulation of cultured rat CCs that cause a pronounced reduction of the threshold of CA release associated with the low threshold of activation of newly recruited Ca^{2+} channels (104, 479) (see below). It is also noteworthy that CCs from mice previously stressed by exposure to rats express a high density of T-type channels as determined by Ca^{2+} current patch-clamp studies (Emilio Carbone, Chiara Calorio, and Daniela Gavello, unpublished observation).

Adrenomedullary function during inflammatory responses

Inflammatory responses involve multiple organs and are mediated largely by cytokines that act hormonally on virtually all tissues via the circulation, as well as locally through the mobile and ubiquitous cytokine-secreting cells of the immune system, which can migrate to various locations during and after infection. Lipopolysaccharide and cytokine receptors have been identified functionally and biochemically on CCs, suggesting a role for the adrenal medulla in responding to inflammation, and potentially coordinating various phases of the immune response (Figures 12 and 13). In fact, this signaling system may hold the key to explaining how stress and immune function mutually modify one another, although definitive experiments in this direction remain to be reported (82, 171).

Two inflammatory cytokines, interleukin type 1 (IL-1) and tumor necrosis factor-alpha (TNF-α), have been shown to regulate CC neuropeptide expression, increasing expression of VIP and decreasing that of met-enkephalin in bovine CCs in primary culture. These cytokines also amplify the effects of cAMP on VIP and substance P biosynthesis (211). The TNF-α receptor is expressed in the bovine adrenal medulla *in vivo* (4, 5), and TNF-α regulates gene transcription and elevates production, of both galanin and secretogranin II (SgII) (5). Bunn and colleagues have postulated a role for TNF-α in VIP biosynthesis and TH induction, based on inflammation-induced elevation in mRNA encoding the neuropeptide prohormone and the TH protein, in the rodent adrenal medulla *in vivo* (82, 171). Additional studies support a physiological role for cytokine regulation of adrenomedullary peptide production, in particular in counter-regulation of inflammation. Thus, cytokine-induced production of galanin (4) may counter-regulate cytokine actions elsewhere in the body via enhancement of glucocorticoid production in the adrenal cortex (483) (see Figure 13). IL-1 and TNF (82), themselves inflammatory cytokines, increase the production of IL-6 mRNA in CCs. As IL-6 has anti-inflammatory properties, its production by the adrenal medulla may also function as a counter-regulator of excess cytokine action associated with the pathogenesis of sepsis.

Interactions between cytokines and CC neuropeptides may also be more or less pronounced in the presence of PACAP and CC autocoids (Figure 13), thus linking anti-inflammatory mechanisms to stress and potentially allowing a better understanding of multiorgan involvement in pathophysiological states including inflammation and hypertension (88).

GABAergic regulation of CA release

Excessive response to stress can be harmful. Therefore, adequate control of CA secretion by CCs is essential for normal physiological function and appropriate stress response. Substances secreted by CCs can either augment or decrease CA exocytosis. Receptors for ATP (P2Y receptors), CA (α-adrenergic receptors), and enkephalin (μ-opioid receptors) coupled to Gi-type G-proteins inhibit Ca_V channels and CA release (148) (see also *Ca^{2+} channel-secretion coupling* above). Conversely, D1 dopamine and β-adrenergic receptors augment CA release by increasing Ca^{2+} influx through L-type Ca^{2+} channels or by phosphorylating components of the exocytosis apparatus (30, 100). A regulatory mechanism mediated by gamma-aminobutyric acid (GABA, the main inhibitory neurotransmitter of the CNS) was also described in the adrenal gland (493).

GABA inhibition in the CNS is mediated by ionotropic $GABA_A$ receptors ($GABA_A$-Rs), permeable to Cl^- ions and metabotropic G-protein-coupled $GABA_B$ receptors, which control cellular excitability by regulating Ca^{2+} and K^+ ion channels (22, 71, 75). When GABA (or the $GABA_A$-R agonist muscimol) is injected intravenously, heart rate and blood pressure increase (75). These cardiovascular actions are prevented by adrenalectomy or a $GABA_A$-Rs antagonist, suggesting stimulation of CA secretion by $GABA_A$-Rs. In the isolated adrenal gland, muscimol increases baseline CA release but reduces synaptically evoked CA secretion (317). This paradoxical effect reflects the "dual action" of $GABA_A$-R activation (273, 439). GABA is stored in dense core granules (274) and it is likely released together with CA, ATP, and opioids in response to physiological stimuli (493). Functional tests with $GABA_A$-R antagonists indicate that GABA is present in the milieu of adrenal medulla slices (14, 273).

Acute application of GABA or muscimol to CCs in culture causes membrane potential depolarization, AP firing, opening of Cav channels, and a transient $[Ca^{2+}]_i$ rise that initiates CA exocytosis (87, 240, 317, 493, 513). GABA-induced adrenal CA secretion is likely due to this excitatory mechanism. Conversely, $GABA_A$-Rs tonically inhibits spontaneous cholinergic transmission (14) and shunts CC membrane electrical resistance, weakening synaptic excitation and impairing AP firing (13, 14, 87, 350). These effects explain the GABA inhibition of synaptically evoked CA release (317).

In neurons, intracellular chloride concentration ($[Cl^-]_i$) results from the interplay between Cl^- accumulation by the $Na^+/K^+/2Cl^-$ cotransporter NKCC1 and Cl^- extrusion by the cation-chloride cotransporter KCC2. Immature neurons display excitatory responses to GABA (55) because of Cl^- efflux due to a high $[Cl^-]_i$, resulting from a greater functionality of NKCC1 (Figure 14). Conversely, in mature neurons, the GABA response is inhibitory because $[Cl^-]_i$ is low due to a dominant activity of KCC2, and Cl^- enters the cell ((336); Figure 14B). In rat adrenal CCs ~44% of $GABA_A$-Rs-mediated response comprises depolarization and $[Ca^{2+}]_i$ elevation (Figure 14C), while in ~26% it includes hyperpolarization and $[Ca^{2+}]_i$ drop (Figure 14D) (13, 14); the remaining CCs are unresponsive. Since muscimol-induced $[Ca^{2+}]_i$ rises are not inhibited by bumetanide (13), NKCC1 is probably not responsible for Cl^- accumulation in rat CCs. This role could be played by the anion-exchanger AE3 or by Pendrin, a member of the SLC26A family of Cl^-/HCO_3^- exchangers that is expressed in adrenal CCs and can modulate CA release (373). Pendrin would be most active in CCs that respond to GABA with membrane depolarization and $[Ca^{2+}]_i$ rise (Figure 14C). The dynamic reciprocal regulation of AE3/Pendrin and KCC2 underlies the variance in $[Cl^-]_i$. It is plausible that the proportion of CCs responding to GABA with excitation or inhibition varies depending on how $[Cl^-]_i$ is controlled (333).

Experiments in isolated rat adrenal gland suggest that GABA regulates CA secretion differently depending on the functional state: Under nonstressful conditions, the activation of $GABA_A$-Rs by endogenous GABA inhibits cholinergic transmission and reduces CC excitability (14), thus preventing excessive CA release. During intense stress, strong synaptic stimulation initially causes a vigorous CA release, which then declines (240). Activation of postsynaptic $GABA_A$-R by released GABA can then depolarize CCs and maintain CA exocytosis even under acute stress. These findings emphasize the importance of GABAergic regulation as another CA secretion controlling agent.

The Stimulus-Secretion Coupling of the Adrenal Medulla and CCs in Disease

A role of adrenal CCs in essential hypertension?

Essential hypertension, the most common manifestation of high blood pressure, is also the most insidious since it leads to cardiac hypertrophy, congestive heart failure, stroke, and retina and kidney damage. It has several possible causes, but an augmented sympathetic tone has been implied in many studies (23, 212). Higher levels of plasma CAs resulting from increased release from sympathetic synaptic terminals and adrenal CCs is a common finding in essential hypertension (147, 251). In the spontaneously hypertensive rat (SHR), an animal model (488) widely used to investigate hypertension, blood pressure rises above 150 mm Hg at 6 to 9 weeks of age, reaching ~200 mm Hg by 15 weeks (376). At 45 weeks of age, SHRs develop cardiac hypertrophy and heart failure (137). Chemical sympathectomy of newborn SHRs delays the onset of hypertension, and when this procedure is combined with demedullation (bilateral removal of the adrenal medulla), adult SHRs become normotensive

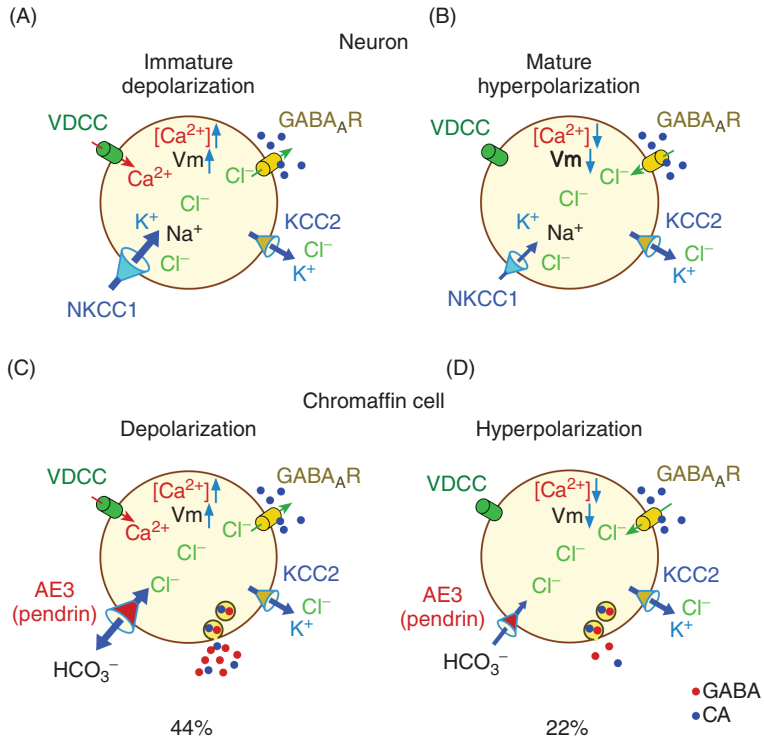

Figure 14 Divergent responses of rat CCs to GABA$_A$ receptor activation. (A) GABA response is excitatory in immature neurons because of a greater functionality of NKCC1. (B) In mature neurons, GABA response is inhibitory because of the dominant activity of the KCC2. (C) GABA$_A$-Rs-mediated response is depolarizing with $[Ca^{2+}]_i$ elevation in ~44% of rat adrenal CCs. (D) in ~26% of CCs, GABA response is hyperpolarizing and causes $[Ca^{2+}]_i$ drop. The scheme also represents the anion-exchanger AE3 (pendrin), which accumulates Cl^- in exchange for intracellular HCO3$^-$ and participates in Cl^- transport into CCs as a replacement for NKCC1. VDDC: voltage-dependent Ca^{2+} channel.

(376). In fact, in young SHR, both EPI and NE plasma levels are elevated compared to age-matched normotensive Wistar-Kyoto rats (WKY) (496), and isolated adrenal glands from SHR secrete more CA than those from normotensive rats in response to depolarizing agents (ACh or high K^+) (385). In cultured CCs, the CA secretion (cumulative charge) measured with single-cell amperometry (see the section titled "Measuring exocytosis") is 2.3-fold higher and the number of spikes 2.4-fold larger in SHR versus WKY CCs (65, 445). Likewise, massive intracellular Ca^{2+} release from the ER or mitochondria triggers long-lasting bursts of spikes, threefold larger in SHR CCs compared to WKY CCs (444). Among the mechanisms suggested to explain the enhanced CA secretion in adult hypertensive SHRs (16-weeks old) are the augmented capacity of the ER Ca^{2+} store due to impaired mitochondrial Ca^{2+} uptake; greater cytosolic and mitochondrial Ca^{2+} transients; larger microdomains of high-$[Ca^{2+}]_i$ at sub-plasmalemmal sites; augmented vesicle pool; and greater quantal size of amperometric events (65, 444–446, 460).

In early hypertensive SHRs (9–12 weeks of age), spontaneous amperometric events are more numerous and of larger mean amplitude in SHR CCs than in WKY. A brief stimulation with high K^+ or caffeine (which causes voltage-gated Ca^{2+} influx or intracellular Ca^{2+} release, respectively) triggers a burst of spikes with greater mean amplitude in SHR CCs. Therefore, CA secretion for both stimuli was approximately twofold higher in SHR CCs (566). Treatment with ryanodine, a specific blocker of RyRs, reduced depolarization-induced CA secretion by 77% in SHR CCs and 10% in WKY, suggesting a greater contribution of Ca^{2+}-induced Ca^{2+} release to CA exocytosis in SHR CCs (Figure 15). Also, $[Ca^{2+}]_i$ signals elicited by a brief stimulation with high K^+ or caffeine were 3.2- and 2.5-fold larger, respectively (566). Together, these findings implicate an enhancement of CA release in the origin of hypertension in this model in SHR and support the notion that a key mechanism involved in the enhanced secretion of CA at early stage of hypertension is a "gain-of-function" of the RyR, an intracellular Ca^{2+} release channel from the ER.

The hypothesis that SHR CCs could have Ca_V currents of greater magnitude was discarded. Electrophysiological recordings showed that this current is either indistinguishable in both strains (565) or reduced by ~39% in SHR CCs

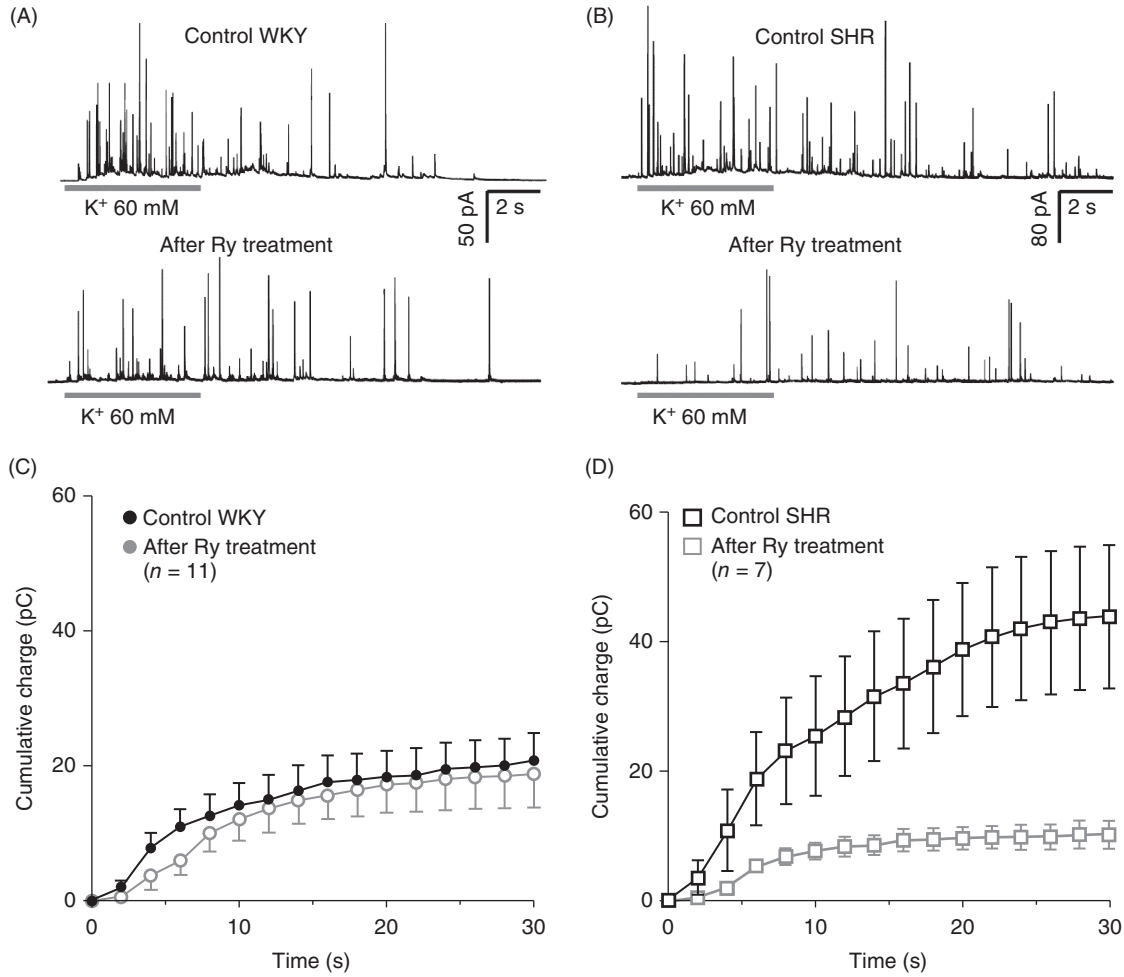

Figure 15 Ryanodine effects on depolarization-induced CA secretion. (A, B) Burst of amperometric spikes elicited by a 5-s-long depolarizing pulse in a WKY (A) and an SHR CCs (B) before (top) and after (bottom) incubation with 10 μM ryanodine. (C, D) Mean cumulative charge in WKY and SHR CCs, respectively, before and after ryanodine treatment. The cumulative charge after RyR blockade was not significantly affected in WKY CCs (20.8 vs. 18.8 pC; $p = 0.274$), but it was drastically reduced in SHR CCs (from 43.9 to 10.1 pC; $p = 0.0001$). The number of cells examined is shown in parentheses. Reused, with permission, from Segura-Chama P, et al., 2015 (566).

compared to WKY (460). A comprehensive analysis revealed that CA hypersecretion in SHR CCs results from the combination of larger depolarization-induced Ca^{2+} transients, more exocytosis events per time unit, and a greater proportion of medium and large amperometric spikes due to a higher CA content per granule (566). A study in prehypertensive SHRs and WKY CCs at 4 weeks of age revealed that all changes found in early hypertensive rats (566) are already present, but to a lesser degree (Peña-del Castillo, JG, in preparation). The cumulative secretion elicited by high K^+ and caffeine is 2- and 1.6-fold larger, respectively, in SHR, compared to WKY CCs. Ryanodine treatment reduces CA secretion by 48% in SHR and by 10% in WKY, and $[Ca^{2+}]_i$ signals elicited by stimulation with either high K^+ or caffeine were 1.4-fold larger in SHC CCs. These data suggest that the "gain-of-function" of the Ca^{2+}-induced Ca^{2+} release mechanism is already present in prehypertensive stages in SHR CCs and that it intensifies with age. It remains to be established to what extent the dysfunctional CA secretion by the adrenal medulla and possibly the SNS are involved in the origin of essential hypertension. Identifying the most likely molecular targets of adrenal and sympathetic hyperfunction in SHR rats leading to hypertension, and their potential dysfunction in human hypertension, is an area in which further advances will have major clinical implications.

Pheochromocytoma

Pheochromocytoma is a tumor arising from CCs in the adrenal medulla and sympathetic paraganglia, which can synthesize and secrete NE, EPI, and, to a lesser extent, DA (21, 617). By acting on their target receptors, these CAs cause significant physiological changes in the body, most often severe hypertension, which can cause devastating cardiovascular and cerebrovascular effects, if untreated (77). Pheochromocytomas are more prevalent than paragangliomas. Yet, the combined

prevalence of these tumors is less than 0.05% in the general population (27, 492). Most pheochromocytomas are benign tumors: the prevalence of malignancy varies between 10% and 15%. In contrast to the normal adrenal medulla, negative feedback of TH on CA synthesis is absent in pheochromocytoma, thus contributing to the uncontrolled and autonomous synthesis of CAs (462). The surgical treatment represents the only modality of ultimate cure, but timely diagnosis and pharmacological treatment is the basis of a successful outcome. Several laboratories have devoted considerable effort to validating markers for pheochromocytoma and especially in distinguishing its benign and malignant forms (378).

While the most common sign of pheochromocytoma is hypertension (90), additional symptoms include episodes of headache, palpitation, anxiety, and sweating related to excess CA secretion (21). The duration of paroxysms varies from a few minutes to 30 min, separated by asymptomatic intervals, from many times per day to a few times per month. Other less well-known symptoms are nausea, vomiting, weight loss, and diabetes mellitus. The secretory profile of pheochromocytoma can be useful in its diagnosis. Extra-adrenal tumors secrete NE and rarely DA (525), while adrenal pheochromocytomas primarily secrete EPI, with or without NE. DA-secreting tumors are rare and occur only in extra-adrenal tumors. Patients with tumors that produce high concentrations of NE are likely to incur sustained hypertension, while patients with elevated levels of EPI often experience paroxysmal and orthostatic hypertension (321). DA-secreting tumors are often asymptomatic (525). Pheochromocytomas can also produce adrenomedullin, vasoactive intestinal polypeptide (VIP), ACTH, NPY, endothelin-1, somatostatin, atrial natriuretic factor, and parathyroid hormone-related peptide. The resulting clinical picture will depend on the combination of increased CA and the amount released of these vasoactive substances (424).

CA metabolism is crucial to protect the organism against excessive CAs. In pheochromocytoma, more than 94% of the metanephrines derive from CA metabolism within tumor cells (195). Production and secretion of metanephrines is continuous and independent from CA secretion. Dopamine is also O-methylated by COMT to 3-methoxytyramine (3-MT), which is also continuously released from the tumor (195, 197). While free metanephrines are excreted in urine, the sulfate-conjugated metanephrines (194) represent 30-fold higher levels than free metanephrines, and are the principal form in which they are finally eliminated in the urine. Some of these metanephrines are oxidized to 3-methoxy-4-hydroxyphenylglycol (MHPG) (194). Both DHPG and MHPG are oxidized in the liver to vanillylmandelic acid (VMA), the major end product of CA metabolism (196). This unconjugated metabolite, excreted in the urine, has been used for many decades as a marker of the presence of CC tumors. However, VMA has insufficient diagnostic accuracy and has been removed from the diagnostic checklist of CC tumors (378).

It is impossible to predict when and how much CA a particular tumor will release during a secretory episode, but it is known that hypertensive paroxysms can be precipitated by physical activity (exercise and postural change) or tumor manipulation (77, 289). One of the few physiological studies conducted on cultured human pheocromocytoma cells (289) revealed that they express densities and proportions of L, N, and P/Q types of voltage-gated Ca^{2+} channels similar to those of normal human CCs. Nonetheless, the extent of downmodulation of Ca^{2+} currents by ATP and opioids is quite heterogeneous in these cells. The pattern of exocytotic events is also heterogeneous; some cells secrete spontaneously and continuously, while others evoke a normal secretory pattern after a brief pulse of high K^+. Cell heterogeneity suggests that normal CCs coexist with pathological CCs that escape the autocrine/paracrine downmodulation of Ca^{2+} channels and hence, may produce abnormal Ca^{2+} signals and CA hypersecretion. Hyperexcitability of CCs is also likely, given the reported peak of CA secretion often observed during tumor manipulation (288). These alterations could underlie the uncontrolled CA peaks suffered by patients with pheochromocytoma, which are responsible for the typical symptoms produced by this tumor. There is much to be learned about the physiopathology of tumoral CCs. This knowledge will certainly provide a basis for better understanding of the pathogenesis and treatment of this disease.

The molecular mechanism regulating CA release during acute, chronic, and intermittent hypoxia

In mammals, low oxygen pressure (hypoxia) is an environmental stressor that triggers CA release from adrenal CCs, depending on the extent and duration of the hypoxic exposure (97, 332). The acute response is initiated by the O_2-sensitive glomus cells of the carotid body that through the activation of SNS triggers the release of CAs from CCs. Acute hypoxia causes sustained cell depolarization, repetitive firing, Ca^{2+} entry, and CA release (73, 239, 449, 482, 550, 602, 615).

In most animal species, adrenal CCs play a critical role during fetal and perinatal life when sympathetic innervation is absent or immature. CCs possess inborn chemosensitivity to O_2 that allows them to control directly the release of CAs from the adrenal gland under nonneurogenic conditions (567, 578). In CCs of fetal and neonatal rodents, the hypoxia-induced release of CAs plays a key role in setting heart rates and oxygen delivery to the growing tissues (529). This is particularly evident during the transition from intra- to extrauterine life when the fetus experiences episodic asphyxia, and the hypoxia-induced release of CAs helps to regulate the cardiac function through the activation of β-ARs and prepare the lungs for air-breathing (567, 578). As the adrenal gland acquires cholinergic innervation, typically during the seventh to tenth postnatal days in rodents (619), the acute hypoxia-sensing mechanism of CCs is gradually suppressed with the same time course (567, 578). Remarkably, direct O_2-sensitivity is reestablished after adrenal gland denervation (567). How this occurs is not yet fully understood. There is likely a developmental loss of hypoxia chemosensitivity in CCs (343, 450, 534, 613, 614) that can

reemerge after prolonged deprivation of neural input in adult rat adrenal glands. However, there is also evidence that a fraction of CCs intrinsically possess an O_2-sensing mechanism regardless of cholinergic innervation, which is also effective in adult rat CCs (239, 315, 375, 449, 602). This issue, and the remodeling of cell function that occurs in response to chronic and intermittent hypoxia, will be explored and clarified in the following sections.

The role of K^+ channel in O_2-sensing in perinatal and mature CCs

The first cellular studies addressing the K^+ channel types involved in the nonneurogenic mechanisms of the hypoxia-induced secretory response in cultured rat CCs date to the late 1990s (449, 450, 614). It appeared evident that the Ca^{2+}-dependent release of CA was triggered by a hypoxia-induced block of K^+ channels that generated membrane depolarization, cell firing, and Ca^{2+} entry through voltage-gated Ca_V channels (449, 613). Early findings suggested the involvement of BK and K_V channels. In voltage-clamp experiments, acute hypoxia reversibly inhibited both Ca^{2+}-dependent BK currents and voltage-gated K_V currents (449, 602, 615). Later findings also implicated SK (346, 375) and K_{ATP} (Kir6.1, Kir6.2) ATP-sensitive K channels (73, 375, 550, 615). Acute hypoxia clearly inhibited SK channels, while activating K_{ATP} channels, most likely due to the transient reduction of ATP during low O_2. In the case of SK channels, voltage-clamp recordings showed that hypoxia reduced the outward K^+ currents of neonatal rat CCs and apamin largely prevented these effects (375). In the case of K_{ATP} channels, their involvement and upregulation were evident in rat CCs when acute hypoxia was tested in the presence of the K_{ATP} channel blocker glibenclamide. The addition of glibenclamide did not affect K^+ current, but the inhibitory action of acute hypoxia was enhanced (Figure 2a in Ref. 550), suggesting that K_{ATP} channels are upregulated only in response to low O_2. Current-clamp recordings confirmed this view. The hypoxia-induced cell depolarization caused burst firing in the control condition that was further enhanced in the presence of glibenclamide (Table 2) (see Figure 2b in Ref. 550). Apparently, K_{ATP} channels play a protective role in the hypoxia-induced response of CCs. These channels hyperpolarize the cell during hypoxia and have a maximal expression at mid-gestation decreasing toward late gestation, while BK channels show an opposite trend. Thus, the hypoxia-evoked CA release is attenuated at mid-gestation due to high K_{ATP} expression and more hyperpolarized membrane potential, but is facilitated at birth as K_{ATP} expression decreases and BK expression increases (73).

Likely, heteromeric TASK-1/3 channels are also involved in hypoxia-induced CA secretion in CCs. TASK-1/3 channels are highly expressed in rat and mouse CCs (316, 389) and their activity is moderately reduced (30%–40% inhibition) by acute hypoxia (351). Given the key role that these channels play in setting the resting potential of rat and mouse CCs, it is likely that the hypoxia-evoked reduction of their resting current is sufficient to induce cell depolarization, burst firing, Ca^{2+} entry, and CA release during acute hypoxia. In conclusion, it seems that, although to different extents, nearly all K^+ channels expressed in mammalian CCs are involved in O_2-sensing, suggesting a common mechanism acting on a protein domain shared by all of the K^+ channels of CCs.

Concerning the O_2-sensing mechanism, significant progress has been made in the past decade to understand the downstream events in the signal transduction cascade regulating the hypoxia-evoked response in CCs, nicely described in recent reviews (401, 482). Briefly, there is strong support for the involvement of complexes I and IV of the mitochondrial electron transport chain (ETC) coupled to the alteration of reactive oxygen species (ROS) (344, 450). Recently, it has been proposed that loss of the mitochondrial subunit *Ndufs2* of complex I, which encodes a protein that participates in ubiquinone binding, is critical for the O_2-sensing mechanism in glomus cells and adrenal CCs (223). An important role is also played by the cytochrome c oxidase subunit IV isoform 2 (*Cox4i2*) and the NADH dehydrogenase (ubiquinone)1 alpha subcomplex, 4-like2 (*Ndufa4l2*) (238). Unresolved and controversial issues still exist concerning the role of ROS generated by complex I in the O_2 signaling pathway. It is not clear, for instance, whether acute hypoxia induces an *increase* (223) or a *decrease* in ROS (238) and how ROS interact with K^+ channels. Further studies will clarify this issue.

Ca_V channel types responsible for hypoxia-induced release of CAs at embryo and perinatal life CCs and their roles

Ca_V channels have a key role in the hypoxia-evoked response of neonatal CCs (see Refs. 482 and 495 for recent reviews). They convert the hypoxia-induced mild depolarization (73, 550) and associated repetitive firing into Ca^{2+} influx that drives vesicle fusion and release of CAs (224, 550, 602). Thus, the question is: which Ca^{2+} channel types are expressed in neonatal CCs and how they contribute to the release of CAs. As nifedipine strongly attenuates the response of neonatal CCs to acute hypoxia, L-type channels would appear to be critical for the regulation of the hypoxia-induced release of CAs (1, 602, 614). Neonatal CCs of rats, mice, and sheep express different percentages of the same Cav channels expressed in adult CCs (L, N, and P/Q) (20, 73, 224, 379, 495) with relative percentages similar to those found in adult CCs (240, 417), except for a net increase in expression of Cav3.2 (T-type) channels in adults (73, 345, 379). T-type channels are typically upregulated in adult CCs under stressful conditions (107, 298, 368, 479), including chronic (103) and intermittent hypoxia (584) (see below and Table 1 in Ref. 417).

Concerning the specific role of Ca_V channel subtypes on the quantal release of CAs measured with carbon-fiber microelectrodes on embryonic and neonatal CCs, two reports indicate the involvement of L-, N-, P/Q-, and T-type channels that contribute differently to the acute hypoxia-evoked response (417). In one case (224), exposure of cultured rat embryo

CCs to 1 min hypoxia elicits transient bursts of amperometric secretory spikes followed by dispersed spikes during the period of O_2 deprivation. This bulk response is blocked 85% by nimodipine and 35% by combined ω-conotoxin GVIA and ω-agatoxin IVA, suggesting that the acute hypoxic response in early life is initially controlled by both Cav1 (L) and Ca_V2 (N- and P/Q-type) channels. However, due to the fast inactivation of $Ca_V2.1$ (P/Q-type) and Ca_V 2.2 (N-type) channels, their contribution is blunted after a few seconds so that the slowly inactivating $Ca_V1.3$ L-type channels remain the main ones responsible for the hypoxia response. In the second case (379), recording of secretory events with amperometry performed in neonate rat adrenal slices reveals a pronounced spontaneous generation of spike events. Acute hypoxia increases the frequency of spikes, and the hypoxia-induced response is triggered by Ca^{2+}-influx through T-type ($Ca_V3.2$) channels, giving rise to what is commonly indicated as the "low-threshold exocytosis" that also occurs in adult rat CCs exposed to chronic cAMP (110, 479) or chronic hypoxia (104, 107). Interestingly, T-type channel expression, as well as CC responsiveness to hypoxia decreases with postnatal maturation, and adrenal medulla denervation restores the sensitivity to hypoxia in parallel with the recruitment of T-type channels.

Ion channel remodeling in CCs following chronic and intermittent hypoxia

Exposure to chronic hypoxia, as it occurs in patients with chronic pulmonary obstructive disease causes elevated sympathetic activity, increased circulating CAs, and hypertension (432). Recent studies have shown that exposure to sustained or chronic hypoxia *in vivo* and in cultured cells leads to a functional remodeling of CCs excitability and CA secretion (89), and this may occur regardless of the increased sympathetic activity (104, 160). In this regard, exposure of cultured adult rat CCs to chronic hypoxia (3% O_2 for 12–18 h) causes a hypoxia-inducible factor (HIF)-dependent upregulation of $Ca_V3.2$ T-type Ca^{2+} channels that results in enhanced CA secretion during prolonged mild depolarizations (2–10 mM KCl) (104). T-type channels are absent in control normoxic conditions (20% O_2), and high-threshold Ca^{2+} currents (L, N, P/Q, and R) are insensitive to low O_2, suggesting that the hypoxia-induced recruitment of T-type channels is the only Ca^{2+} source that during chronic hypoxia sustains vesicle secretion at "low-threshold" voltages. This unique channel remodeling is similar to that occurring with another stressor stimuli (417). Interestingly, an HIF-2α-dependent increased expression of $Ca_V3.2$ T-type channel activity is also reported in PC12 cells following exposure to chronic hypoxia (160).

Chronic hypoxia also causes an upregulation of the brain-derived neurotrophic factor (BDNF)-TrkBs signaling pathway in CCs that leads to an increased voltage-gated Ca^{2+} entry and CA secretion (562). TrkBs are usually expressed in CCs and are upregulated during chronic hypoxia. Addition of BDNF causes increased burst firing, elevation of intracellular Ca^{2+}, and enhancement of CA release. Addition of the selective blocker of T-type Ca^{2+} channels TTA-P2 attenuates the BDNF-induced response, suggesting a key role of T-type channels in the upregulation of the Ca^{2+}-dependent CC response during chronic hypoxia. Since chronic hypoxia and stressors in general cause increased levels of circulating plasma BDNF (278), the facilitation of the BDNF-TrkB signaling pathway provides an alternative non-neurogenic mechanism that may contribute to the enhanced CA release during chronic hypoxia.

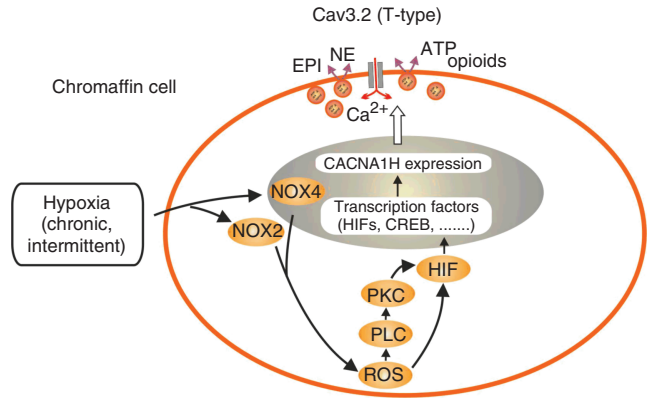

Figure 16 Cell signaling pathways leading to CACNA1H gene expression and Cav3.2 channels recruitment during chronic or intermittent hypoxia. Schematic pathway of the activation of transcription factors (HIF, CREB, etc.) and CACNA1H gene expression through a NOX, ROS, PLC, and PKC cascade leading to Cav3.2 channels recruitment during chronic/intermittent hypoxia. Adapted, with permission, from Mahapatra S, et al., 2012 (417).

Recurrent apnea with chronic intermittent hypoxia occurs in patients with sleep-disordered breathing and is associated with an increased sympathetic activity, enhanced levels of circulating CAs, and increased risk to develop hypertension and stroke (572). In adult rats, exposure to intermittent hypoxia leads to the potentiation of CA secretion from CCs during acute hypoxia. The mechanism involves a PKC-dependent increase of the RRP of SGs and an HIF-2α-dependent cell function remodeling (521). A similar potentiation of CA secretion in response to acute hypoxia is evident in neonatal CCs after exposure to intermittent hypoxia. The effect is associated with an increase of voltage-gated Ca^{2+} entry and intracellular Ca^{2+} stores (585) driven by a NOX-ROS-PLC-PKC signaling pathway, culminating in HIF-dependent upregulation of NADPH oxidase. The result is an increased expression of both $Ca_V3.1$ and $Ca_V3.2$ T-type channels and RyR_2 and RyR_3 (Figure 16).

Altered CC exocytosis by mutated proteins linked to human neurodegenerative diseases

A limited but increasing number of studies support the hypothesis that altered synaptic neurotransmitter release in various brain nuclei during the progression of neurodegenerative diseases is paralleled by changes in secretion in the sympathoadrenal axis (157). Below, we address how

proteins associated with neurodegeneration in various neurological diseases, including Parkinson's, Huntington's, and Alzheimer's diseases, autism, and amyolateral sclerosis, may function, or contribute to dysfunction, including the stages of exocytotic CA release from CCs (for complete details, see the section titled "Measuring exocytosis").

Altered CA exocytosis by the expression of proteins associated with neurodegenerative diseases

α-*Synuclein* The protein α-synuclein is located at presynaptic nerve terminals under physiological conditions. However, duplication and triplications of the WT human α-synuclein gene are present in familial forms of Parkinson's disease (PD) and dementia with Lewy bodies (577). Diminution of spontaneous synaptic responses (563), and of neurotransmitter release due to a reduction in size of the vesicle recycling pool (475), occurs in neurons overexpressing human α-synuclein. Also, this overexpression leads to synaptic dysfunction and diminished exocytosis in the striatum (258, 571). Pathology associated with α-synuclein has also been found in the adrenal medulla of PD patients and mouse CCs endogenously expressing the protein (234). Furthermore, the overexpression of α-synuclein in mouse CCs decreases CA release by acting at a late exocytotic step (372) that is associated with an acceleration of exocytosis by promoting fusion pore dilation (399). Thus, it seems that striatal synaptic pathology linked to α-synuclein also exists in adrenal medullary CCs, causing altered fusion pore kinetics and decreased exocytosis.

Huntingtin Expansion of CAG trinucleotide repeats (CAG repeats) in the N-terminal part of the huntingtin gene is the main pathological feature of HD. Huntingtin-associated protein 1 (HAP1) is the first interacting partner of huntingtin, and the degree of binding correlates with the length of the polyglutamine (CAG) repeats and primarily localizes to synaptic vesicles (382). Peripherally, HAP1 also localizes in secretory vesicles of endocrine cells, including adrenal medullary CCs (177, 384). This generated the hypothesis that HAP1 could contribute to the regulation of exocytosis. Adrenal CCs from mice with the HAP1 gene deleted discharged fewer amperometric secretory spikes and total secretion was decreased, upon stimulation with high K^+; this correlated well with a smaller RRP and a smaller fraction of docked vesicles, indicating that HAP1 reduces full fusion exocytosis by affecting vesicle docking and control of fusion pore stabilization (414). HAP1 also decreases endocytosis by interacting with some proteins of the exocytotic machine and binding to clathrin (415).

Altered excitability and exocytosis in mouse models of neurodegenerative diseases

The APP/PS1 mouse model of Alzheimer's disease

Senile plaques of amyloid-beta (Aβ) and neurofibrillary tangles due to tau hyperphosphorylation are the pathogenic hallmarks of Alzheimer's disease (AD) (275). Synaptic deficits correlate with this disease (568). Presenilin 1 (PS1) is the catalytic subunit of the multimolecular complex ϒ-secretase; PS1 contributes to the regulation of Ca^{2+} movements from the ER (474) and of neurotransmitter release (688). Mutations in the amyloid precursor protein (APP) and PS1 have been linked to early onset AD and to alterations of Ca^{2+} signaling and synaptic transmission. *APP/PS1* mice carry the Swedish mutation and the human PS1 mutation A246E (66). These mice develop an AD-like disease consisting of early Aβ plaques (66), diminished long-term potentiation (266), impairment of hippocampal synaptic transmission (524), and decreased synaptic plasticity (246). About 60% of amperometric secretory spikes generated with K^+ depolarizing pulses in CCs are preceded by an observable "foot." In aged mice, four of the characteristics of single exocytotic events were substantially smaller in *APP/PS1* mice with respect to wild-type mice, namely, peak current (Imax; −30%), decay time (−40%), $t_{1/2}$ (−45%), and quantal size (−50%). Thus, the fusion pore stabilization, expansion, and closure are faster in *APP/PS1* mice, but CA secretion is less. Although these mice exhibited brain Aβ deposition, Aβ pathology was not observed in their adrenal medullary tissue (156).

The SOD1^{G93A} mouse model of amyotrophic lateral sclerosis

Selective loss of motor neurons in amyotrophic lateral sclerosis (ALS) patients leads to paralysis, respiratory insufficiency, and death 3 to 5 years after diagnosis (546). Around 10% of patients have a mutation of glycine to alanine at codon 93 at Cu^{2+}/Zn^{2+}-dependent superoxide dismutase 1 (SOD1^{G93A}). In model mice, this mutation produces ALS paralytic symptoms at postnatal day 90, mimicking the human disease onset (267). Pathogenic features of ALS include augmented glutamate release (141, 339, 607), disturbed Ca^{2+} handling, overproduction of free radicals, and motor neuron death by apoptosis (651).

In adrenal CCs from SOD1^{G93A} mice at ages P90-P120, ACh elicited a 47% higher exocytosis burst secretion compared to WT mice. The kinetic analysis of single-vesicle amperometric events revealed notable differences in SOD1^{G93A} CCs. There was a 36% lower rise rate and 61% slower decay with 55% wider half-width and 52% higher quantal size, giving rise to single-vesicle exocytotic spikes with a slower release rate but greater CA release (92).

The R6/2 and R6/1 mouse models of Huntington's disease

HD is an autosomal dominant neurodegenerative disease with clinical symptoms of hyperkinetic involuntary movements, progressive dementia, aggressiveness, and paranoid psychosis. The disease is associated with mutated huntingtin, consisting of an anomalous expansion of CAG repeats giving rise to extended polyglutamine stretch and resulting in the formation of misfolded huntingtin aggregates that accumulate in the nucleus and the cytoplasm. These pathological features

are associated with neuronal loss in the caudate nucleus and putamen, in cortical layers III, IV, and VI and the lateral tuberal nucleus of the hypothalamus (365, 653). Mutated huntingtin aggregates and neuronal death are the two relevant markers of human HD that have been modeled in mice. For instance, transgenic mice R6/1 and R6/2 express the human huntingtin gene with, respectively, around 115 and 150 CAG repeats (425). In comparison with other HD mouse models, the R6/2 is the one that develops widespread huntingtin inclusions in the brain and early phenotypic symptoms (383). The R6/1 model reproduces several features of HD in older animals (272, 547, 548). Synaptic dysfunction and altered neurotransmitter release have been found in these HD mice models. For instance, electrically evoked dopamine release is attenuated in brain slices of 6-week-old R6/2 mice (327). The same group found that K^+ stimulation triggered a secretory response with similar spike number but lower vesicle quantal size (328) in CCs of R6/2 mice 3-months old. In a recent study performed in R6/1 mice, several alterations in CCs were reported; some of them were already present in 2-month-old mice at a phenotypic pre-disease stage, but they were more pronounced in 7-month-old mice when motor deficits were already established. CCs from R6/1 mice exhibited the following features: (i) mutated huntingtin overexpression as nuclear aggregates; (ii) smaller CC size with decreased dopamine-β-hydroxylase, indicating a decreased number of chromaffin SG; (iii) reduced adrenal medulla CA content; (iv) reduced peak Na_V currents; (v) membrane hyperpolarization and decreased ACh-evoked APs; (vi) diminished $[Ca^{2+}]_c$ transients with faster Ca^{2+} clearance; (vii) decreased quantal secretion with smaller single-vesicle quantal size; and (viiii) faster kinetics of the exocytotic fusion pore, pore expansion, and closure. These data suggest that the primary event in these alterations is the deposition of mutated huntingtin in the nucleus of adrenal CCs of R6/1 mice that could be responsible for less Na_V channel expression and function, giving rise to decreased cell excitability, altered Ca^{2+} handling and exocytosis, and eventually, cell damage and loss of CCs. These data suggest that in HD, the sympathoadrenal axis, which is tightly controlled by cortical neurons via hypothalamus, brain stem, and spinal cord is also significantly affected, predicting dysfunctional stress responses in HD patients (435).

The autistic TS2-neo mouse model carrying the G406R missense mutation on Cav1.2 channel

Timothy syndrome (TS) is a rare multiorgan channelopathy characterized by cardiac arrhythmias, long QTs, immune deficiencies, and autism spectrum disorder (ASD) (586, 587). TS is associated with a *de novo* single point mutation in the pore-forming subunit of $Ca_V1.2$ L-type Ca^{2+} channels (*CACNA1C*) and exists in two major forms (TS1 and TS2), depending on whether the point mutation appears on exon 8a (587) or exon 8 (586). The two exons code for the IS6 helix of $Ca_V1.2$ channel controlling the voltage-dependence of activation and inactivation. In the TS2, one mutation occurs at gly406 (G406R) within exon 8 that is highly expressed in the brain and the heart (80%) and to a lesser degree in the adrenal glands (586). The mutation causes reduced channel inactivation and shifts the voltage dependence of activation toward more negative potentials, thus causing increased Ca^{2+} influx during rest and cell activity. TS2 patients with the G406R mutation exhibit ASD-type behaviors (586).

The recent availability of the autistic TS2-neo mouse (41) made it possible to study the origins of neuronal firing and Ca^{2+} signaling mistuning that might generate autism. CCs possess high densities of L-type Cav1.2 channels (46, 426, 429) and are thus an excellent cell model to study the role of Cav1.2 channels on AP firing and CA secretion (100, 389). Interest in the effects of the TS2 mutation on CC function is intensified by a recent case report of a 2-month-old TS patient, whose post-mortem autopsy revealed remarkable bilateral adrenal glands dystrophy, possibly caused by the increased intracellular Ca^{2+} associated with the $Ca_V1.2$ channel mutation that occurred mainly in the adrenal medulla (340).

Recently, it has been reported that L-type currents in TS2-neo mouse CCs exhibit slower inactivation, voltage-dependent activation and inactivation shifted toward negative potentials, and large "window" current at rest (91). The increased "window" current is a likely cause of the increased resting Ca^{2+} that lowers the density of functioning Na_V channels, promotes AP switching from tonic to bursting firing mode alters cell morphology and reduces both mitochondrial metabolism and CA secretion. Extended cytoplasmic vacuolization and cell swelling is also observed by high-resolution electron microscopy in intact adrenal glands of TS2-neo mice. The findings of Calorio et al. (91), provide evidence that CCs are an excellent cell model for studying the effects of the Cav1.2 G406R mutation on AP firing and on Ca^{2+} signaling associated with autism.

A hypothesis concerning the alterations undergone by the sympathoadrenal axis in neurodegenerative diseases

Evidence is accumulating to support the hypothesis that some of the pathogenic features occurring in the CNS of patients suffering from certain neurodegenerative disease are also expressed in the sympathoadrenal axis. Those alterations may occur for the following reasons: (i) the sympathoadrenal axis may undergo impaired stress responses imposed by the limitations of body functions associated with neurodegeneration, (ii) "propagation" via cortico-hypothalamic-sympatho-adrenal axis of pathogenic synaptic dysfunctions occurring in the brain, (iii) mutant proteins linked to neurodegenerative diseases are expressed in CCs themselves, altering cell excitability, ion currents, Ca^{2+} signaling, and exocytosis, and (iv) leading to chronic alterations of blood pH, hormones, and their metabolites or hypoxic conditions that may further change CC function (see above). These four pathogenic pathways may ultimately give rise to altered exocytotic release at sympathetic neurons, and of NE and EPI at adrenal CCs, thus leading to poor adaptation to stressful conflicts in patients with neurodegenerative diseases.

Conclusion

Being the amplifying arm of the cortico-hypothalamic-sympatho-adrenal axis during the fight-or-flight response, the study of the physiological aspects of adrenal CCs has fundamental and intrinsic interest. Additionally, due to their accessibility and easy preparation of primary cultures from embryos, neonates, or adult mammals, CCs have been used widely as biological models to study exocytosis in fine detail, both ultrastructurally and molecularly. Ever since 1961 when Douglas and Rubin pioneered the discovery that Ca^{2+} entry was the trigger for ACh-mediated explosive release of CAs from perfused cat adrenal glands, we have learned much about cellular excitability, ion channels, Ca^{2+} signaling, SG pools, exocytosis, and endocytosis, both in normal and in animal models of disease, from the CC.

By now, a quite complete list of ion channels has been identified and characterized in CCs from various mammalian species, including humans. However, although the role of most channels has been defined in physiological processes (i.e. cell excitability, exocytosis, and endocytosis), only a few studies have approached how certain pathological states (i.e. hypoxia, hypertension, neurodegenerative diseases, and diabetes) affect these physiological parameters. It is not surprising that, as part of the sympathoadrenal axis that regulates body homeostasis, the adrenal medullary CCs are affected in various disorders, particularly brain disease. There is, therefore, ample scientific motivation to investigate the alterations undergone by CCs in animal models of disease as well as in humans. Recently, ion currents from CCs have been successfully recorded with automated patch-clamp technology and instrumentation (514). Given their relevance as proven models of CNS neurons, CCs could be a suitable preparation for high-throughput screening for the discovery and development of new drug candidates targeting therapeutically relevant ion channels.

The regulation of the rapid exocytotic release of CAs into the circulation during stress is particularly critical for two reasons: first, EPI is the most powerful secreted molecule of the organism and its release must be adapted to the degree of stress; and second, if extensively released, EPI may give rise to hypertensive crises and cardiac arrhythmias. Regulation of exocytosis has various control steps both centrally through afferent sympathetic output to CCs and at the level of the splanchnic-nerve-CC synapse, and in regulating the firing rate of the CC itself. There are also local regulatory loci, for example the activity of Ca_V channels by neurotransmitters acting on surface receptors on CCs. A second important type of regulation, particularly relevant under conditions of chronic stress occurs intracellularly and rests on the rate of secretory vesicle transport and the refilling of the exocytotic machinery with new vesicles. Future experiments should focus this problem in models closer to physiology, namely, in adrenal slices and *in vivo*. As proteins and ion channels are remodeled in cultures of CCs, efforts should be made to clarify this issue using the more complex preparation of adrenal slices.

The simple and classical view that the adrenal medulla releases CAs under acute and chronic stress in response to ACh release from the splanchnic nerve supported the investigation of the detailed cellular physiology underlying the function of this endocrine tissue for many years, and continues to do so. This view has been vastly expanded over the past 20 years, the adrenal medulla is now viewed as a more complex, and more integrative, stress transducer. Basal secretion, under the influence of ACh, is seen as important to cardiovascular function as well. Additional first messengers besides ACh, including GABA, PACAP, bradykinin, angiotensin, histamine, cytokines, and others, provide regulation of the CC and integration of inputs from the immune as well as the nervous system during stress to produce a complex output, including proteins, peptides, and CAs, that likely affects sensory neurons, and perhaps even the brain, as well as liver, heart, vasculature, and other CA target organs. The secretory products of CCs also have important, yet-to-be-discovered paracrine, autocrine, and hormonal roles, and this is especially true for CgA and its processed peptides including catestatin. The roles of ACh at the splanchnicoadrenomedullary synapse may require reassessment, especially regarding a potential role in modulation of gap junctions, nAChR- and mAChR-mediated gene regulation, and subtle but important regulation of the tempo of PACAP-induced CA release during both acute and chronic stress.

For over half a century, the CC has been a unique model to study basic mechanisms of cell excitability, ion channels, Ca^{2+} signaling, exocytosis, and endocytosis, all linked to basic principles of neuronal communication. We predict that CCs, particularly those of transgenic murine models of disease, and after genetic manipulation of SNARE and other proteins of the secretory machinery will continue to be an invaluable tool to explore those mechanisms with complementary genetic and molecular biology techniques, and with electrophysiological and neuropharmacological approaches. The future of CCs as a neuronal model is assured.

The abbreviations used throughout this review are listed in Table 3.

Acknowledgements

We are very grateful to all members of the CC club (*International Symposium of Chromaffin Cell Biology*) who have devoted so much of their research effort to the chromaffin cell. This group meets every 2 years at different locations worldwide in an outstanding scientific and friendly atmosphere. This work has been partially funded by grants from i) CONACyT Laboratorio Nacional de Canalopatías 299051 and PAPIIT AG200119 (DGAPA-UNAM) to A.H-C. ii) MH002386 to L.E.E., iii) SAF 2016-78892R from MINECO, Spain, European Union Horizon2020 Research and Innovation Programme under the Marie Skoldowska-Curie Grant Agreement No. 766124, and Fundación Teófilo Hernando, Spain to A.G.G., iv) Ministerio de Ciencia, Innovación y Universidades BFU2017-82618-P to R.B. and v) Telethon Foundation, Italy (GGP 15110) and local grants from the University of Torino to E.C.

Table 3 List of Abbreviations

Abbreviation	Definition	Abbreviation	Definition
AADC	Aromatic amino acid decarboxylase	DCRD	Distal C-terminal regulatory domain
AE3	Anion exchanger	EGFP	Enhanced green fluorescent protein
Aβ	Amyloid beta-protein	Egr-1	Early growth response protein 1
AC	Adenylate cyclase	EPI	Epinephrine
ACh	Acetylcholine	EPAC	Exchange protein activated by cAMP
ACTH	*Adrenocorticotropic hormone*	ER	Endoplasmic reticulum
AD	Alzheimer's disease	ERG	Ether-à-go-go-related gene
AHP	Afterhyperpolarization	ERK	Extracellularly regulated kinase
AP	Action potential	GABA	γ-Aminobutyric acid
ALS	Amyotrophic lateral sclerosis	$GABA_A$-R	$GABA_A$ receptor
APP	Amyloid precursor protein	GPCR	G-protein-coupled receptor
AR	Adrenergic receptor	HAP1	Huntingtin-associated protein 1
ASD	Autism spectrum disorder	HCMDs	High calcium microdomains
ATP	Adenosine triphosphate	HD	Huntington's disease
BK	Big-conductance potassium channels	HIF	Hypoxia-inducible factor
BAM22P	Peptide (aa210-231) of proenkephalin-A	Kv	Voltage-gated potassium channels
BDNF	Brain-derived neurotrophic factor	IBMX	3-Isobutyl-1-methylxanthine
β-AR	Beta-adrenergic receptor	IL-1	Interleukin type 1
CAs	Catecholamines	$InsP_3$	Inositol tris-phosphate
Cav	Voltage-gated calcium channels	$InsP_3R$	Inositol tris-phosphate receptor
$[Ca^{2+}]_i$	Intracellular calcium concentration	IRP	Immediately releasable pool
$[Cl^-]_i$	Intracellular chloride concentration	ISI	Interspike interval
CC	Chromaffin cell	K_{ATP}	ATP-sensitive K channel
CDI	Calcium-dependent inactivation	KCC2	Cation-chloride cotransporter
CGRP	Calcitonin gene-related peptide	LDCV	Large dense core vesicles
CGs	Chromaffin granules	L-Dopa	Levorotatory form of dopa, L-3,4-dihydroxyphenylalanine
CgA	Chromogranin A	mAChR	Muscarinic cholinergic receptor
cGMP	Cyclic guanosine monophosphate	MAO	Mono amine oxidase
cAMP	Cyclic adenosine monophosphate	MHPG	3-Methoxy-4-hydroxyphenylglycol
CICR	Calcium-induced calcium release	MAPK	Mitogen-activated protein kinase, also called ERK,
CNS	Central nervous system		extracellular signal-regulated kinase
CPE	Carboxypeptidase processing enzyme	mNCX	Mitochondrial electroneutral Na^+/Ca^{2+} exchanger
COMT	Catechol-*O*-methyltransferase	MICU	Mitochondrial Ca^{2+} uniporter
CREB	cAMP response element-binding protein	MCCs	Mouse chromaffin cells
DA	Dopamine	nAChR	Nicotinic cholinergic receptor
DR1	Dopamine receptor type 1	Nav	Voltage-gated sodium channels
DBH	Dopamine-beta-hydroxylase	NE	Norepinephrine

(*continued*)

Table 3 (*Continued*)

Abbreviation	Definition	Abbreviation	Definition
NCS-1	Neuronal calcium sensor-1	SG	Secretory granule
NCX	Na^+/Ca^{2+} exchanger	SHR	Spontaneously hypertensive rat
NCS	Neuritogenic cAMP sensor	SNARE	Soluble NSF-attachment receptor complex
NKCC	$Na^+/K^+/2Cl^-$ cotransporter	SNS	Sympathetic nervous system
NO	Nitric oxide	Sp1	Specificity protein 1
NOS	NO synthase	SK	Small-conductance potassium channels
NPY	Neuropeptide Y	SOCCs	Store-operated calcium channels
NSF	*N*-Ethylmaleimide-sensitive factor	SOD	Superoxide dismutase
PACAP	Pituitary adenylate cyclase-activating polypeptide	SR	Sarcoplasmic reticulum
PAC1R	PACAP type 1 receptor	SRP	Slow releasable pool of vesicles
PAM	Peptidylglycine alpha-amidating monooxygenase	TEA	Tetraethylammonium
PD	Parkinson's disease	TH	Tyrosine hydroxylase
PDE	Phosphodiesterase	TS	Timothy syndrome
PIP_2	Phosphatidylinositol (4,5)-bisphosphate	TIRFM	Total internal reflection fluorescence microscopy
PKA	Protein kinase A	TNF-α	Tumor necrosis factor-alpha
PKC	Protein kinase C	TrkB	Tyrosine receptor kinase B
PKG	cGMP-dependent protein kinase	TTX	Tetrodotoxin
PLB	Phospholamban	VAMP	Vesicular monoamine transporter
PMCA	Plasma membrane Ca^{2+}-ATPase or Ca^{2+} pump	VAChT	Vesicular acetylcholine transporter
PNMT	Phenylethanolamine-*N*-methyltransferase	VNUT	Vesicular nucleotide transporter
PS1	presenilin 1	VIP	Vasoactive intestinal polypeptide
Rapgef	Rap-activating guanine-nucleotide-exchange factor	V-ATPase	Vesicular proton pump
ROS	Reactive oxygen species	VMA	Vanillylmandelic acid
RP	Reserve pool of vesicles	VMAT	Vesicular monoamine transporter
RRP	Readily releasable pool of vesicles	WT	Wild type
RyR	Ryanodine receptor	WKY	Wistar-Kyoto rats
SERCA	Sarco-endoplasmic reticulum Ca^{2+}-dependent ATPase		

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