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Roles of Na^+, Ca^{2+}, and K^+ channels in the generation of repetitive firing and rhythmic bursting in adrenal chromaffin cells

Christopher J. Lingle[1], **Pedro L. Martinez-Espinosa**[1], **Laura Guarina**[2], and **Emilio Carbone**[2]

[1]Department of Anesthesiology, Washington University School of Medicine in St. Louis, 660 S. Euclid Ave., St. Louis, MO 63105, USA

[2]Department of Drug Science, Laboratory of Cellular and Molecular Neuroscience, N.I.S. Centre, Corso Raffaello 30, 10125 Torino, Italy

Abstract

Adrenal chromaffin cells (CCs) are the main source of circulating catecholamines (CAs) that regulate the body response to stress. Release of CAs is controlled neurogenically by the activity of preganglionic sympathetic neurons through trains of action potentials (APs). APs in CCs are generated by robust depolarization following the activation of nicotinic and muscarinic receptors that are highly expressed in CCs. Bovine, rat, mouse, and human CCs also express a composite array of Na^+, K^+, and Ca^{2+} channels that regulate the resting potential, shape the APs, and set the frequency of AP trains. AP trains of increasing frequency induce enhanced release of CAs. If the primary role of CCs is simply to relay preganglionic nerve commands to CA secretion, why should they express such a diverse set of ion channels? An answer to this comes from recent observations that, like in neurons, CCs undergo complex firing patterns of APs suggesting the existence of an intrinsic CC excitability (non-neurogenically controlled). Recent work has shown that CCs undergo occasional or persistent burst firing elicited by altered physiological conditions or deletion of pore-regulating auxiliary subunits. In this review, we aim to give a rationale to the role of the many ion channel types regulating CC excitability. We will first describe their functional properties and then analyze how they contribute to pacemaking, AP shape, and burst waveforms. We will also furnish clear indications on missing ion conductances that may be involved in pacemaking and highlight the contribution of the crucial channels involved in burst firing.

Keywords

Sodium channels; Potassium and calcium channels; Action potential; Burst firing; Chromaffin cell excitability; Catecholamine release

Correspondence to: Emilio Carbone.

Introduction

Chromaffin cells (CCs) of the adrenal medulla are sympathetically innervated neuroendocrine cells that secrete catecholamines (CAs) into the general circulation in response to stress. Typically, the trigger for CA secretion is the activity of the splanchnic nerve whose nerve terminals in the adrenal medulla release acetylcholine (ACh) sufficiently close to CCs that fast synaptic potentials can be observed, at least in some cells [72]. The immediate response to ACh release is activation of nicotinic receptors (nAChRs) [47, 108] which both depolarize CCs and permit Ca^{2+} influx through nAChR [146]. This in turn produces cell depolarization, action potential (AP) discharges, and CA secretion. In some species, ACh also activates muscarinic AChRs (mAChRs) [60, 97, 108] that also contribute to secretion through the stimulation of stored cytosolic Ca^{2+} [59, 64]. In some case, mAChR stimulation induces secondary depolarization by opening non-selective cation channels [70, 71] or blocking TASK-1 potassium channels [69] that increase AP firing. The details and relative importance of mAChR activation in CA secretion remains incompletely understood and may vary appreciably among species.

Despite the potentially simple role that CCs play as relay elements that couple splanchnic nerve activity to CA secretion, CCs exhibit a rich “palette” of ion channels. The diversity of ion channels found in CCs mirrors that found in neurons, permitting extensive variation in patterns of electrical activity that may occur regardless of the activity evoked by neural input. If CCs are essentially relay elements that connect splanchnic nerve activity to nAChR-evoked CA secretion, why should there be such a rich variety of ion channels? Despite the central importance of neurogenically evoked release of CAs [38], one possibility is that intrinsic electrical activity of CCs may under some circumstances also contribute to CA secretion. Consistent with this idea, recent work has begun to reveal that CCs can exhibit a variety of patterns of intrinsic excitability, including a slow-wave burst activity [5, 56, 58, 90, 94, 130] that may be of potential importance in CA secretion.

Here, we provide a brief summary of the Na^+, Ca^{2+}, and K^+ ion channels available to influence CC excitability and a detailed overview of the types of intrinsic excitability that have been observed in CCs together with its mechanistic underpinnings. We will also discuss possible implications for CA secretion and highlight areas of future investigation that need to be addressed.

The “palette” of ion channels

Despite being a relatively homogenous population of cells, CCs exhibit a surprisingly rich array of ion channels and it is possible that there are still some missing elements that will be soon uncovered.

Na^+ channels (Nav1.3, Nav1.7)—CCs of essentially all mammalian species exhibit a robust voltage-dependent Na^+ (Nav) current [48, 81, 130] that appears to be entirely TTX-sensitive. The Na^+ current in CCs has been reported to arise from Nav1.7 [75, 133, 134], but evidence on this point is limited. Recent work, using quantitative PCR and western blots, suggests a contribution of both Nav1.3 and Nav1.7 in mouse CCs (MCCs), with a predominance of the Nav1.3 isoform [130]. However, both the steady-state inactivation and

activation behavior of the mouse Nav whole-cell currents seems consistent with a single type of channel [130]. Based on studies of Nav1.3 and Nav1.7 expressed heterologously, both channels share generally similar functional properties, but the voltage at which Nav1.3 is half-inactivated is right-shifted compared to that for Nav1.7 [34, 65, 132]. In pancreatic α- and β-cells, Nav1.3 and Nav1.7 are both expressed, with Nav1.3 more abundant in α-cells and Nav1.7 more abundant in β-cells. In these cells, steady-state inactivation curves require fits with double Boltzmann functions but single Boltzmann functions with the proper shift in both Nav1.3 KO and Nav1.7 KO mice [143]. This suggests that, if both Nav1.3 and Nav1.7 were functionally present in CCs, it should be possible to discern this in steady-state inactivation protocols. Yet, in MCCs, Nav currents seem most consistent with dominant Nav1.3 channels. In fact, preliminary results confirm that Nav current is completely absent in CCs from Nav1.3 KO mice (Martinez-Espinosa and Lingle, unpublished).

One complication to the view that Nav1.3 and Nav1.7 current can be distinguished in native cells is that Nav channel gating properties, including the steady-state inactivation curves, are critically influenced by the particular auxiliary β subunits with which they are expressed [67, 144]. Furthermore, steady-state inactivation of a variety of Nav channels is influenced by fibroblast growth factor homologous factors (FGFs) [55, 78, 110, 138]. An additional complexity is that exposure of cultured bovine CCs to tumor necrosis factors results in up-regulation of Nav1.7 message and protein, suggestive that particular physiological circumstances may up- or down-regulate specific Nav channel components [120]. Clearly, additional work is required to clarify the potential contributions of different Nav channels in CCs.

Irrespective of the molecular identity of CC Nav channels, a unique feature of Nav currents revealed in both MCCs and RCCs is a slow recovery from fast inactivation occurring on the order of 10's to 100's of ms [81, 130], with slower and more incomplete recovery at potentials near resting potentials (− 40, − 50 mV). Such slow inactivation has a time course which suggests that the intrinsic gating of CC Nav current may effectively modulate Na^+ channel availability and thus may alter firing frequency [77, 118] and burst firing [130]. If the process of Nav slow inactivation could itself be regulated by neuromodulators, this would provide an additional nuance to how CC firing rates and mode of firing can be controlled.

Ca^{2+} channels (Cav1, Cav2, Cav3)—Since the early days of patch-clamp recordings [11, 48, 68], the pharmacological diversity, gating properties, and modulation of voltage-dependent Ca^{2+} current in CCs from many species have been the subjects of extensive investigation [1, 9, 10, 12, 13, 18, 21, 24, 28, 32, 50, 79, 80, 85, 86, 88, 91, 100, 102, 104, 105, 109, 116, 127]. Essentially all known subtypes of high voltage-activated Ca^{2+} current, including both Cav1.2 and Cav1.3 L-type channels, the Cav2.1 P/Q-type channel, the Cav2.2 N-type channel, and the Cav2.3 R-type channel have been described not only in rat [107], mouse [4, 86], cat [1], and bovine [9, 10, 85] chromaffin cells, but also in human [105]. In addition, stressful and age-related conditions unmask the presence of Cav3.2 low-voltage, inactivating T-type Ca^{2+} channels in CCs [18, 25–27, 66, 76, 99].

Because of interest in how cytosolic Ca^{2+} transients may couple to exocytosis, CCs have been explored extensively as a model system to try to elucidate critical Ca^{2+}-dependent steps required for secretion [42, 51]. As part of such efforts, attention has been focused on whether specific Cav channel subtypes may have specialized roles in coupling to secretion of CAs or play other subtype-specific roles. In this regard, evidence on this point varies. In mouse adrenal slices, R-type Cav channels have been linked to rapid secretion [4]. Other work has implicated a close colocalization of P/Q-type channels to the immediate releasable pool of vesicles in mouse CCs [6, 7] while others have found an effective coupling of T-type as well as of L-type Cav channels to exocytosis in rat CCs [24] but not specific colocalization to the secretory machinery [54]. Most work on excitation-secretion coupling in CCs of various species suggests weak coupling between all Cav channel types and secretory vesicle localization [30, 43, 73, 121]. Consistent with this is also a study using modeling buffered Ca^{2+} diffusion near the membrane to mimic secretion in bovine CCs. According to the model, the amount of exocytosis that arises from secretory granules colocalized to Cav channels is estimated to be ~10% of the total [74]. Experimental data are best simulated when ready releasable granules are randomly distributed within a regular grid of Cav channels with mean interchannel distances of 300–600 nm.

Cav channels also play a central role in the regulation of CC excitability arising from the interplay of inward Cav currents and the associated activation of Ca^{2+}-dependent outward currents either through nearby BK channels or more distant SK channels [98, 128]. For some neurons and neuroendocrine cells, it has been proposed that mechanisms may exist enabling close coupling of Cav and BK channels. In support of this idea, Cav2.2 [93] and Cav2.1 channels [139] have been shown to couple to BK channel activation. In CCs, all Cav channels can drive BK currents but the L-type appears preferentially colocalized to the BK channels, particularly when using brief depolarizing steps [92, 107]. With longer depolarizing stimuli, both secretion [85] and activation of BK channels are similarly activated by the bulk elevation of Ca^{2+} arising from all Cav channels [85, 107].

In neurons and neuroendocrine cells, Cav channels can also be effectively modulated by neurotransmitters and neuromodulators by interacting with G protein-coupled receptors (GPCRs) which exert critical autocrine/paracrine control on synaptic transmission and neuroendocrine secretion (for a review see [142]). Chromaffin cells follow this general rule as they express a large variety of GPCRs that are mainly targeted by the released products of their own granules [3]. This gives a unique autocrine character to the Cav channel modulation, particularly in view of the structural arrangement of CCs that are organized in a compact epithelial fashion with a gap of ~200 A° between adjacent cells to form a “bar-like” structure facing blood vessels in the intact adrenal gland [33]. Cav2.1 (P/Q-type) and Cav2.2 (N-type) channels are effectively inhibited by ATP [35, 49], opioids [2, 21], and histamine [37], which exhibits a voltage-dependent facilitation during strong depolarization. Cav1 (L-type) channels are either inhibited through a voltage-independent PTX-sensitive mechanism by α- and β-AR agonists [23, 28, 61] or up-regulated by a cAMP/PKA intracellular pathway [28, 88], similar to the β-AR-mediated up-regulation of cardiac Cav1.2 channels [15]. Thus, apart from the Cav1.2/Cav1.3 up-regulation by β-AR agonist stimulation, most pathways of Cav channel modulation serve as a negative feedback inhibition to regulate catecholamine secretion in CCs. The voltage-dependent inhibition is removed by 15–20% during

stimulation with AP trains of high frequency (45 Hz) [36], but the voltage-independent inhibition of L-type channel persists either during stimulation with high frequency AP trains or repeated burst activity. The existence of an effective autocrine inhibition of Cav2 and Cav1 channels during physiological conditions is most evident when measuring the time course of Cav currents in clusters of CCs [62] or in CCs belonging to adrenal gland slices [63]. Stopping bath perfusion, the Cav currents appear inhibited due to the locally released neurotransmitters (ATP, opioids, A, NA) that can autocrinally modify the currents by slowing the activation and lowering the amplitude. In contrast, continuous perfusion helps recover the normal Cav current activation and size by clearing the released molecules. How such modulation may truly affect spontaneous firing, rhythmic bursts, and secretion has never been investigated either in slices or in isolated CCs.

Ca^{2+}-dependent, voltage-independent SK-type K^+ channel—CCs were among the first cells in which a voltage-independent, but Ca^{2+}-dependent, K^+ current was initially identified in both rat [97, 98] and bovine CCs [8] This current is sensitive to the bee venom toxin apamin and mediates a robust activation of outward current following release of cytosolic Ca^{2+} stores [97]. Single channels of the SK type are of small conductance and are half activated at about 0.7 μM cytosolic Ca^{2+} [103]. As in other cells possessing these channels [17, 44, 45], the SK channels of CCs are only activated following sustained elevation of cytosolic Ca^{2+} [98]. Mouse CCs express all the identified SK channel subtypes: SK1, SK2, and SK3 [131]. Interestingly, SK activation slowly builds up between successive APs and addition of apamin increases the spontaneous firing rate in most CCs. This suggests that there is enough SK activation between spontaneous APs to prolong the interspike interval (ISI). Intriguingly, the percentage increase in firing frequency produced by apamin is inversely correlated with the basal firing frequency, supporting the view that SK channel activation contributes significantly to determining the ISI, especially in cells firing at low frequencies or not firing at all [131].

A peculiarity of SK channels is that they respond to relatively small and persistent increases in cytosolic Ca^{2+} [46]. This allows them to play a negative feedback role in response to Ca^{2+} influx to sculpt firing frequency in CCs. SK currents build-up markedly during high frequency firing or prolonged bursts and help terminating the firing or the burst [131]. In this way, SK channels are the main K^+ channel type that activate during mild depolarization near rest or during the ISI. The increase is proportional to the amount of Ca^{2+} entering the cell and thus proportional to the number of repeated APs or the duration of a burst. In this way, SK channels act as an effective “brake” of firing cells ensuring more regular firing and AP of maximal amplitude during sustained cell activity.

Ca^{2+}- and voltage-dependent BK-type K^+ channels—CCs were one of the first cell types in which BK channels were first described [95]. Although there is variability in the absolute current density of Kv currents in CCs, with appropriate Ca^{2+} elevations and sufficient depolarization, BK current is typically the most prominent outward current in CCs in all mammalian species examined [82, 94, 98]. A unique feature of CC BK current, whether in rat, cow, or mouse, is that it can be either inactivating or non-inactivating, as established in both excised-patch single channel recordings and macroscopic current

recordings [40, 92, 94, 117, 118]. In some CCs, the whole-cell BK current is essentially completely inactivating (termed BK_i current), while in other cells, there is very little inactivation at all and has been termed BK_s for sustained BK current [40, 82, 94, 118, 129], with other cells exhibiting an intermediate behavior [40]. BK_i and BK_s currents are also evident in whole-cell recordings in other cell types including anterior pituitary cells [125, 126].

BK inactivation in CCs depends on the presence of a regulatory β_2 subunit [136, 140] which contains a cytosolic N-terminus that mediates a pore occlusion type of inactivation [141]. Up to four β_2 subunits can be present in an individual BK channel, and this occurs both with heterologous expression of α and β_2 subunits [137] and in native CCs [40, 94]. Genetic deletion of the β_2 subunit completely abolishes inactivation of BK current in mouse CCs [94]. The functional role of BK channel inactivation per se remains unclear [119], and during a train of APs at 10 Hz, little cumulative BK channel inactivation will occur. The absence of cumulative inactivation arises since the typical inactivation time constant at physiological voltages and cytosolic Ca^{2+} is probably slower than 35 ms, reflecting an average of about 2 β_2 subunits per channel [40, 94]. However, in addition to producing inactivation, the regulatory β_2 subunit also shifts the range of voltages of activation in comparison to WTchannels [140]. In comparison to BK channels lacking β_2 subunits, with a channel with a full set of 4 β_2 subunits, the shift would be about − 60 mV to more negative voltages with 10 μM cytosolic Ca^{2+}. It is this shift in the effective channel gating range that results in different firing between cells with inactivating BK current and cells without [94, 118, 119]. In the absence of the β_2 subunit, BK channels will activate a bit more rapidly but to a lower open probability at a given Ca^{2+} [94]. Because of this weaker activation, peak AP amplitude is somewhat increased, AP repolarization is slowed, and the AHP is reduced. This is thought to slow the rate of recovery of Nav channels from inactivation, thereby reducing the contribution of Nav channels in the next AP eventually resulting in AP block with enough cumulative inactivation [77]. How the properties of BK channels may impact on burst firing will be considered further below.

Given the importance of BK channels in influencing AP properties and firing rates in CCs, any mechanism that modulates BK channels may have critical effects on firing and CA secretion. This topic deserves more attention, but some interesting examples have been identified. An opioid receptor, most likely μ-type, has been shown to enhance BK channel activation in bovine CCs in a membrane delimited [124], but apparently G-protein-independent fashion [123], potentially providing a mechanism of opiate receptor-mediated inhibition of CA secretion. Arachidonic acid and other metabolites have also been shown to robustly enhance BK currents in bovine CCs [122]. BK currents in bovine and rat CCs are also affected by glucocorticoids in complex ways, although perhaps at non-physiological concentrations [83]. BK currents are also up-regulated by increased levels of leptin (the “satiety hormone”) that via PI3K inhibits the tonic firing of resting hippocampal neurons [52, 115] and mouse CCs [53]. In mouse CCs, the same potentiating effect of leptin on BK channels stabilizes the firing rate during sustained stimulation.

Slowly (Kv1–3) and fast inactivating (Kv4) voltage-dependent K^+ channels—In early work on BCCs, two distinct Kv channel types were identified [96] based primarily on

single-channel conductance and rates of activation and inactivation. A more recent study confirms the existence of these Kv currents when BK channels are blocked by charybdotoxin [114]. The transient K^+ current component (I_A; Kv4) is prominent and has half-maximal activation at − 14.2 mV in nominally Ca^{2+}-free solution. Inactivation is nearly complete after 100 ms (τ_{inact} = 25 ms), and recovery from inactivation is fast. About 85% of the channels recover with a time constant of 37 ms. All this suggests that transient Kv channels in CCs can play a role in the regulation of AP trains during sustained stimulation. Kv channels are also expressed in rat [98] and mouse CCs [92] when BK current is inhibited. In the rat, the whole-cell Kv current requires voltages above − 40 mV to be activated. When K^+ current activated at + 80 mV is compared under conditions in which BK current is absent, total Kv current density (pA/pF) in mouse CCs is about 3–4-fold higher than that in rat CCs [94], consistent with a stronger contribution of Kv channels than BK channels to AP repolarization in mouse in comparison to rat [129].

Other K^+ channels: Kv7 (KCNQ) and Kv11 (ERG) channels and K_{2P} (TASK)—A number of studies have provided evidence of other CC K^+ channels, which might be of importance in the regulation of cell excitability, but for which information remains limited. First, an M-current channel (Kv7; KCNQ) inhibited by extracellular histamine has been observed in bovine CCs [135]. Kv7 are "low-threshold" K^+ channels that are already open at rest, activate very slowly with membrane depolarization, and do not inactivate during prolonged depolarizations. Kv7 are highly expressed in postganglionic sympathetic neurons [20] and brain [89] and are blocked by mAChR activation through a membrane-delimited PIP2 depletion mechanism mediated by Gq/PLC-β [39]. Their existence in CCs together with the presence of mAChRs is suggestive of a potential role of Kv7 channels in cell depolarizations induced by mAChR activation, but data on this issue are still unclear and partly contradictory [101]. Second, a current with gating properties and pharmacological sensitivities characteristic of an ERG (Kv11) current has been described in rat CCs [58]. Block of ERG with the selective blocker WAY-123,398 causes marked cell depolarization; increased firing frequency; and, in some case, depolarization block, suggesting an important role of this channel in the regulation of spontaneous CC excitability. Finally, a background pH-sensitive K^+ current termed TASK-1-like has been observed in rat CCs [69]. TASK-1 and TASK-3 channels are two-pore "leak" channels (K_{2P}) also expressed in mouse CCs that contribute to the membrane potential and firing activity of cells at rest [56]. In rat CCs, TASK-1 channels are shown to be blocked by the activation of mAChRs and are thus postulated as one of the targets of the mAChR stimulatory action that causes increased CA secretion [69]. Although much less is known about these K^+ currents than many of those described above, each of them have properties that under pathophysiological circumstances may impact on CC excitability.

Blending the "palette"—As noted above, the relative contribution of Na^+, Ca^{2+}, and K^+ channels within CCs appears to vary substantially among species. For example, Kv conductance contributes a much larger fraction of total K^+ conductance in MCCs than in RCCs [94]. BK channels in RCCs [40], exhibit more robust inactivation than in MCCs [94], indicative that, on average, there are more regulatory β_2 subunits per BK channel in rat cells than in mouse. Cav channels are also differentially expressed among animal species [51].

For example, bovine CCs express low densities of Cav1 channels (20%) while rat and mouse CCs possess higher densities (40–50%) that condition their basal firing activity [5, 61, 92]. Even among CCs within a given species, the particular composition of the underlying channels may vary. For example, in both RCCs [40] and MCCs [94], BK channel inactivation varies considerably among different cells. Similarly, resting mouse CCs respond differently to apamin, suggesting differential densities of expressed SK channels that regulate basal firing rates [130]. Another important consideration is that various physiological circumstances may up- or down-regulate particular currents by re-modeling the expression of specific Cav channels in regard to gender [29], age [76, 102], hypoxia [25], and stress [57].

How different current components may vary among species, among cells within a species, or with physiological conditions poses an important challenge to the identification of the ion channels that regulate the firing in any cell. Even within a simple cell like a CC, the attempt to define a single archetypic explanation of how different patterns of firing are generated may not be simple. Despite these cautionary remarks, our goal below is to try to summarize results that identify the contributions of specific ion channel components to different types of spontaneous electrical activity found in CCs, with a particular focus on rhythmic slow wave bursting activity. In regard to slow wave bursts, we will assess whether there are common features among different manipulations that increase the likelihood of burst occurrence and point to future issues that must be addressed in order to assess whether such activity is of physiological relevance to adrenal and sympathetic function.

Types of spontaneous electrical activity in adrenal chromaffin cells

Beside the widely accepted role that splanchnic nerve-evoked depolarization plays on CA secretion in adrenal CCs, these cells exhibit a number of distinct non-neurogenic electrical activities when isolated and plated in culture. Most typically, they exhibit simple repetitive spontaneous AP firing of about ~1 Hz (Fig. 1a) but also show occasionally slow wave bursts (Fig. 1b). Recent work shows that various manipulations may increase the likelihood of observing burst firing [56, 130]. An example is given in (Fig. 1c). In addition to these cases, inspection of various published records suggests that rhythmic firing patterns in CCs obtained under various conditions may exhibit a number of other waveforms that vary in terms of frequency and duration of firing and duration of period of inactivity (Fig. 2a–d). These examples suggest that CCs are capable of much more complex patterns of rhythmic activity than typically considered. Depending on the prevalence of different patterns of rhythmic activity within the CC population, one or more of these patterns of activity might be expected under some circumstances to contribute to a non-neurogenic secretion of CAs. To what extent such non-neurogenic CC activity may contribute to CA secretion independent of splanchnic nerve activity remains unknown. However, the existence of such neuronal-like bursting activity suggests that understanding how prevalent it may be, when it occurs, and how it may be regulated is of key importance to understand the pathophysiological role of the adrenal medulla.

Repetitive action potential firing in CCs—The ability of CCs to generate APs in response to depolarizing current injection or application of ACh was first established about

40 years ago [16, 19] using sharp electrode recordings. At that time, extracellular recordings suggested that CCs may exhibit some spontaneous firing activity but at relatively modest frequencies (0.1 Hz) [19]. With the application of patch-clamp methods to CCs, the primary focus was on identification of various current components that may participate in nerve-evoked depolarization and which may influence the Ca^{2+}-influx required for secretion. Yet, multiple papers throughout the past 25 years have confirmed that CCs do fire spontaneously, either in current clamp recordings or in recordings of spontaneously occurring Ca^{2+} transients [14, 58, 97]. In some cases, records of spontaneous activity exhibited considerable rhythmic complexity [58], perhaps suggestive of bursting (Fig. 2b).

Over the past 10 years, attention has been given to the origins of spontaneous AP firing in CCs led primarily by one of our groups using KO and KI mice generated by the Striessnig group [106, 111]. The primary focus of this body of work has been the identification of the currents that control the timing of repetitive AP firing [87, 90, 92, 129, 131]. Through a comparison of WT and Cav1.3 KO mice and by pharmacological dissection of the currents, active during the ISI and preceding the upswing of an individual AP, both a Cav1.3-mediated inward current and a BK-mediated outward current are active during the ISI. This results in an essentially stable level of net current close to zero [92]. In the Cav1.3 KO CCs, both the inward and outward currents are absent, reflecting the requirement that Cav1.3 supports BK activation. Interestingly, when BK conductance is blocked either by 1 μM paxilline or 5 mM TEA, current during the ISI appears as a slowly activating inward current, which is absent in Cav1.3 KO cells. The intrinsic time-dependence of the Cav1.3 current activation would suggest that its activation is a key timing element essential to initiate each subsequent AP. The relatively unchanging membrane potential during most of the ISI apparently arises primarily from coupling of Cav1.3 activation to BK activation serving to hold a cell near a net zero current level.

Is Cav1.3 a pacemaking channel?—The biophysical properties of Cav1.3 are consistent with it playing a more important role in repetitive firing in comparison to other Cav currents or Nav currents in CCs. KO of Cav1.3 results in a ~9 mV positive shift in the g(V) for L-type current activation [87]. The derived g(V) for Cav1.3 activation suggests that Cav1.3 channels begin to open around − 50 mV, right within the range suitable for a Cav1.3 contribution to the onset of inward current during the ISI, and about 10 mV more negative than the range of activation of Cav1.2 and other Cav subtypes.

Overall, it is evident that Cav1.3 contributes significantly to the pacemaker current in CCs, but is Cav1.3 the only determinant of repetitive firing in CCs? Several factors suggest that there are additional contributors to pacemaking that remain to be identified. A requirement for any pacemaking mechanism is that some time-dependent changes in conductance are expected to occur during the ISI that leads to a net inward current sufficient to produce regenerative activation of other inward currents. In this regard, it is not clear how the combination of Cav1.3 and BK activation acts to time a gradual increase in net inward current sufficient to elicit each sequential AP. In addition, 30% of Cav1.3 KO CCs continue to fire spontaneously.

Clearly, there must be determinants other than Cav1.3 that are permissive for repetitive firing. However, at present, no obvious inward current has been observed in the Cav1.3 KO cells during the ISI when sequential AP-clamp waveforms are employed [92]. Given the known heterogeneity of different current components among CCs in both rat and mouse, it perhaps would be interesting to compare currents during the ISI in Cav1.3 KO mice between cells that are quiescent and those that are still capable of firing repetitively. One possibility is that, in some cells, Cav1.2 current density might be sufficient and cell resting potential depolarized enough that activation of Cav1.2 may lead to repetitive APs. If that were the case, AP clamp waveforms in Cav1.3 KO cells might reveal such nifedipine-sensitive inward current activation during the ISI, but perhaps at slightly more depolarized holding potentials. To date, that has not been observed. However, if Cav1.2 were able in some cases to substitute for Cav1.3 in pacemaking, one would expect that L-type current inhibition would always prevent spontaneous firing in CCs. Indeed, there are cases in which CCs exhibit repetitive firing, even when all L-type Ca^{2+} channels are blocked by nifedipine [86, 92], suggestive that some pacemaker activity can persist even in the total absence of L-type current. Together, these considerations suggest that Cav1.3 alone is not the necessary and sufficient component required to support repetitive firing in CCs, although when it is present, it is probably the major source of inward current leading to AP initiation. Overall, however, Cav1.3 does appear to be of fundamental importance in pacemaking in a variety of neurons and its functional properties do make it well-suited to contribute in a similar way in CCs [32, 86, 127–129].

Other missing conductances sustaining spontaneous CCs firing?—Another factor to consider about the mechanisms that underlie spontaneous firing in CCs is that the normal CC resting potential is about − 45 to − 55 mV. This relatively positive V_m suggests that CC membranes likely express a substantial depolarizing resting cation flux. It is unlikely that the intrinsic fast inactivating Nav channels in CCs contribute to persistent Na^+ current [130]. Here, we mention two further possibilities that may be worth considering in regard to unknown factors that might help drive repetitive firing. First, although there are no supporting data yet, there might be non-selective cation currents or even variants of Nav or Cav channels that, at least in some CCs, may help drive firing. Second, another unexplored possibility that might be particularly applicable to CCs is the possibility that stochastic activity of one of the known CC ion channels may drive firing. A number of studies have considered the idea that simple stochastic activity of ion channels near cell resting potential may drive firing particularly in cells of high input impedance [41, 112, 113]. The idea is that, either via openings or closings of single channels or via voltage-fluctuations associated with stochastic behavior of population of channels with some average open probability, occasional events or ensemble fluctuations may be sufficient to drive V_m to a level that initiates an AP. In CCs, a classic early paper did, in fact, demonstrate the ability of an opening of single acetylcholine channel to elicit an AP [47]. In a situation in which stochastic activity may drive firing, there will not be any obvious time-dependent change in current during an ISI. Stochastic fluctuations would potentially initiate an AP with some time-dependent probability based on how often an opening or ensemble of activity of sufficient duration to elicit AP onset might occur. Such a “timing mechanism” may result in a distribution of interspike interval durations that would not be as tightly timed as ISIs

defined by other mechanisms. With respect to CCs, such a proposal is entirely speculative, but given the high input impedance of CCs (3–5 GΩ) and the broad distribution of ISIs observed in spontaneously firing CCs [131], perhaps the possibility may merit future attention. Random fluctuations of about 4–6 mVof baseline activity are clearly evident in the V_m recording of Cav1.3DCRD$^{HA/HA}$ KI mouse CCs in which Cav1.3 Ca^{2+}-dependent inactivation is accelerated by favoring Ca^{2+}-calmodulin binding to the C-terminal regulatory domain of the channel (see Fig. 10a in [111]). The mutated CCs exhibit more negative resting potentials in which the random oscillations are clearly visible but are unable to trigger APs due to their subthreshold levels.

Slow wave bursting in CCs

Recent work has shown that some repetitive slow wave bursting activity can be observed in about 15% of WT mouse CCs (e.g., Fig. 1b), either grown in culture [130] or in adrenal medulla slices [94]. Furthermore, three distinct kinds of manipulations have been shown to enhance or unmask this slow wave bursting. In one case, in mice in which the β_2 auxiliary subunit of BK channels is genetically deleted, almost all CCs exhibit repetitive slow wave bursting (Fig. 3b) which occurs at a frequency (~1 Hz) similar to the spontaneous AP frequency (Fig. 3a) of WT neurons [94]. In the second case, by reducing Na^+ channel availability by either slow depolarizations that induce partial Nav channel inactivation or application of low concentrations of TTX (Fig. 3c), bursting activity is unmasked [130]. 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 and a nearly 7-fold increase in CA secretion. Cell depolarization is attributed to the block of pH-sensitive TASK-1/3 and BK channels [56] (Fig. 3d).

Slow wave bursting is characterized by an initial AP that is of somewhat reduced peak amplitude, which then exhibits a slower than normal decay. In some cases, the slow repolarization leads to a persistent pedestal of depolarized potential upon which a repetitive series of truncated, presumably Ca^{2+}-dependent action potentials, occurs. The number of secondary APs can vary from burst to burst, but in terms of potential physiological function, it is probably the plateau of potential that is most likely to influence cytosolic Ca^{2+} concentrations.

Deletion of BK-β_2 subunit causes slow wave bursts in mouse CCs—What are the alterations in CC conductances that might allow slow wave bursts to occur? In contrast to the case of β_2-subunit deletion, complete loss of the BK channel by KO of the pore-forming α subunit does not result in bursting activity [129], suggesting that the presence of the BK channel lacking the β_2 subunit is essential to bursting [94]. The precise shape of the AP appears important in determining the ability to burst, and this is influenced by whether BK channels have β_2 subunit or not. Since the specific gating shift produced by β_2 subunits depends essentially linearly on the average number of β_2 subunits in the population [137], the gating range of BK channels in β_2 KO mouse CCs is expected to be about 20–30 mV right shifted compared to those cells having about 1.5–2 β_2 per channel as estimated for mouse CCs [94]. The shift influences how much BK channels can contribute to repolarization and after-hyperpolarization (AHP) in CCs. In addition to this, the β_2-

containing BK channels activate more slowly and turn off more slowly. Thus, in the absence of β_2, although the remaining BK channels require more Ca^{2+} to be activated, those that are activated will turn on more rapidly, thereby slowing the rising phase of the AP and lowering the AP peak [94]. This will diminish not only Nav activation but also Cav and Kv activation, all of which in turn will attenuate the normal falling phase of the AP. The diminished activation of Kv channels (which are abundant in mouse CCs) will reduce their contribution to fast repolarization, while the broader AP will sustain Cav activation during the falling phase. It is therefore proposed that the slowing of the AP decay in the β_2 KO cells results in a balance of Cav and BK activation that somehow sustains the plateau of voltage underlying the slow-wave [94].

Reduced Nav channel availability unreveal bursts firing in mouse CCs— Manipulations that reduce Nav channel availability also favor bursting or doublet/triplet firing [130]. Is there any similarity in the underlying basis of bursting between manipulations in Nav availability compared to β_2 subunit KO? Similar to APs in the β_2 KO CCs, APs occurring during reduction of Nav availability are of reduced peak amplitude and broader time course. This also allows for more prolonged Ca^{2+} current activation during the AP while the reduced AP amplitude also reduces Kv activation perhaps allowing for secondary spikes to occur on the slowed falling phase of the AP. In fact, [130] show, using a burst as a voltage-clamp waveform, that the altered waveform of the first AP results in sufficient slowing of the AP that, during the time normally corresponding to an AHP, inward Ca^{2+} current exceeds that of BK current, thereby providing the inward current necessary to generate a voltage plateau and a secondary AP. But, in this case, normal inactivating BK channels are presumably present in such cells, so one might wonder why the β_2-containing BK channels are not sufficient to mediate the AHPs that might terminate a burst. This point will require future study.

Block of TASK-1/3 and BK channels by acidic pHo induces burst firing— Lowering the pHo favors either sporadic or sustained burst firings [56]. Low pHo causes marked CC depolarization (10–15 mV), slowed and lower APs that mainly derives from the block of pH-sensitive TASK-1 channels (100% block at pHo 6.6 [69]) and block of BK channels (60% at pHo 6.6) with minor or no blocking effects on Nav, Cav, and Kv channels. Burst firing in fact can be mimicked by applying mixtures of TASK1 and BK channel blockers (A1899, paxilline) to mouse CCs. Interestingly, burst firing occurs during both spontaneous resting activity or during strong step depolarization that induces sustained activity. In both cases, burst firing is converted back into repetitive firing of single APs by adding sufficient doses of nifedipine, indicating that L-type Cav channels together with BK channels play a key role in the generation and maintenance of plateau potential that sustains the bursts. Low pHo or mixtures of TASK-1 and BK blockers are also very effective in enhancing CA secretion by nearly an order magnitude with respect to control CCs [56].

Further conditions for bursts firing—Overall then, in all three cases that induce bursting: β_2 subunit absence, reductions of Nav current, and simultaneous block of TASK-1 and BK channels by low pHo, similar slowing of AP duration and reduction of AP peak are observed. This is associated with a slowing of the AP repolarization phase, which apparently

permits a balance of Cav and BK channel activity that maintains a more positive membrane potential, allowing secondary spikes. Although these similarities suggest that the underlying temporal sequence of conductances that induces bursts may be similar in each case, this will require further investigation. A further important observation is that burst firing occurs irrespective of whether mouse CCs belong to adrenal slices [94] or are maintained in cultures for 2 days after dissociation [56, 128].

Irrespective of the origins of bursting behavior, the fact that conditions have been identified in which very robust stereotypic slow-wave bursting can be observed raises the possibility that physiological factors may also unmask bursting under some circumstances. As we learn more about the timing of conductances that may underlie burst generation, it will be possible to ask in a more focused way how modulation of different endogenous conductances might lead to rhythmic activity in CCs. Are there physiological factors (hypoxia, muscarinic stimulation, circulating hormones) that may increase the likelihood of occurrence of bursting, by up- or down-regulation of specific conductances? Similarly, might the expression of β_2 subunits in CCs be regulated? Given the heterogeneity in β_2 subunit expression among CCs [40, 82, 118], it would not be surprising if mechanisms that regulate β_2 abundance might exist (e.g., [31, 84]).

Potential implications of non-neurogenic firing for CA secretion from CCs

The evidence of non-neurogenic excitability of CCs only takes on physiological significance if important alterations in CA secretion from CCs occur as a consequence of non-neurogenic stimuli. From amperometric recordings, single APs are only weakly able to elicit exocytosis in cultured rat CCs [145]. Exocytotic events coupled to APs are occasionally observed only at frequencies higher than 1 Hz. Similarly, only minor increases in membrane capacitance associated with fusion of exocytotic vesicles are observed in cultured rat CCs in response to stimulation with 20 APs applied at 1 Hz, while secretion is 5- to 10-fold higher when 20 APs are applied at 10 Hz [42]. This suggests that the spontaneous ~1 Hz AP firing observed in CCs, either in dissociated cell culture or in slices, is unlikely to impact significantly on blood CA levels. However, these same studies and others [42, 56, 85, 130, 145] indicate clearly that burst-type patterns of stimulation, whether from higher frequency AP firing or from sustained depolarization, leads to marked increases in CA secretion. Although the specific ability of naturally occurring slow wave burst waveforms to elicit CA secretion has not yet been evaluated [22, 42, 85, 145], there are already good indications that slow wave bursts will increase the amount of Ca^{2+} entering the cell and, thus, will be more effective then spontaneous AP activity in eliciting Ca^{2+}-dependent secretion [56, 130].

Conclusions, unsolved questions, and future investigations

Like neurons, CCs are capable of exhibiting a variety of complex patterns of rhythmic activity. Is the complexity of conductances and firing behaviors in CCs an ancestral property that derives from their neuronal sympathetic origins or are such features of physiological importance to CCs? We expect that future work will reveal the physiological importance of non-neurogenic firing mechanisms in CCs that could have implications also for better understanding the firing patterns of peripheral and central neurons. Some specific questions related to CCs remain unanswered and could drive interesting future investigations:

1. What are the specific conductances and their timing of activation that drive burst firing in CCs?
2. Are there physiological circumstances, such as muscarinic or histaminergic receptor activation, that might acutely alter specific conductances leading to burst firing?
3. Are there modulatory pathways that might regulate BK channel β_2 subunit expression or BK function, thus altering the tendencies of CCs to exhibit bursting?
4. Are naturally occurring burst waveforms effective at producing secretion?
5. Does CCs bursting lead to tonic elevations of blood CAs?

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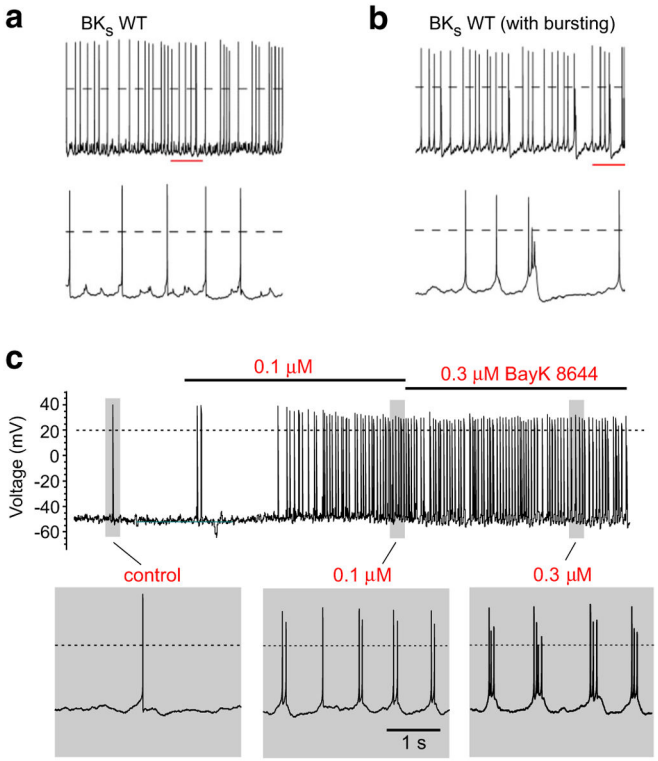

Fig. 1.

Types of spontaneous activity in mouse CCs. **a** Spontaneous repetitive firing in a CC with largely non-inactivating BK current from a mouse adrenal slice at lower (*upper*) and faster (*bottom*) time base. **b** Spontaneous firing and bursts in a CC with non-inactivating BK current. Modified from [94]. **c** Sporadic spontaneous activity in a mouse CC in control conditions that switches in burst firing during application of increasing doses of the L-type channel agonist BayK8644 (0.1 and 0.3 μM). Modified from [56]

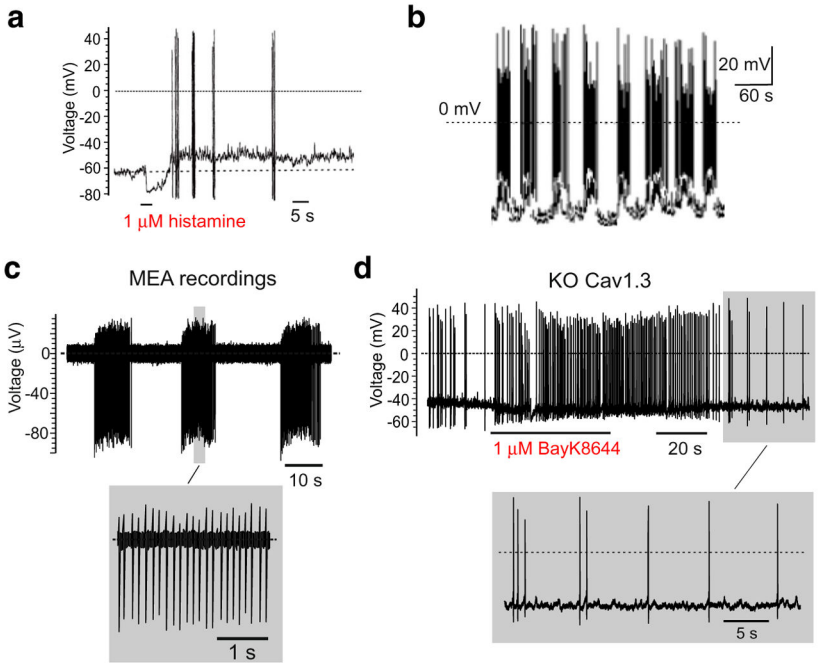

Fig. 2.

Rhythmic firing patterns in bovine, rat and mouse CCs. **a** Rhythmic groupings of APs seen in bovine CCs with perforated patch recordings following application of histamine. Modified from [135]. **b** Rhythmic paroxysms seen in a study on ERG currents using perforated patch recordings from rat CCs. Modified from [58]. **c** Examples of groupings of spontaneous high frequency firing separated by prolonged silent periods in MEA recordings from rat CCs. Modified from [129]. **d** Rhythmic groupings of APs observed after washout of BayK8644 in mouse CC. Modified from [92]

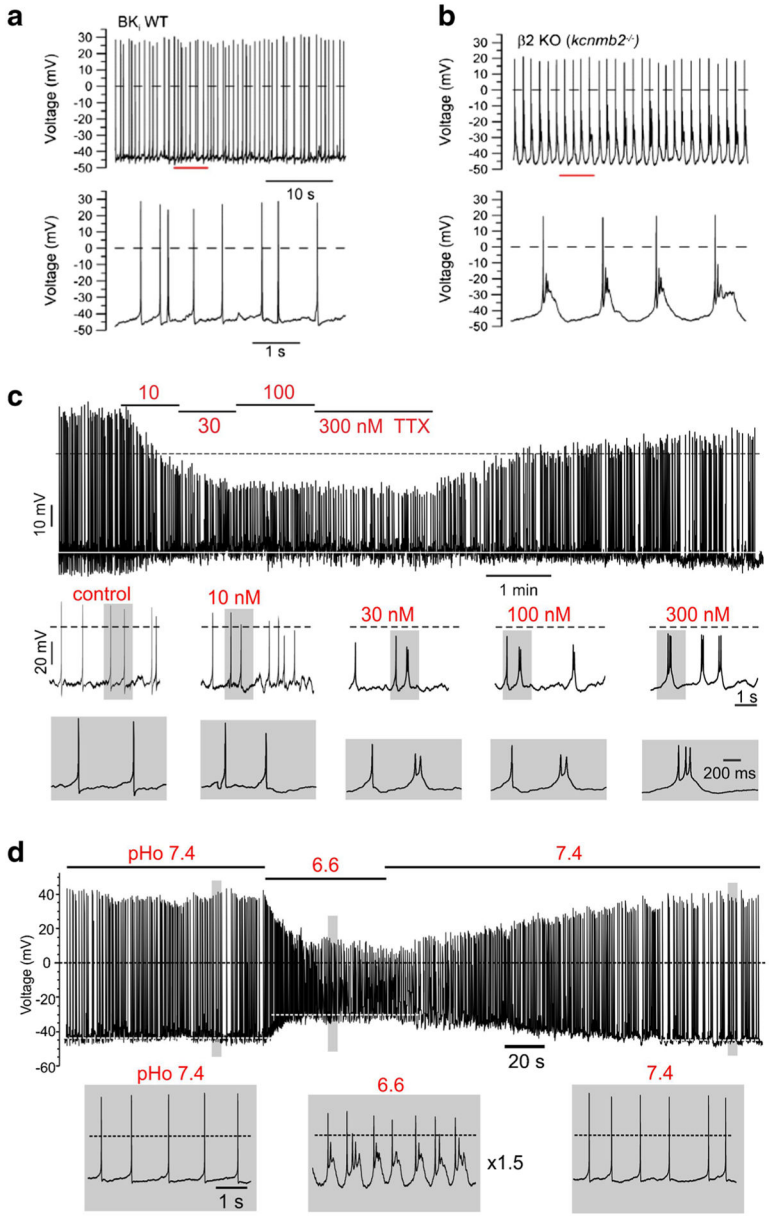

Fig. 3.

Unmasking of slow wave burst activation by β_2 KO, TTX, or acidic pHo in mouse CCs. **a** Spontaneous repetitive firing in a CC with largely BK_i current from a mouse adrenal slice at lower (*upper*) and faster (*bottom*) time base. **b** Slow wave bursting in a CC (mouse adrenal slice) from a β_2 KO mouse. Modified from [94]. **c** Various concentrations of TTX were sequentially applied to a spontaneously firing CC reducing AP amplitude. **d** Selected records show induction of slow wave bursting with reductions in Nav current by TTX. Modified from [130]. **e** Slow wave bursts induced by a marked depolarization induced by acidic pHo (6.6). The cell depolarization, AP amplitude, and mode of firing fully recover on returning to physiological pHo (7.4). Modified from [56]