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Review

Cav1.3 and Cav1.2 channels of adrenal chromaffin cells: Emerging views on cAMP/cGMP-mediated phosphorylation and role in pacemaking ☆

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ABSTRACT

Voltage-gated Ca^{2+} channels (VGCCs) are voltage sensors that convert membrane depolarizations into Ca^{2+} signals. In the chromaffin cells of the adrenal medulla, the Ca^{2+} signals driven by VGCCs regulate catecholamine secretion, vesicle retrievals, action potential shape and firing frequency. Among the VGCC-types expressed in these cells (N-, L-, P/Q-, R- and T-types), the two L-type isoforms, Cav1.2 and Cav1.3, control key activities due to their particular activation–inactivation gating and high-density of expression in rodents and humans. The two isoforms are also effectively modulated by G protein-coupled receptor pathways delimited in membrane micro-domains and by the cAMP/PKA and NO/cGMP/PKG phosphorylation pathways which induce prominent Ca^{2+} current changes if opposingly regulated. The two L-type isoforms shape the action potential and directly participate to vesicle exocytosis and endocytosis. The low-threshold of activation and slow rate of inactivation of Cav1.3 confer to this channel the unique property of carrying sufficient inward current at subthreshold potentials able to activate BK and SK channels which set the resting potential, the action potential shape, the cell firing mode and the degree of spike frequency adaptation during spontaneous firing or sustained depolarizations. These properties help chromaffin cells to optimally adapt when switching from normal to stress-mimicking conditions. Here, we will review past and recent findings on cAMP- and cGMP-mediated modulations of Cav1.2 and Cav1.3 and the role that these channels play in the control of chromaffin cell firing. This article is part of a Special Issue entitled: Calcium channels.

Contents

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1. Introduction

L-type calcium channels (LTCCs; Cav1) belong to the family of voltage-gated Ca^{2+} channels (VGCCs) that are permeable to Ca^{2+} and sensitive to membrane voltage. VGCCs include also the Cav2 (N, P/Q, R) and Cav3 types (T) [1] and are often classified as high-voltage (HVA; L, N, P/Q and R-type) and low-voltage activated (T-type) [2–4]. LTCCs are hetero-oligomers consisting of a pore-forming α_1-subunit of 190–250 kDa in association with auxiliary β-, $\alpha_2\delta$- and γ-subunits [5]. The α_1-subunit is composed of four membrane-spanning domains (I–IV) linked together in a single polypeptide chain. Each domain contains six putative transmembrane segments (S1–S6) plus a "P" loop that dips partially into the pore to form the pore lining [5]. Like the other VGCCs, LTCCs open readily during membrane depolarization and allow Ca^{2+} to enter the cell. LTCCs, thus, regulate cell excitability and control a variety of Ca^{2+}-dependent physiological processes, like: excitation–contraction coupling in cardiac, skeletal and smooth muscles, gene expression, synaptic plasticity, brain aging, hormone secretion, vesicle retrieval and pacemaker activity in heart, neurons and neuroendocrine cells [6–13].

Presently, four genes are found to code for the Cav1.1, Cav1.2, Cav1.3 and Cav1.4 subunits [1]. Of these, Cav1.1 and Cav1.4 are mainly expressed in skeletal muscle, pituitary cells and the retina, whereas Cav1.2 and Cav1.3 are widely distributed throughout the central nervous system, sensory and endocrine cells, atrial myocytes and cardiac sino-atrial node cells [12,14–18]. All four Cav1 isoforms are highly sensitive to 1,4-dihydropyridine (DHP) blockers (Ca^{2+} antagonists) or activators (Ca^{2+} agonists), which are the most commonly used compounds for identifying functional LTCCs. The blocking potency of 1,4-DHPs differs depending on the holding potential [7,19–21] but their separation on this basis is complex and is rarely used for quantitative assays. This justifies the continuous search for new selective blockers for Cav1.2 and Cav1.3 [16,22], which are the most widely expressed Cav1 isoforms.

2. Cav1.2 and Cav1.3 as multitask channels controlling excitability, secretion and endocytosis in chromaffin cells

Cav1.2 and Cav1.3 channels are highly expressed in chromaffin cells of the adrenal medulla [23–27] where they control excitability, secretion of catecholamines and vesicle retrieval. Excitability is primarily controlled by Cav1.3 which activates at very low voltages and inactivates slowly in a voltage-dependent manner (measured in Ba^{2+}) with respect to Cav1.2 and other high-threshold channels (N, P/Q, R) [7,28–30]. Depending on the C-terminal regulatory domain [30], Cav1.3 exhibits weak or pronounced Ca^{2+}-dependent inactivation (CDI). Assuming that at the pacemaker potential (−50 to −40 mV) the intracellular level of Ca^{2+} is rather low, it is clear that CDI contributes little to the total inactivation of the channel at these potentials. This enables Cav1.3 to carry most of the pacemaker's current that sustains chromaffin cell's spontaneous activity [10–13,18] and to drive sufficient SK current activation that helps in adapting the firing rate to a sustainable frequency during prolonged depolarizations (spike-frequency adaptation, SFA) [31]. In addition, Cav1.3 has been shown to be tightly coupled to fast inactivating BK channels, suggesting also a key role in the control of the AP shape [11,12]. Concerning the second main activity, it is widely accepted that catecholamine secretion is particularly sensitive to L-type currents in rat (RCCs) and mouse chromaffin cells (MCCs) [10,32–37]. In RCCs L-type channels carry nearly half of the total Ca^{2+} current and are responsible for the corresponding exocytosis [36,38]. Concerning vesicle retrieval, LTCCs have a direct control of compensatory and excess endocytosis, which are strongly attenuated when LTCCs are blocked [39–41]. At present, however, it is not clear yet if both isoforms have distinct roles in this process.

In addition, Cav1.2 and Cav1.3 are shown to be effectively modulated by G-protein coupled receptors in membrane micro-domains [42–45], or up- and down-regulated by cAMP/PKA and NO/cGMP/PKG pathways, respectively [10,46–49]. These two mechanisms act opposingly on channel gating (see [50–52] for reviews) and give rise to Ca^{2+} current changes of one order of magnitude under proper stimulation of one pathway and inhibition of the other [49]. All these peculiarities highlight the strategic role that Cav1.3 and Cav1.2 exert on chromaffin cell functioning.

3. The autocrine modulation of LTCCs: a feedback for regulating catecholamine secretion

Chromaffin cells constantly release catecholamines, opioids and ATP during low and sustained sympathetic stimulation (see [51–53] for reviews). As a consequence, Ca^{2+} channels experience basal and stimulus-induced autocrine modulations, which affect their activation–inactivation gating and probability of channel opening, altering in this way the amount of Ca^{2+} entry into the cell. This autocrine effect is mediated by the adrenergic, opioidergic and purinergic autoreceptors highly expressed in chromaffin cells and coupled to G-proteins. This occurs in isolated chromaffin cells [42,44,48,49] but is more prominent in cell clusters [54] and in adrenal gland slices [45], where the released neurotransmitters can accumulate more effectively between closely packed chromaffin cells.

3.1. Direct and remote modulation of LTCCs

For Cav2.1 (P/Q) and Cav2.2 (N) channels the autocrine modulation driven by purinergic, opioidergic and adrenergic receptors is mostly inhibitory and voltage-dependent [42,44,45,55]. Cav2.1 and Cav2.2 channel activation is markedly delayed, but short pre-pulses to positive voltages can fully remove the Giβγ-mediated delay and channel activation returns to normal immediately following depolarization [43,56–62]. In contrast to this, LTCC modulation is mainly voltage-independent and can occur through two distinct pathways (see [52] for a review): 1) by quickly inhibiting LTCCs gating via PTX-sensitive G-proteins (*direct mode*); or 2) by slowly up-regulating LTCCs activity through a cAMP/PKA-mediated phosphorylation (*remote mode*), similar to the up-regulation of cardiac Cav1.2 channels during β_1-AR-mediated sympathetic stimulation. The direct modulation is fully resolved in membrane micro-patches and derives from the interaction of G-protein subunits with LTCCs within membrane micro-domains where Ca^{2+} channels, membrane autoreceptors and coupled G-proteins coexist [44–46,58]. The remote modulation is more complex and involves the presence of functional adenylate cyclases, an effective cAMP-mediated PKA up-regulation and LTCCs phosphorylation (see [51] and [52] for a review). The cAMP/PKA mediated up-regulation of LTCCs exists in BCCs ([46]), RCCs ([48]) and MCCs ([10]) and is thus a general property of chromaffin cells, related to the expression of β-ARs in these and other animal species [63,64].

Although not directly related to the neurotransmitter-mediated modulation described above, LTCCs are also regulated by a remote mechanism which originates from the autocrine NO/cGMP/PKG pathway which tonically inhibits LTCCs and catecholamine release in BCCs [47,65] and MCCs [49]. Chromaffin cells possess sufficient levels of neuronal-like NO-synthase and guanylyl cyclase to synthesize NO and cGMP [65] which can tonically down-regulate L-type currents and catecholamine release. A marked depression of L-type currents occurs also when NO-donors or permeable forms of cGMP are exogenously applied to isolated BCCs [47] or MCCs [49]. The action is PKG-dependent, develops slowly by reducing the open channel probability and causes no changes to the time course of L-type current activation. The NO/cGMP/PKG-mediated down-regulation of LTCCs exerts an opposite action to the cAMP/PKA-mediated up-regulation, closely resembling the effects that the two pathways exert on cardiac Cav1.2 channels.

As underlined elsewhere [49,66,67], the existence of the two modulatory pathways in MCCs, which express equal densities of functional Cav1.2 and Cav1.3 channels [11,13], raises key questions concerning LTCC modulation:

- Are Cav1.2 and Cav1.3 equally prone to cAMP/PKA and cGMP/PKG phosphorylation at basal conditions and during stimulation?
- Do the PKA- and PKG-mediated phosphorylations act independently on LTCCs?
- To what extent the two opposing mechanisms could synergistically affect the size of Cav1.2 and Cav1.3 Ca^{2+} currents?

The answers to these questions are not obvious given the little amount of information available on cAMP/PKA and cGMP/PKG modulation of native Cav1.3 channels (see Ref. [5]). In the following paragraph we briefly summarize the new findings that recently appeared on the sensitivity of Cav1.2 and Cav1.3 to cAMP/PKA and cGMP/PKG-mediated phosphorylation.

3.2. Cav1.2 and Cav1.3 are equally modulated by cAMP/PKA and cGMP/PKG

Given the lack of selective blockers for Cav1.2 and Cav1.3, a suitable approach to quantify the effects of PKA/PKG activators and inhibitors on the two Cav1 isoforms is to use knock-out (KO) mice strains missing either one of the two isoforms. Comparing the effects on WT and Cav1.3$^{-/-}$ KO mice and normalizing the effects by the size of L-type currents furnishes an immediate estimate of PKA/PKG actions on Cav1.2 in Cav1.3$^{-/-}$ MCCs and Cav1.2 + Cav1.3 in WT MCCs. In MCCs, the estimate is further simplified since Cav1.2 and Cav1.3 carry the same quantity of Ca^{2+} currents [11,13]. An equal modulation of the two channels (either positive or negative) would turn out in a double effect on WT with respect to Cav1.3$^{-/-}$ MCCs. Following this approach it has been possible to show that Cav1.2 and Cav1.3 are equally modulated by cAMP and cGMP in an opposing manner [49].

The cAMP/PKA pathway is already active at basal levels where both isoforms are equally down-regulated by the PKA blocker H89 (Fig. 1a) and are fully up-regulated by the adenylyl cyclase activator forskolin which causes a current increase proportional to the L-type current size in both WT and Cav1.3$^{-/-}$ MCCs (Fig. 1b). Similar findings are reported for the LTCCs of RCCs [48], BCCs [46] and are comparable to the up-regulatory action of cAMP/PKA on cardiac Cav1.2 channels [68,69]. This proves unequivocally that Cav1.3 is effectively up-regulated by the cAMP/PKA pathway, in good agreement with works reporting the existence of: 1) PKA phosphorylation sites in the C-terminal tail of recombinant Cav1.3 channels [70], 2) co-localization of the neuronal Cav1.3 C-terminal with the A-kinase anchoring protein 15 (AKAP15) and β_2-AR in the mouse brain [71] and 3) a PKA and cAMP-mediated up-regulation of Ca^{2+} currents carried by the Cav1.3 long C-terminal splice variant [72] and the recombinant 180 kDA Cav1.3 isoform [73]. These two latter works also suggest that at variance with Cav1.2, AKAPs are not specifically required for reconstituting PKA-mediated up-regulation of Cav1.3 in heterologous expression systems, in apparent contradiction with the observation that AKAP15 co-localizes with neuronal Cav1.3 α1 subunit [71]. The presence of AKAPs is critical for the reconstituted PKA-modulation of Cav1.2 in tsA201 cells. In fact, in vivo C-terminal proteolytic processing cleaves a distal segment of the C-terminal (DCT) regulatory domain which acts as an auto-inhibitory module when non-covalently bound to the truncated Cav1.2 channel [74]. The DCT domain is critical for reconstituting the PKA-modulation in expression systems. DCT binds to AKAP15 and allows PKA to mediate the disinhibition of the auto-inhibitory signaling complex composed by the Cav1.2 truncated forms, AKAP15 and DCT [74].

As for cAMP/PKA, also the cGMP/PKG pathway is already active at basal levels, with the main difference that it down-regulates the L-type currents when activated in MCCs [49] and BCCs [47,75]. Also in this case, comparing the effects of PKG inhibitors (KT 5823) and cGMP analogues (8-pCPT-cGMP) on WT and Cav1.3$^{-/-}$ MCCs, Cav1.2 and Cav1.3 are shown to be equally down-regulated at rest

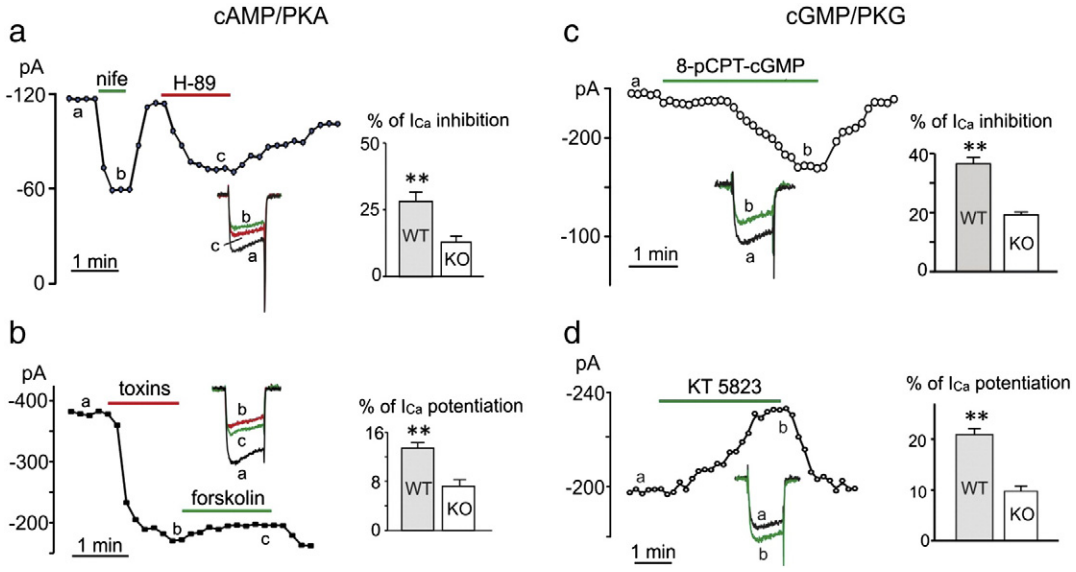

Fig. 1. Ca^{2+} current modulation by cAMP activators, cGMP analogues and PKA- and PKG-blockers in mouse chromaffin cells. a, inhibitory effect of the PKA blocker H-89 (5 μM) on the total Ca^{2+} currents of a WT MCC viewed by plotting the peak Ca^{2+} current amplitude elicited by voltage steps of 20 ms to +10 mV versus time (holding potential −50 mV). The test of H-89 was preceded by an assay of the blocking potency of 3 μM nifedipine to quantify the size of L-type currents. The bars to the right show the percentage of total Ca^{2+} current inhibited by H-89 in WT MCCs (27.7%; n = 8), which is about twice the amount blocked in Cav1.3$^{-/-}$ MCCs (12.8%; n = 8). b, the potentiating action of forskolin (100 μM) on the L-type currents remaining after blocking N-, P/Q- and R-type channels with a mixture of ω-Ctx-MVIIC (10 μM), ω-Ctx-GVIA (3.2 μM) and SNX 482 (0.4 μM) in a WT MCC. The percentage of L-type current potentiation induced in WT MCCs (13.4%; n = 9) is almost twice of that induced in Cav1.3$^{-/-}$ MCCs (7.2%; n = 8). c, inhibitory effect of the cGMP membrane permeable analogue 8-pCPT-cGMP (1 mM). The action of 8-pCPT-cGMP was selective for LTCCs and the percentage of current inhibition was 36.5% for WT MCCs (n = 4) and 19.2% for Cav1.3$^{-/-}$ MCCs (n = 6). d, the potentiating effects of the PKG-blocker KT 5823 (1 μM) on the Ca^{2+} currents of a WT MCC. On average the percentage of potentiation was 20.9% for WT MMCs (n = 11) and 9.7% for Cav1.3$^{-/-}$ MCCs (n = 7). The four panels were adapted from Ref. [49].

by basal levels of active PKG (Fig. 1d). Elevations of cGMP cause a proportional down-regulation of L-type currents in WT and Cav1.3$^{-/-}$ MCCs (Fig. 1c). Thus, as for Cav1.2, also Cav1.3 is effectively down-regulated by cGMP/PKG. This agrees with data on cochlear inner hair cells reporting a NO/cGMP-mediated down-regulation of Cav1.3 currents [76].

3.3. cAMP- and cGMP-mediated phosphorylation act opposingly and independently on LTCCs gating

The existence of two opposing modulations mediated by cAMP and cGMP in neuroendocrine cells, closely resembling those acting on the Cav1.2 isoform of ventricular myocytes, opens interesting questions about the role that these two modulatory pathways are likely to play in chromaffin cell functioning. A number of functional and structural evidence supports the view that the two systems act independently on both channel isoforms (or auxiliary β-subunits). The first set of evidence comes from protein microsequencing and peptide mapping studies which suggest that the two kinases phosphorylate different serine sites of the Cav1.2 α_1 and β subunits. PKA phosphorylates Cav1.2 α_1-subunit at Ser1700 (cardiac) and Ser1901 (neuronal) sites [70,74,77,78] and Cav1.2 β-subunit at Ser 478/479 and Ser 459 [79–81], while PKG phosphorylates Cav1.2 α_1-subunit at Ser533 [77,82,83] and Cav1.2 β subunit at Ser 496 [82]. The same could occur for the phosphorylating action of PKA and PKG on Cav1.3 α_1-subunit in which PKA is shown to phosphorylate Ser1964, Ser1743 and Ser1816 [84,85], but nothing is known about the phosphorylation sites of PKG. PKA and PKG phosphorylate also Ser 1928 [74,83], which is also phosphorylated by PKC [86], but this serine residue is shown not to be functionally linked to Cav1.2 channel gating modification [87]. Apart from this singularity, all the other sites sensitive to PKA and PKG phosphorylation appear to be distinct. It should be noticed, however, that the existence of distinct phosphorylation sites for PKA and PKG does not rule out the possibility that they may mutually interfere on channel gating.

The second and most convincing evidence in favor of an independent action of the two pathways is the synergist effect that cAMP and cGMP can produce when opposingly modulated (one activated and the other inhibited) to give either minimal or maximal values of L-type currents differing by one order of magnitude (18% vs. 180% of control value). Fig. 2 illustrates the effects of the two opposing synergistic regulations of L-type currents. In one case, the application of the cAMP/PKA activator forskolin followed by the selective PKG inhibitor KT 5823 leads to a nearly doubling of the L-type current (Fig. 2 bottom-right), while in the other case the sequential application of the PKA-blocker H89 and the cGMP analogue, 8-pCPT-cGMP, leads to a reduction of about 80% of the same current (Fig. 2 top-right). The cumulative PKA- and PKG-mediated modulations of L-type currents summarized in Fig. 2-left are among the most impressive changes of neuronal L-type current amplitude that can be driven by intracellular modulatory pathways. They are strikingly similar to the changes observed for the cardiac Cav1.2 currents when separately enhanced by cAMP or down-regulated by cGMP [5,69,88,89], which set the systolic strength and sino-atrial node frequency during sympathetic and parasympathetic regulation of heart beating.

3.4. Functional relevance of Cav1.2 and Cav1.3 modulation on chromaffin cell functioning

Recent works on MCCs, BCCs and RCCs [46,48,49] point to a converging idea that cAMP/PKA and cGMP/PKG pathways are already active at rest due to the basal activity of the two cyclases (AC and GC). AC is mainly activated by PACAP [90], Ca^{2+} entry and G protein subunits that are activated by the basal activity of hormones and neurotransmitters released by sympathetic neurons [91], surrounding capillaries [92,93] and by the autocrine activity of chromaffin cells [42,48,57,58]. This latter is most probably the main cause of the high basal level of cAMP in culture conditions (2.2 mM) that rises 2- to 3-fold during β_1-AR stimulation and/or PDE-4 selective inhibition [10]. 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 chromaffin cells [65,94]. Under these conditions, cGMP/PKG appears to work as a 'break' to limit the potentiating effects

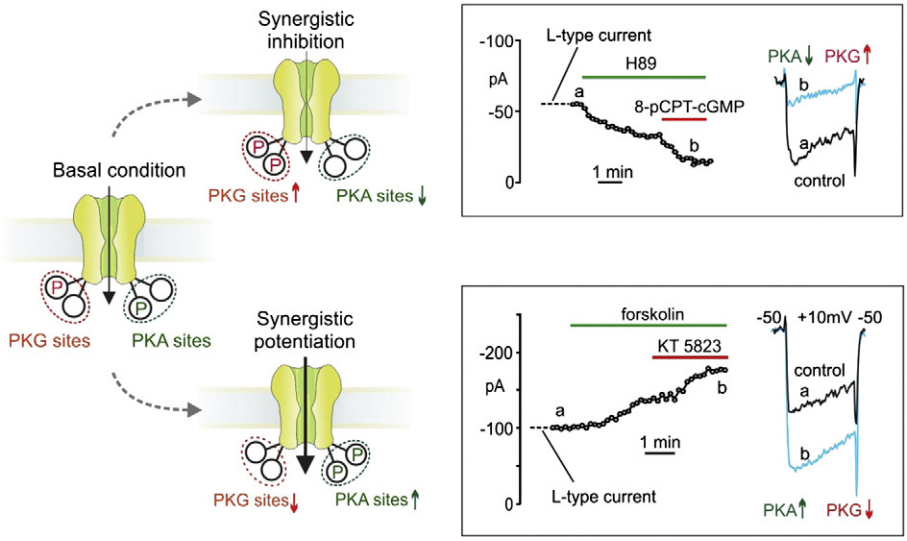

Fig. 2. The synergistic effects of cAMP/PKA and cGMP/PKG pathways in WT MCCs. To the left is shown a schematic representation of Cav1 channel α1 subunit with two PKA and PKG phosphorylation sites (P) during *basal conditions*, *synergistic potentiation* and *synergistic inhibition*. Basally, both PKA and PKG P-sites are partially phosphorylated and un-phosphorylated. Phosphorylation and dephosphorylation driven by up- and down-regulation of PKA and PKG proceed independently of each other to reach two extreme conditions in which either two PKA P-sites are dephosphorylated and two PKG P-sites are phosphorylated (minimal Cav1 current; top right) or the PKA and PKG P-sites are oppositely phosphorylated–dephosphorylated (maximal Cav1 current; bottom right). The location and the number of P-sites at the intracellular side are arbitrary. The two panels to the right show the time course of synergistic down-regulation of LTCCs induced by the sequential application of H-89 and 8-pCPT-cGMP (top) and the synergistic up-regulation of LTCCs by sequential application of forskolin and KT 5823 (bottom). In both cases the WT MCCs were pretreated with a toxin mixture containing ω-Ctx-MVIIC, ω-Ctx-GVIA and SNX 482 to block N-, P/Q- and R-type channels (see Fig. 1b). The drawing and the experimental data are adapted from Ref. [49].

of cAMP/PKA and helps setting the resting levels of Cav1.2 and Cav1.3 currents.

A *synergistic potentiation* of LTCCs could occur during sustained sympathetic stimulations that releases PACAP and induces massive secretion of adrenaline from chromaffin cells, which would further raise the levels of cAMP/PKA through the autocrine activation of the β_1-ARs present in RCCs and MCCs [10,48]. The increased Ca^{2+} entry during high-frequency stimulation could in turn activate the cGMP-specific Ca^{2+}-calmodulin-dependent PDE (PDE1) that regulates the resting levels of cGMP [65,95]. Thus, activation of a cGMP-specific PDE that lowers cGMP/PKG levels and the parallel increase of PKA during PACAP release and β_1-AR stimulation could markedly enhance Cav1.2 and Cav1.3 currents. This would sustain the rapid increase of firing activity and catecholamine release that ensure the fast activation of the 'fight-or-flight response' in chromaffin cells.

A reversed action (*synergistic inhibition*) could occur if, as in most mammalian ventricular myocytes, chromaffin cells possess cGMP-activated PDE2 isoform that hydrolyzes cAMP [96]. Any robust up-regulation of the NO/cGMP/PKG pathway under these conditions would enhance cGMP and down-regulate cAMP which would rapidly depress Cav1.2 and Cav1.3 channel gating. The existence of other PDEs acting on cAMP beside PDE4, is supported by the findings that in MCCs the non-specific PDE blocker IBMX increases basal cAMP levels more potently than the PDE-4-specific blocker rolipram [10].

The Cav1.3 up- and down-regulation by PKA and PKG described here could have also key physiological significance if extrapolated to the Cav1.3 channels of other tissues where the channel is highly expressed and functional [12]. In cardiac sino-atrial and atrio-ventricular node cells, Cav1.3 contributes to the pacemaker's current controlling heart beating [8,97] and, thus, a β_1-AR or a NO/cGMP/PKG-driven modulation of its gating could either accelerate or decelerate the heart rate. Effective modulations driven by the cAMP/PKA and cGMP/PKG pathways could occur also to the Cav1.3 channels of cochlear inner hair cells [98] and dopaminergic neurons of substantia nigra pars compacta [99] which control key functions of hearing sensory transduction and motor control.

4. Cav1.3 as pacemaker channels in chromaffin cells

The first recordings of AP firing in chromaffin cells date back to 1976. Current injection or application of ACh was able to make gerbil chromaffin cells fire when measured in acute isolated slices or primary cultures [100]. Neither the absence of extracellular Ca^{2+}, nor the block of VGCCs by Co^{2+} was able to induce a cessation of firing in their preparation, indicating a key contribution of voltage-gated Na^+ channels to spontaneous firing. Data on RCCs, published in the same year, were also reported by Brandt et al. [101]. Interestingly, these authors reported that by means of extracellular recordings, RCCs fired in a slow but spontaneous manner (0.05–0.1 spikes/s). This was not observed in recordings where the cell was impaled by a sharp micropipette, presumably due to damage of the cell membrane that typically results in a lower input resistance. A later study by Kidokoro and Ritchie [102] strengthened the existing evidence that RCCs possess the prerequisites to spontaneously fire APs. They measured spontaneously discharged APs by extracellular recordings in physiological conditions. It has to be mentioned that Brandt et al. [101] measured APs in 10 mM extracellular Ca^{2+} that might elevate tonic Ca^{2+}-activated K^+ currents which could interfere with cell's excitability. An increased divalent cation concentration in the bath is known to lead to a positive shift of the activation threshold of voltage-gated channels [103,104]. This thus implicates that the cell has to bridge a bigger voltage gap in order to trigger an AP. Under physiological conditions as used by Kidokoro and Ritchie [102], the ability to generate spikes was abolished by Co^{2+} (a non-selective VGCC blocker) and not by TTX, suggesting a key role for VGCCs in spontaneous spike generation in RCCs. Until recently, the general belief was that pacemaker channels were either belonging to the HCN family, the low-threshold persistent Na_v channels or the low-voltage activated (LVA; T-type) Ca^{2+} channels. One of the criteria for a channel to contribute to the pacemaker is in fact that it has to open at low membrane potentials and to carry inward cation currents. This was the reason why HVA Ca^{2+} channels were excluded "by definition". Dopaminergic neurons with an L-type dependent pacemaker mechanism were in fact considered rather an exception [105].

4.1. Nav1 channels of chromaffin cells do not fulfill the requirements of pacemaker channels

The Na^+ currents of chromaffin cells are typically transient in nature and mainly TTX-sensitive [13,106,107]. All inward current ascribed to Na^+ can be blocked by TTX at concentrations as low as 100 nM [108]. Klugbauer et al. [109] were the first showing that BCCs express high densities of the $Na_V1.7$ isoform. It is even believed that the $Na_V1.7$ channel that originally was called PN1 (peripheral nervous system specific) is the only isoform expressed in chromaffin cells [106,107]. $Na_v1.7$ in fact shows a peculiar expression pattern with high levels of expression in chromaffin cells, sympathetic ganglia and Aβ- as well as C-type dorsal root ganglion (DRG) neurons [107,110–112].

$Na_V1.7$ channels produce fast activating and inactivating currents that generally recover slowly from fast inactivation [107]. This slow recovery from inactivation is generally believed not to permit and sustain prolonged high-frequency firing of excitable cells. $Na_V1.7$ channels, however, respond to slow depolarizing ramps due to the slow onset of closed state inactivation and could trigger boosting depolarizations to bring V_{rest} closer to V_{thresh} [110]. Data from our lab unequivocally indicate that Na_V1 block by 300 nM TTX does not block spontaneous membrane oscillations of MCCs [12,13]. AP-clamp experiments prove clearly that Na^+ currents in MCCs are confined to the AP-upstroke phase, showing a rather complete inactivation during the broad Ca^{2+}-dependent spike waveforms [31] (Fig. 3). When MCCs are forced into fast firing patterns (e.g. by blocking SK channels)

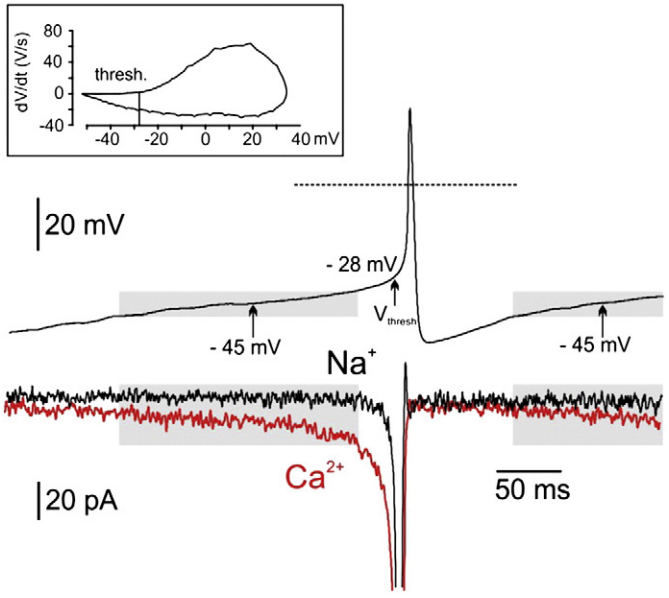

Fig. 3. Contribution of Cav1 and Nav1 channels to MCCs action potentials. *Top*, representative AP of a WT MCC. The inset represents a phase plane plot (dV/dt vs V) of the respective AP waveform. *Bottom*, overlapped Nav1 (black) and Cav1 currents (red) supporting MCCs pacemaking. The gray regions indicate the currents that flow in between membrane potentials of −40 and −50 mV. Notice that Nav1 currents contribute specifically to the spike upstroke while Cav1 currents support the slow potential rise of the pacemaker phase.

we observed a continuous and steady decrease of the spike amplitude, spike threshold and dV/dt_{max} which are all indicative of a reduced Na_V channel availability [31]. This was not found to interfere with the ability of MCCs to elicit spontaneous APs, indicating that fast inactivating Na^+ currents fulfill only a minor role in MCCs pacemaking.

4.2. LTCCs as pacemaker channels in chromaffin cells

In contrast with TTX, nifedipine (3 μM) just like Cd^{2+} is very effective in blocking MCCs spontaneous activity [10–12,50]. A property that is not limited to chromaffin cells but is shared with several central neurons in which LTCCs are responsible for pacemaking (see [12] for a review). Given that MCCs and RCCs express only Cav1.2 and Cav1.3 channels [11,16,24] the pacemaking of these cells is obviously restricted to either one of the two channels. Cav1.3 in particular exhibits biophysical features that are critical for pacemaking. The channel possesses a low-threshold of activation (−50 mV in 2 mM Ca^{2+}), fast activation kinetics and relatively slow inactivation characteristics [6,113]. The interest in Cav1.3 as a pacemaker channel grew substantially after it was shown that it contributes to the spontaneous firing patterns of substantia nigra (SN) dopaminergic neurons [9,114]. Given this, Marcantoni et al. [11] investigated the exact nature of the LTCC-dependent pacemaker of MCCs. Since no Cav1 selective DHPs are available yet, this information was provided by experiments conducted on Cav1.3$^{-/-}$ mice. Strikingly, Cav1.3 deficiency leads to a loss of firing in the majority of MCCs. The small portion of cells that maintained their spontaneous activity, fired at elevated frequencies and were characterized by depolarized resting membrane potentials of −40 to −45 mV. WT-MCCs typically fluctuate around a V_{rest} of −45 to −50 mV that perfectly coincides with the activation threshold of Cav1.3 channels and is about 9 and 25 mV more negative than that of Cav1.2 and Nav1 channels, respectively [13]. Especially at V_{rest} the input resistance is notably elevated (4–5 GΩ) ensuring significant membrane depolarizations, even upon small inwardly directed Ca^{2+} currents (2–3 pA). Voltage- and Ca^{2+}-dependent inactivation of Cav1.3 is moreover minimal around the pacemaker potential, favoring the development of persistent Ca^{2+} currents that cover the interspike intervals as is evident for WT MCCs (Fig. 3). This pacemaker current in fact is strongly attenuated in Cav1.3$^{-/-}$ MCCs, unequivocally proving its direct involvement in MCCs pacemaking. Impeding Ca^{2+} ions to flow in the cell through LTCCs leads to a cell depolarization instead of an expected hyperpolarization. This apparent anomaly can be explained by predicting a specific coupling of LTCCs (Cav1.3 in particular) to Ca^{2+}-activated K^+ channels that fine-tune MCCs pacemaking.

4.3. MCCs pacemaking: a healthy balance between Cav1.3 inward Ca^{2+} currents and Cav1.3-driven outward K^+ currents

As mentioned before, Ca^{2+} ions serve a multitude of roles in cellular physiology, ranging from neural growth and brain plasticity to cell death. Ca^{2+} is furthermore able to affect gene expression and trigger exocytosis of neurotransmitters [115]. The “power” of Ca^{2+} as an effective second messenger partly resides in the huge concentration gradient difference that is maintained across the cell membrane. Ca^{2+} outside the cell is 10^4 times more concentrated as the unbound Ca^{2+} inside. Thus, cells that make use of a Ca^{2+}-dependent pacemaker need enhanced metabolic controls in order to guarantee efficient Ca^{2+} handling [114,116]. An additional way to reduce the total amount of Ca^{2+} influx is by shortening the AP duration and slowing down the pace of firing by activating Ca^{2+}-activated K^+ channels. For MCCs in fact the “drive” of pacemaking (Cav1.3) is strongly coupled to BK and SK channels that act as a “brake” in these cells [11,12,31]. Cav1.3 deficiency results in a strong and almost complete block of a transient outward current that covers the region in between the spike peak and the antipeak. This current was shown to reflect a paxilline-sensitive fast-inactivating BK current, which is characteristic of MCCs [11,50], RCCs [117] and BCCs [118]. Loss of this BK current typically results in broader APs and reduced fast after-hyperpolarization phases [11].

Although the acute application of paxilline leads to an increase of the firing frequency (as is the case of a fraction of spontaneously firing Cav1.3$^{-/-}$ MCCs), BK$^{-/-}$ MCCs did exhibit reduced firing frequencies [12]. Similarly, Purkinje neurons of BK$^{-/-}$ mice show reduced firing frequencies as compared to WTs [119]. Upon stimulation we observed furthermore that BK loss reduces the onset of instantaneous firing frequency and fastens the time course of spike frequency adaptation of MCCs (Vandael & Carbone, unpublished data). A similar phenomenon has been observed for hippocampal pyramidal neurons [120]. A possible explanation could be that more Ca^{2+} enters during the broader APs and triggers more prominent SK currents. Thus, even though Cav1.3 deficiency leads to an aberrant activation of BK channels, this phenomenon is not likely the only reason of the observed increased firing frequencies of the spontaneously active Cav1.3$^{-/-}$ MCCs. Moreover, Cav1.3$^{-/-}$ MCCs typically respond to BayK 8644 with a sudden strong rise in the firing frequency followed by a depolarization block, a phenomenon that was not always observed in MCCs lacking the BK channels [12]. All BK$^{-/-}$ MCCs could be forced into depolarization block when BayK 8644 was co-applied together with apamin, a potent SK channel blocker. This thus indicates that Cav1.3$^{-/-}$ MCCs show an aberrant activation of both BK and SK channels.

4.4. A functional coupling between Cav1.3 (the motor) and SK channels (the brake) drives MCC firing

The abovementioned issues were the drive for studying the role of Cav1.3 in SK channel activation. SK channels were first identified in BCCs by Marty and Neher [121], and later in RCCs by Neely and Lingle [122] but their role on chromaffin cell excitability remained elusive. We have recently shown that MCCs express SK1-3 channels that contribute to a tonic current that slows down basal firing frequencies [31]. Interestingly, the resting firing frequency does reflect the amount of SK channels that contribute to MCCs pacemaking. A negative correlation in fact exists between the basal frequency and its percentage increase induced by apamin [31] (Fig. 4). In other words, the more SK channels are available in a chromaffin cell the lower is its resting firing frequency. Fig. 4 shows two examples of MCCs displaying low and high firing frequencies and how apamin affects more markedly the MCC firing slowly. In line with this, SK channels contribute to spike frequency adaptation upon current injection (Fig. 5a, c). In brief, SK channels give rise to a gradual increase in interspike interval duration when MCCs are stimulated. Spike frequency adaptation furthermore enhances the availability of Na_v channels, necessary to maintain stable AP waveforms. As suggested by Engel et al. [123], SK currents might shunt the membrane resistance, meaning that more current is required to induce a certain change in voltage. This also implicates that cells activating a sufficient amount of SK channels can deal with stronger current inputs as compared to cells with little SK activation. In this regard we found that Cav1.3$^{-/-}$ MCCs show a remarkable lower degree of spike frequency adaptation (Fig. 5a, c) and an earlier switch into depolarization block as compared to WTs [31]. Both findings suggest that Cav1.3$^{-/-}$ MCCs have difficulties in triggering SK currents. It is thus tempting to hypothesize that the observed increased firing frequencies of the subgroup of spontaneously active Cav1.3$^{-/-}$ MCCs can be attributed to a reduced degree of SK activation.

When SK currents are measured during a train of APs it is striking that they build up during the interspike intervals in WT MCCs and that this phenomenon is strongly attenuated in Cav1.3$^{-/-}$ MCCs (Fig. 5b, d). Nifedipine was moreover able to induce a complete

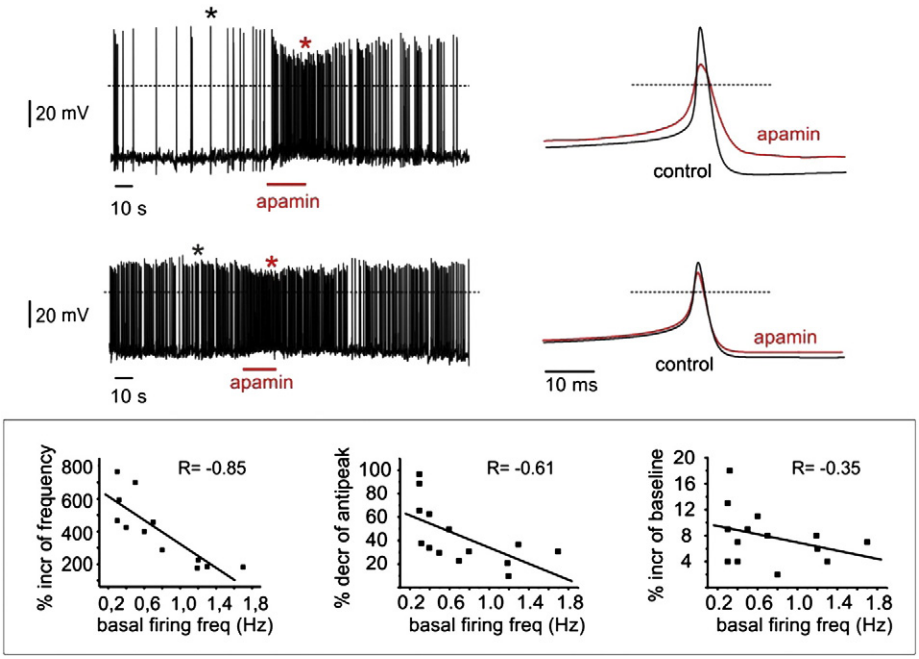

Fig. 4. SK channels slow down the MCC pacemaker cycle. *Top left*, representative current clamp recordings of spontaneously firing MCCs without current injection. Notice that apamin (200 nM) leads to stronger increases of firing rate in cells with low basal firing frequencies as compared to cells with elevated basal firing rates. *Top right*, close-ups of the APs indicated by the asterisks (left). Again the effects of apamin on the AP shape are more evident in cells that show low basal firing rates. *Bottom*, graphical representation of the degree of correlation between the basal firing rate and the percentage increase (or decrease) on frequency (left), after hyperpolarization (middle) and baseline (right) induced by apamin.

block of the interspike interval specific SK current, enforcing the idea that LTCCs are critical in triggering SK channels activation in MCCs. Voltage-clamp experiments on slow SK tail currents pointed out although that LTCCs and non-LTCCs contribute equally to SK activation. Indeed, a train of APs is not at all comparable to a square pulse voltage-clamp protocol. Analyzing the total amount of Ca^{2+} charge flowing in the cell during the firing cycle we concluded that Cav1.3 shows a major contribution as compared to the other HVA channels. Already the interspike interval is in charge of 50% of the total Ca^{2+} charge surging into the cell and is largely absent in Cav1.3$^{-/-}$ MCCs. Thus, Cav1.3 is coupled in a functional rather than in a molecular manner to SK channels in MCCs.

Noteworthy is the overlap between the outward Cav1.3-dependent SK current and the inwardly directed Cav1.3 current itself during the pacemaker cycle (Fig. 5b, d). The sum of both has its implications on the rate of depolarization and consequently on the duration of the interspike interval. MCCs are thus a perfect example of a spontaneously active cell type where the "motor" of the system (Cav1.3) controls the "brake" (SK) in order to prevent over-excitation. These findings furthermore indicate that modulation of the "brake" could have as important consequences on catecholamine release as modulation of the "motor".

5. L-type channels and secretion: any specific role for Cav1.2 and Cav1.3?

In chromaffin cells, the different VGCCs (L, N, P/Q, R, T) contribute to exocytosis proportionally to their density of expression and gating properties [33–36,124–128]. Secretion is not particularly linked to any specific Ca^{2+} channel type and either deletion or up-regulation of one of them causes a proportional change in secretion. For instance, Cav2.1 deletion causes a loss of the P/Q-type currents with a compensatory increase of L-type currents and secretion [129]. Similarly, when up-regulated by cAMP [130] or chronic hypoxia, [131] T-type channels contribute to low-threshold exocytosis with the same Ca^{2+}-dependence of L-type channels [38].

As shown above, Cav1.2 and Cav1.3 represent the final target of different modulatory pathways mediated by direct activation of G protein-coupled autoreceptors or remote pathways involving PKA and PKG activation. In principle, by acting on the two isoforms, any of these signaling loops can exert also a potent modulatory effect on the exocytotic response. In fact, the L-type current increase induced by cAMP stimulation only accounts for 20% of the total secretory response, suggesting an additional down-stream effect on the secretory machinery [36]. It is also interesting to notice that, due to their slower and less complete time-dependent inactivation, LTCCs (in particular Cav1.3) are favored in triggering exocytosis with respect to other HVA Ca^{2+} channels during sustained stimuli. Nevertheless, the contribution of Cav1.2 and Cav1.3 to exocytosis remains proportional to the quantity of Ca^{2+} ions entering the cell, suggesting that there is no preferential co-localization of LTCCs to secretory granules [51].

Although the critical role of LTCCs in triggering exocytosis is well established [36,53,132,133] there are no clear indications of a possible distinct role of Cav1.2 and Cav1.3 to exocytosis, despite the different inactivation kinetics and voltage range of activation of the two isoforms [7]. Preliminary observations show that deletion of the Cav1.3 subunit in MCCs lowers the amount of exocytosis at very negative potentials (−50 to −30 mV in 10 mM Ca^{2+}) (Navarro V, Carbone E, Carabelli V, unpublished data), indicating that besides sustaining action potential firing, Cav1.3 preferentially contributes to exocytosis at low membrane potentials [66]. This is in good agreement with what recently reported by Peréz-Alvarez et al. [26], in which secretion of Cav1.3$^{-/-}$ mice is partially reduced at very negative potentials. In this way, Cav1.3 contributes to the low-threshold exocytosis similar to the T-type Cav3.2 channel when expressed during cAMP/PKA elevations [38,127] or chronic hypoxia conditions [131].

6. L-type channels and endocytosis: are Cav1.3 and Cav1.2 equally involved?

Another open question to solve concerns the role of Ca^{2+} and Ca^{2+} channels in the retrieval of synaptic vesicles during endocytosis.

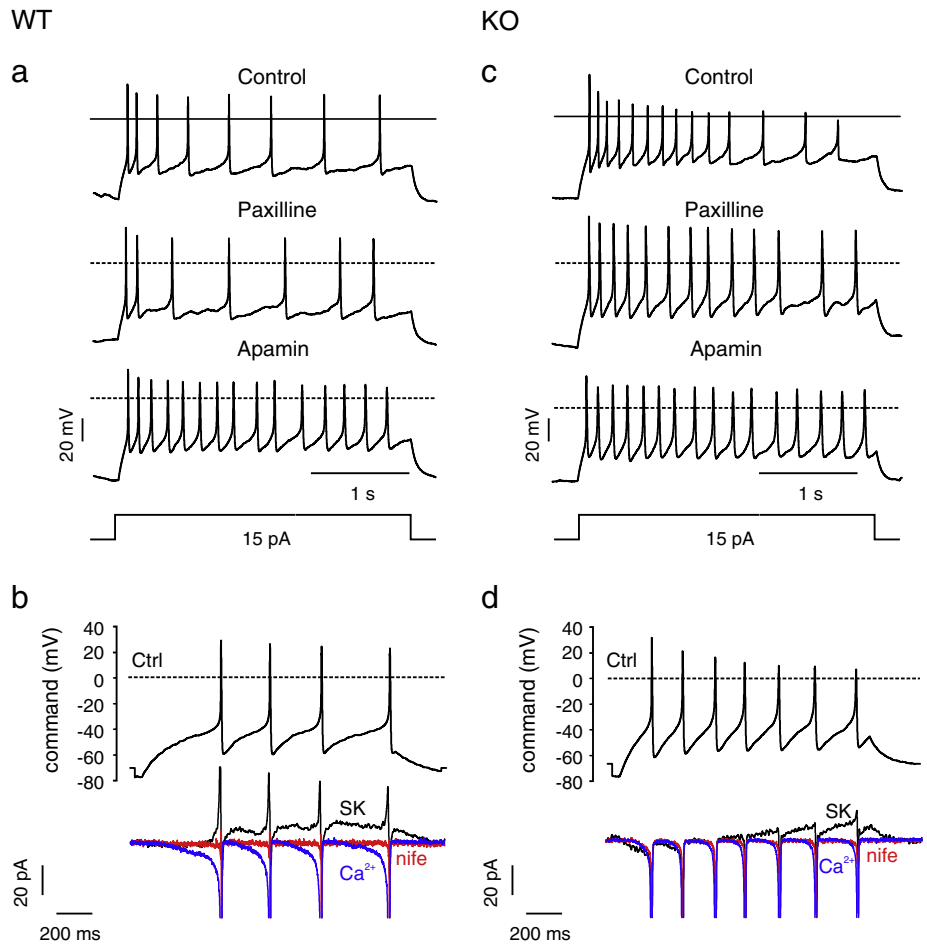

Fig. 5. Spike frequency adaptation of MCCs is dependent on Cav1.3-driven SK channels. a, representative current clamp traces of a MCC after 15 pA current injection in control (top), after block of BK channels by 1 μM paxilline (middle) and after block of SK channels by 200 nM apamin (bottom). Notice that apamin and not paxilline reduces the naturally occurring spike frequency adaptation of WT MCCs. b, representative AP-clamp traces of a WT MCC. The top trace represents the AP train recorded in response to a 15 pA current stimulus. To the bottom are illustrated the SK currents (black) and the total Ca^{2+} currents (blue) that flow during the spike train. Red traces are recorded in the presence of 3 μM nifedipine to block the L-type currents. Notice that the L-type Ca^{2+} current perfectly overlaps with the interspike interval specific outward SK current. c and d, same as in a and b but referring to Cav1.3$^{-/-}$ MCCs. Notice that the reduced spike frequency adaptation of Cav1.3$^{-/-}$ MCCs can be ascribed to the inability of these cells to build up sizeable SK currents (adapted from Ref. [31]).

Neuroendocrine chromaffin cells exhibit different types of endocytosis, according to cell activity and stimulation protocols. Square pulse depolarizations cause exocytosis followed by a decline in membrane capacitance, which can reach the pre-stimulus level (*compensatory* endocytosis) or fall even below (*excess* endocytosis) [134,135]. Transition between these two modes appears to be regulated by intracellular Ca^{2+}: the retrieval being accelerated and potentiated by increasing Ca^{2+} levels. It is interesting that in chromaffin cells endocytosis is also supported by barium [136] by the activation of kinase/phosphatase-mediated pathways [134,137] and by additional pathways of vesicle recycling independent of dynamin and calmodulin [40] In bovine chromaffin cells, the mechanism of action involves sphingosine and originates at the intracellular site [138]. The phenomenon is lost when applying repeated stimuli [139].

Concerning the role of Ca^{2+} channels in sustaining endocytosis, a preferential coupling of LTCCs to endocytosis has been recently proposed: Ca^{2+}-entry through LTCCs in bovine chromaffin cells is more effective in triggering endocytosis than exocytosis [39] (see also [41] for a recent review). Even at the mammalian neuromuscular junction, endocytosis is mainly sustained by LTCCs, while P/Q-type channels trigger exocytosis [140]. Block of LTCCs by DHPs decreases endocytosis and directs newly formed synaptic vesicles to a slow-release vesicle pool during high-frequency stimulation. According to the proposed model, in the absence of functioning LTCCs, endocytosis cannot be sufficiently fast to balance exocytosis causing vesicular membrane to accumulate at the presynaptic surface.

Given the main role of LTTCs in controlling compensatory and excess endocytosis, the next issue is whether Cav1.2 or Cav1.3 has a preferential control on vesicle retrieval. One argument in favor of Cav1.3 is its slower and less complete time-dependent inactivation with respect to Cav1.2. The delayed inactivation of Cav1.3 could be physiologically relevant for sustaining prolonged Ca^{2+} influxes that support normal endocytosis. This can be certainly clarified by comparing the endocytotic responses to Ca^{2+} loads in WT and Cav1.3$^{-/-}$ MCCs and by checking whether the hypothesis of an equal role of Cav1.2 and Cav1.3 on vesicle retrieval is satisfied. Under these conditions, a nearly half reduction of functional LTCCs, as occurred in Cav1.3$^{-/-}$ MCCs, should cause a proportional decrease of endocytosis. Finally, also the coupling of Cav1.2 and Cav1.3 to calmodulin, the Ca^{2+} sensor of different forms of endocytosis [141], could be a further molecular target of differential regulation of the endocytosis that needs to be tested.

7. Concluding remarks

The importance of Cav1.2 and Cav1.3 channels in the control of chromaffin cell firing, catecholamine secretion and vesicle retrieval is of growing interest and supported by an increasing number of

up-coming papers [12,18,41,67]. Cav1.3 and Cav1.2 contribute to shape the AP waveform and support the pacemaker current. Cav1.3, in particular, activates BK and SK channels that in turn set the frequency of spontaneously firing cells and help in adapting the spike frequency during prolonged depolarization. At present, there are no indications of any preferential coupling of either one isoform to the exo- and endocytotic machinery. Since both channels are equally modulated by PKA- and PKG-mediated pathways and may undergo drastic up and down autocrine regulations, it is expected that both isoforms play a critical role in chromaffin cell functioning.

Future works will certainly help to identify how the voltage-dependence of activation–inactivation gating and modulatory pathways acting on these Cav1 channels may fine-tune cell firing and Ca^{2+}-dependent excitation–secretion coupling. New findings on the functional role of these two LTCCs on chromaffin cells activity will be beneficial not only for understanding stress-related pathologies associated to the adrenal gland [67] but also for resolving the origin of diseases of central neurons, sensory and cardiac cells where the two LTCCs are highly expressed and play vital roles [12,16].

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