Channels 4:6, 440-446; November/December 2010; © 2010 Landes Bioscience

$Ca_V1.3$ as pacemaker channels in adrenal chromaffin cells

Specific role on exo- and endocytosis?

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Key words: L-type calcium channels, BK channels, pacemaking currents, action potential firing, Ca^{2+}-dependent secretion, cAMP, cGMP, hypoxia

Voltage-gated L-type calcium channels (LTCCs) are expressed in adrenal chromaffin cells. Besides shaping the action potential (AP), LTCCs are involved in the excitation-secretion coupling controlling catecholamine release and in Ca^{2+}-dependent vesicle retrieval. Of the two LTCCs expressed in chromaffin cells ($Ca_V1.2$ and $Ca_V1.3$), $Ca_V1.3$ possesses the prerequisites for pacemaking spontaneously firing cells: low-threshold, steep voltage-dependence of activation and slow inactivation. By using $Ca_V1.3^{-/-}$ KO mice and the AP-clamp it has been possible to resolve the time course of $Ca_V1.3$ pacemaker currents, which is similar to that regulating *substantia nigra* dopaminergic neurons. In mouse chromaffin cells $Ca_V1.3$ is coupled to fast-inactivating BK channels within membrane nanodomains and controls AP repolarization. The ability to carry subthreshold Ca^{2+} currents and activate BK channels confers to $Ca_V1.3$ the unique feature of driving Ca^{2+} loading during long interspike intervals and, possibly, to control the Ca^{2+}-dependent exocytosis and endocytosis processes that regulate catecholamine secretion and vesicle recycling.

Introduction

LTCCs are hetero-oligomers consisting of a pore-forming α_1-subunit interacting with accessory subunits (β and α_2-δ) to form a functional channel complex. LTCCs belong to the large family of voltage-gated Ca^{2+} channels that are permeable to Ca^{2+} and include the N, P/Q, R and T-types.[1] Like the other Ca^{2+} channels, LTCCs open readily during membrane depolarization and allow Ca^{2+} to enter the cell. In this way, LTCCs can both regulate cell excitability and trigger a variety of Ca^{2+}-dependent physiological processes, such as: excitation-contraction coupling in all muscle types, gene expression, synaptic plasticity, brain aging, hormone secretion[2] and pacemaker activity in heart, neurons and endocrine cells.[3-6] Presently, four genes are identified coding for the $Ca_V1.1$, $Ca_V1.2$, $Ca_V1.3$ and $Ca_V1.4$ subunits.[1] Of these, $Ca_V1.1$ and $Ca_V1.4$ exhibit specific expression profiles that are restricted to skeletal muscle, endocrine pituitary cells and the retina, whereas $Ca_V1.2$ and $Ca_V1.3$ are widely expressed throughout the central nervous system, sensory and endocrine cells, atrial myocytes and cardiac pacemaker cells.[7,8]

$Ca_V1.2$ and $Ca_V1.3$ channels are widely expressed in the chromaffin cells of the adrenal medulla[6,9-11] and possess key properties that are conditional for the control of chromaffin cell activity. First, $Ca_V1.2$ and $Ca_V1.3$ channels markedly contribute to catecholamine secretion.[12-15] In rat (RCCs) and mouse chromaffin cells (MCCs) they are responsible for nearly half of the total Ca^{2+} current and the corresponding exocytosis.[12,16-20] Second, compensatory and excess endocytosis are strongly attenuated when LTCCs are blocked.[21] Third, LTCC gating can be either up or downregulated by autocrinally released neurotransmitters coupled to membrane-delimited G protein dependent receptors or cGMP/PKG and cAMP/PKA pathways.[20,22-27] Fourth, $Ca_V1.3$ channels activate at very low voltages and inactivate slowly with respect to $Ca_V1.2$ and other high-threshold channels (N, P/Q, R).[2,28,29] This enables $Ca_V1.3$ to carry pacemaker currents sustaining chromaffin cell spontaneous activity.[6,15,20] Finally, $Ca_V1.3$ is tightly coupled to fast inactivating BK channels, suggesting a key control of AP firings and shape.[6]

All these peculiar properties of $Ca_V1.2$ and $Ca_V1.3$ channels highlight the strategic role that these two channels exert on chromaffin cell activity. Full understanding of the function of $Ca_V1.2$ and $Ca_V1.3$ on adrenal medulla physiology could help solving neuronal and cardiovascular pathologies deriving from stressful conditions that develop during prolonged and elevated levels of circulating catecholamine's.

Why Chromaffin Cells Fire Spontaneously?

Bovine, rat, mouse and human adrenal chromaffin cells fire spontaneously when cultured in vitro[6,20,30-39] or maintained in slices of the adrenal gland.[40,41] The percentage of firing chromaffin cells varies considerably (from 20 to 80%) depending on the animal species, the intracellular physiological solutions

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Submitted: 10/20/10; Accepted: 10/20/10
DOI: 10.4161/chan.4.6.12866

and methods of cell isolation. In spite of this, chromaffin cells fire spontaneously regardless of the patch-clamp technique used for AP recording: whole-cell, cell-attached and perforated-patch recording modes.

Spontaneous activity of chromaffin cells can also be monitored by extracellular recording techniques. When plated on a multi-electrode array (MEA) recording system[38] the spontaneous activity is maintained. This proves that AP firing is a genuine phenomenon of chromaffin cells persisting in the absence of splanchnic nerve stimulation and not related to cell damage induced by the patch-clamp electrode. With the MEA system, the intact cell simply adheres to the TiN microelectrodes[42] and the extracellular AP firing is recorded in a non-invasive manner. Indeed, the firing frequencies and the firing modes (tonic versus bursts) monitored by the MEA (**Fig. 1**) match those intracellularly recorded in perforated-patches.[37,38]

Given that chromaffin cells are contacted by multiple cholinergic innervations that effectively control their activity by splanchnic nerve discharges, an unsolved question is why in vivo adrenal chromaffin cells should possess spontaneous AP activity. A possible explanation could be that chromaffin cells are packed together and electrically coupled by gap-junctions[41,43] to form groups of cells that synchronously release the content of secretory granules in nearby blood capillaries.[44] Under these conditions, the spontaneous firing of one or a group of chromaffin cells could warrant the basal release of catecholamines of several electrically coupled cells. Tonic or burst firing, as shown in **Figure 1**, could be also at the basis of the adrenal gland response to increased blood levels of histamine, acetylcholine, angiotensin II (ATII) and K$^+$ ions. Histamine and acetylcholine are known to increase the firing rate of spontaneous APs[34,36,37] and the same is likely to occur during postprandial hyperkaliemia and enhanced blood levels of ATII that is known to increase the levels of circulating catecholamines. All these events could occur independently of the neurogenic control of chromaffin cell activity and could be sustained by the electrical synchronism through gap-junctions.[43]

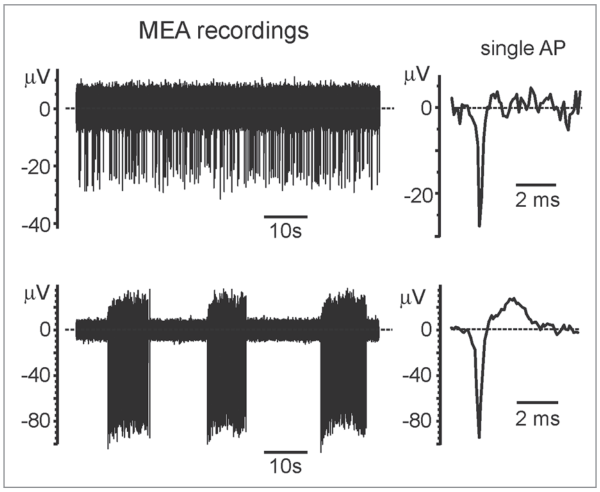

Figure 1. Two types of extracellularly recorded AP firings in rat chromaffin cells (RCCs) using MEAs. Note the µV vertical scale and the different firing modes: tonic (top trace) and bursting (bottom trace). To the right are shown single AP events. RCCs were bathed in Tyrode solution (2 mM Ca^{2+}).

Ca$_V$1.3 Expression and Pacemaking: Ca^{2+} Versus Na$^+$ Subthreshold Currents

All the above arguments justify a detailed analysis of the pacemaker current sustaining chromaffin cell firing, but surprisingly enough there are only few reports on the ion channels controlling chromaffin cell pacemaking. The few studies available focus on the main role of TTX-sensitive Na$^+$ channels in sustaining the AP upstroke,[30,32] and voltage-gated Ca^{2+} channels contributing to AP activity[30,35,40] or shaping APs through their coupling to BK channels.[45] The critical role of voltage-gated Ca^{2+} channels in pacemaking MCCs is best illustrated in **Figure 2**, where addition of 300 nM TTX reduces both the AP overshoot and undershoot with little effect on the firing frequency. In the presence of TTX, there are oscillatory potentials of smaller amplitude that do not overshoot and are effectively blocked by Cd^{2+} (200 µM). This is similar to what is reported for *substantia nigra* pars compacta (SNc) dopaminergic neurons and is indicative of a main role of Ca^{2+} channels in sustaining both the AP upstroke and the firing frequency.[46] In the case of SNc neurons, the block of firing after Co^{2+} application causes hyperpolarization due to the block of a dominant subthreshold Ca^{2+} current.[46] In MCCs and RCCs this is not always the case, in some cells the block of firing by Cd^{2+} causes slight depolarizations, as illustrated in **Figure 2**, indicating a parallel block of Ca^{2+}-activated K$^+$ currents. As in SNc[46-50] and other neurons,[51-56] the persisting firing in the presence of TTX can be effectively blocked by nifedipine (3 µM) and accelerated by BayK 8644 (1 µM) (**Fig. 2**, bottom), suggesting a main role of LTCCs in controlling AP firing in these cells.

Evidence for the existence of an L-type pacemaker current in MCCs comes directly from AP-clamp experiments[20] when K$^+$ and TTX-sensitive Na$^+$ channels are blocked. **Figure 3** shows the time course of these currents in a WT MCC, which is very similar to that recorded in SNc[46] or suprachiasmatic neurons[55] and is highly suggestive of a contribution of Ca$_V$1.3 currents. However, as for SNc neurons and cardiac sino-atrial node cells,[3-5] a direct role of Ca$_V$1.3 in pacemaking MCCs was uncovered by using Ca$_V$1.3$^{-/-}$ KO mice.[6] Deletion of Ca$_V$1.3 reduces drastically the amplitude of the pacemaker L-type current (**Fig. 3**, bottom) and the fraction of firing cells (from 80% to 30%). **Figure 3** (bottom) shows how small the average L-type pacemaker currents are in Ca$_V$1.3$^{-/-}$ KO MCCs and how BayK 8644 can effectively restore them. This reminds of the restored L-type-dependent bursting activity of silent mid-brain spiny neurons in Ca$_V$1.3$^{-/-}$ KO mice after addition of BayK 8644.[57]

Figure 3 shows also that Na$^+$ pacemaker currents are nearly absent in MCCs when compared to the sum of Ca^{2+} and Ca^{2+}-activated K$^+$ currents recorded from the same cell. The former are obtained by subtracting TTX-insensitive from control currents and the latter from a similar procedure using Cd^{2+} (200 µM).

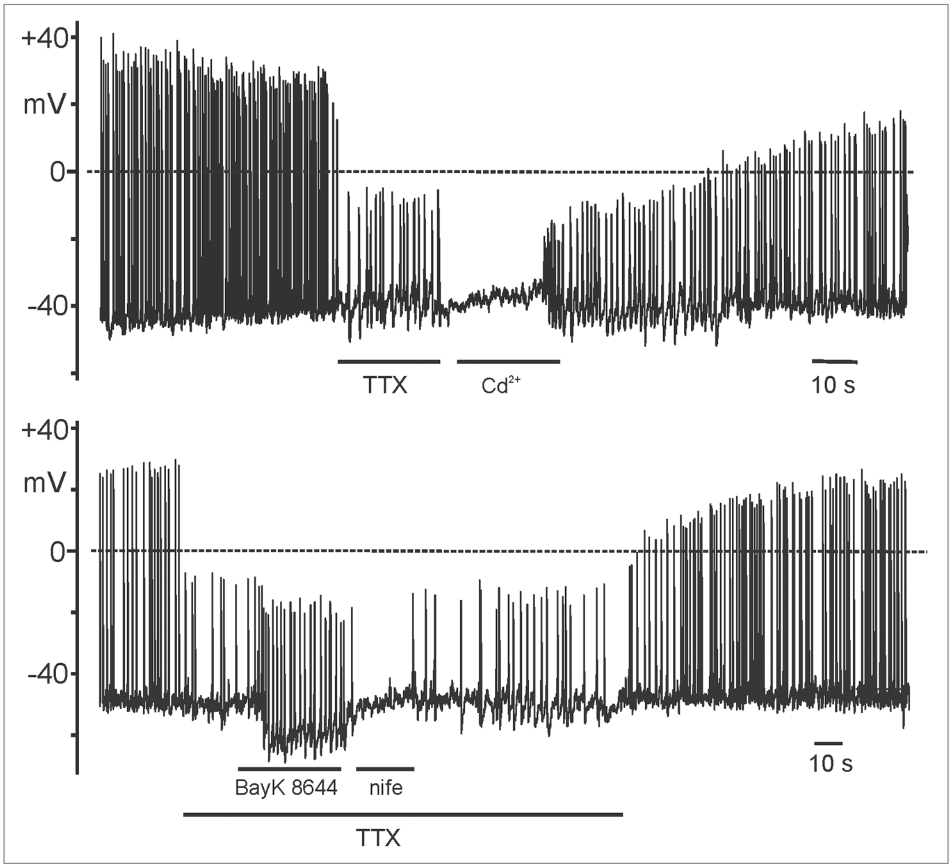

Figure 2. Spontaneous firing in MCCs persists in the presence of TTX (0.3 μM). AP firing is blocked by both Cd^{2+} (200 μM) and nifedipine (3 μM). BayK 8644 (1 μM) increases the firing rate and the after-hyperpolarization amplitude. Recording conditions are those described in Marcantoni et al.[6]

Ca_V1-BK Crosstalk Affects Cell Firing and AP Shape

RCCs and MCCs are shown to express two different BK channel subtypes that can be distinguished according to their inactivation kinetics: a fast inactivating and a slowly inactivating subtype.[58] The fast inactivating BK channel is typically expressed in chromaffin cells and is involved in tonic cell firing. The slowly inactivating BK channel has gating properties similar to central neurons and smooth muscle BK channels[59] and gives rise to phasic firings.[58] Chromaffin cells expressing fast inactivating BK channels are further characterized by deeper AHPs and higher charybdotoxin-sensitivity.[58] Fast inactivating BK channels possess slower deactivation kinetics that might contribute to long lasting AHP, necessary for recovering voltage-gated Na^+ and Ca^{2+} channels to initiate the following AP during sustained firing.[58]

In MCCs we have recently shown that the AP repolarization phase could be delayed by either blocking BK channels with paxilline, or LTCCs with nifedipine, proving the existence of a tight coupling between L-type and BK channels.[6] The main difference between the two blockers is that paxilline increases the firing frequency while nifedipine decreases or blocks the firing.[6] A similar selective coupling is reported in RCCs.[45] In MCCs there is also evidence that the coupling involves specifically Ca_V1.3. Deletion of Ca_V1.3 channels in $Ca_V1.3^{-/-}$ KO MCCs causes more depolarized interspike (resting) potentials and produces prolonged plateau depolarizations in response to BayK 8644.[6] Two properties that cannot be explained by simply silencing Ca_V1.3, but rather by assuming that Ca_V1.3 is effectively coupled

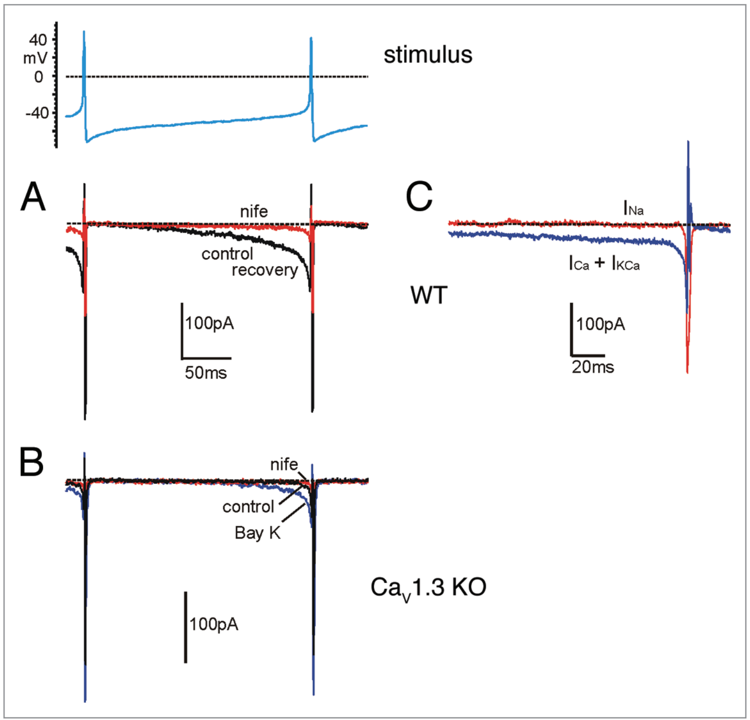

Figure 3. (A and B) Pacemaker Ca^{2+} currents in WT and Ca_V1.3 KO MCCs before (control) and during application of 3 μM nifedipine (nife). In (A) is shown also the Ca^{2+} current after washing nifedipine (recovery). In (B) 1 μM BayK 8644 was applied to test the presence of Ca_V1.2 channels. On the top is shown the AP train stimulus used for the AP-clamp recording. K^+ and Na^+ currents were blocked by adding 135 mM TEA and 0.3 μM TTX to the bath containing 2 mM Ca^{2+}. Both parts are adapted from Marcantoni et al.[6] (C) Comparison of Na^+ and Ca^{2+} + K^+_{Ca} currents in a WT MCC. TTX-sensitive Na^+ currents (red trace) were obtained by subtracting TTX-resistant (0.3 μM TTX) from control currents. The Ca^{2+} + K^+_{Ca} current (blue trace) was obtained by adding 200 μM Cd^{2+} to the TTX-resistant currents and subtracting the remaining component.

to Ca^{2+}-activated BK channels. This also suggests that $Ca_V1.3$ sustains the subthreshold pacemaker current in MCCs (and possibly in RCCs) and that the highly expressed BK channels counterbalance the inward Ca^{2+} current with an outward K^+ current component that decelerates the firing.[6]

Functional Coupling of $Ca_V1.3$ to Fast Inactivating BK Channels in MCCs

In line with the above arguments, it is evident that the BK channels contributing to the repolarization phase of the AP are mostly activated by the Ca^{2+} entering the cell during the interspike. Since this current is mainly carried by $Ca_V1.3$, this explains why nifedipine can effectively control both the frequency and the shape of the AP in spontaneously firing chromaffin cells. A better view to this functional coupling is obtained by directly measuring the K^+ currents flowing during an AP and testing their block by nifedipine (**Fig. 4**). The K^+ current rises and falls very quickly during the AP. The majority of this current is carried by voltage-gated K^+ channels and the BK channels activated by the Ca^{2+} entering the cytoplasm during the interspike. This Ca^{2+} is mainly carried by $Ca_V1.3$ in WT cells and by the other Ca^{2+} channels in $Ca_V1.3^{-/-}$ cells. Thus, the percentage of K^+ current blocked by nifedipine furnishes a direct estimate of the effective coupling between $Ca_V1.3$ and BK channels. **Figure 4** shows that in the case of WT MCCs, nifedipine blocks more than 60% of the outward K^+ currents while in $Ca_V1.3^{-/-}$ MCCs the DHP blocks only a small fraction in spite of the large inward Ca^{2+} current (blue trace).

Given the strong coupling between $Ca_V1.3$ and BK channels, a second interesting issue to solve is how close the two channel subunits are. This can be done using well calibrated Ca^{2+} buffers that limit the diffusion of Ca^{2+} ions beyond membrane nano- or microdomains in which voltage-gated Ca^{2+} channels and BK channels operate. Marty and Neher[60] were the first to use this approach in bovine chromaffin cells. They found that internal solutions containing BAPTA were more effective in blocking the Ca^{2+}-dependent K^+ currents than EGTA containing solutions. Following the same approach and using a double-pulse protocol to quantify the amount and type of BK currents, Chris Lingle and coworkers could formulate a quite realistic picture of how L-type and BK channels are coupled in RCCs.[61,62] The experimental data are consistent with a model in which BK channels are located between 160 and 50 nm from the Ca^{2+} channels that fuel them.[63] More precisely, 30 to 40% of fast inactivating BK channels in RCCs are insensitive to EGTA buffers and are therefore positioned sufficiently close to LTCCs (between 50 and 160 nm) to be influenced by the Ca^{2+} influx through these channels during brief depolarization steps.[63] The remaining channels are far apart (>160 nm) and their activation is fully prevented by saturating EGTA.

We followed a similar approach to evaluate the coupling between $Ca_V1.3$ and BK channels in MCCs, measuring the BK currents by using a voltage-clamp protocol consisting of two consecutive pulses. During the first pulse of 400 ms the cell is stepped from a negative holding potential (-70 mV) to a positive test potential (+80 mV) that overrates the Ca^{2+} reversal potential and produces large driving forces for K^+ ions. In this way, the voltage-gated K^+ channels (K_V) are fully activated for the entire duration of the pulse. During the second pulse the cell is first stimulated by a Ca^{2+} preloading step to +10 mV for 90 ms to achieve maximal Ca^{2+} entry and then stepped to the positive test potential. The resulting current is a mixture of transient BK and sustained K_V currents and the difference between the two K^+ currents with and without Ca^{2+} preloading represents the BK portion.[58,61]

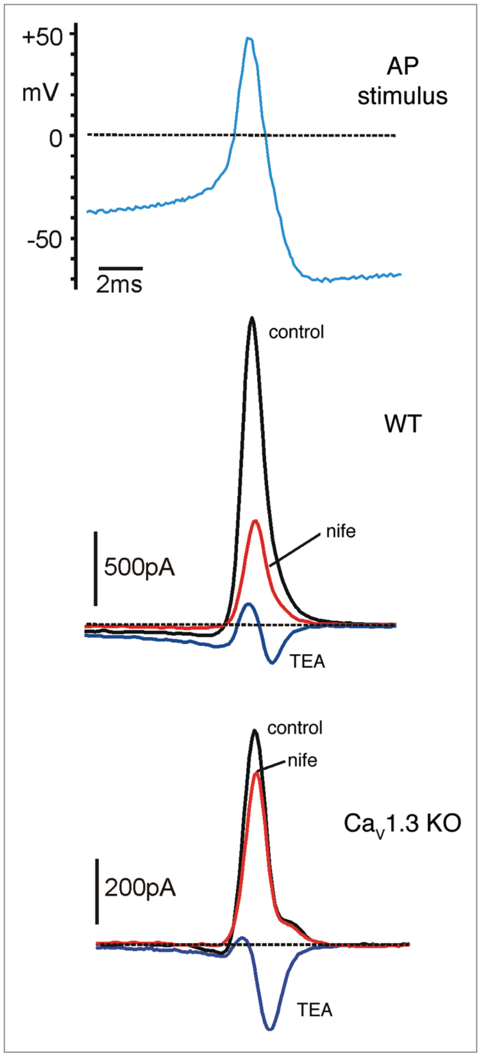

Figure 4. Different block induced by nifedipine on BK currents activated during an action potential in WT and $Ca_V1.3$ KO MCCs. The two MCCs were voltage-clamped using the same AP waveform (top trace) in the presence of 300 nM TTX. In WT MCCs 3 μM nifedipine blocks most of the K^+ outward current while in $Ca_V1.3$ KO the block is strongly attenuated (red trace). Blue traces are Ca^{2+} currents measured in 135 mM TEA and 0.3 μM TTX (adapted from Marcantoni et al.[6]

In the case of MCCs, prolongation of the Ca^{2+} preloading step has different effects on WT and $Ca_V1.3$ KO MCCs (**Fig. 5**). WT MCCs possess BK currents with relatively fast inactivating kinetics that increase in amplitude with increasing preloading steps.[6] In contrast, KO MCCs mainly show slowly inactivating BK currents which turn on with long preloading steps.[6] When the same experiment is repeated in the presence of

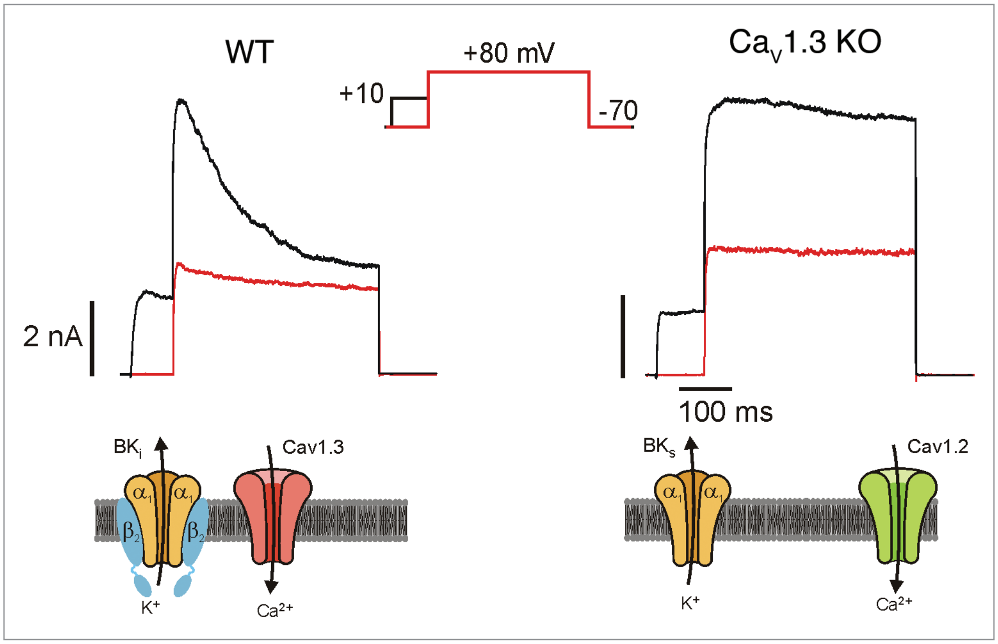

Figure 5. BK currents are mainly fast inactivating in WT MCCs and slowly inactivating Ca$_V$1.3 KO MCCs coupling. The inset shows the pulse protocol used to measure the currents (adapted from Marcantoni et al.[6]). To the bottom are illustrated the main conclusions of the experiments, i.e., Ca$_V$1.3 is closely coupled to fast inactivating BK channels while Ca$_V$1.2 and the other Ca$_V$ channels are weakly coupled to the slowly inactivating BK channels.

20 µM EGTA-AM, WT MCCs display their fast inactivating BK currents while the slowly inactivating channels are apparently lost.[6] These data suggest that, in agreement with the Prakriya and Lingle's model, fast inactivating BK channels are preferentially coupled to Ca$_V$1.3 (between 50 and 160 nm) while slow inactivating BK channels are more distant[6] (>160 nm). Incubating WT and KO MCCs with 20 µM of BAPTA-AM the BK currents are functionally abolished indicating that the fast inactivating BK channels are close but not physically coupled to Ca$_V$1.3,[6] (**Fig. 5**, bottom left). On the other hand, Ca$_V$1.2 is likely to be significantly distant from slowly inactivating BK channels in Ca$_V$1.3$^{-/-}$ MCCs.

LTCC-Secretion Coupling in Adrenal Chromaffin Cells

In chromaffin cells, the different Ca^{2+} channel subtypes (L, N, P/Q, R) contribute to exocytosis proportionally to their density of expression and gating properties.[12,17-19,64-68] Secretion is not particularly linked to a specific Ca^{2+} channel type and either deletion or upregulation of one of them causes a proportional change to secretion. For instance, Ca$_V$2.1 deletion causes a loss of the P/Q-type currents with a compensatory increase of L-type currents and secretion.[69] Similarly, when upregulated by cAMP[14] or chronic hypoxia,[70] T-type channels contribute to low-threshold exocytosis with the same Ca^{2+}-dependence of L-type channels.

In RCCs and MCCs, LTTCs represent the final target of different modulatory pathways mediated by the activation of either G protein-coupled membrane autoreceptors[23-25,27] and intracellularly PKA and PKG[26] or PDE type-4.[20] In principle, by acting on LTCCs, any of these signaling loops can exert a potent modulatory effect also on the exocytotic response. In fact, the L-type current increase induced by prolonged cAMP stimulation only accounts for 20% of the total secretory response, suggesting an additional down-stream effect on the secretory machinery.[12] It is also interesting to notice that, due to their slower and less complete time-dependent inactivation, LTCCs are favored in triggering exocytosis with respect to other HVA Ca^{2+} channels during sustained stimuli. Nevertheless, the contribution of LTCCs to exocytosis remains proportional to the quantity of Ca^{2+} ions entering the cell, suggesting that there is no preferential co-localization of Ca$_V$1 channels to secretory granules.[15]

Although the critical role of LTCCs in triggering exocytosis is well established,[12,13,71,72] there are no clear indications of a possible distinct role of Ca$_V$1.2 and Ca$_V$1.3 to exocytosis, despite the different inactivation kinetics and voltage range of activation of the two isoforms.[2] Preliminary observations show that deletion of Ca$_V$1.3 subunit in MCCs lowers the amount of exocytosis at very negative potentials (-50 to -30 mV in 10 mM Ca^{2+}) (Navarro V, Striessnig J, Carbone E, Carabelli V, unpublished data), indicating that besides sustaining action potential firing, Ca$_V$1.3 preferentially contributes to exocytosis at low membrane potentials. In this way, Ca$_V$1.3 contributes to the low-threshold exocytosis similarly to the T-type Ca$_V$3.2 channel.[14,67,70]

LTCC-Mediated Endocytosis in Chromaffin Cells: A Specific Role for Ca$_V$1.3?

Another open question to solve concerns the role of Ca^{2+} and Ca^{2+} channels in the retrieval of synaptic vesicles during endocytosis. 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).[73,74] 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,[75] by the activation of kinase/phosphatase-mediated pathways[73,76] and by additional pathways of vesicle recycling independent of dynamin and calmodulin.[77] In bovine chromaffin cells, the mechanism of action involves sphingosine and originates at the intracellular site.[78] The phenomenon is lost when applying repeated stimuli.[79]

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.[21] Even at the mammalian neuromuscular junction, endocytosis is mainly sustained by LTCCs, while P/Q-type channels trigger exocytosis.[80] Block of LTCCs by DHPs decreases endocytosis and directs newly formed synaptic vesicle 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 Ca$_V$1.2 or Ca$_V$1.3 has a preferential control on vesicle retrieval. One argument in favor of Ca$_V$1.3 is its slower and less complete time-dependent inactivation with respect to Ca$_V$1.2. The delayed inactivation of Ca$_V$1.3 could be physiologically relevant for sustaining prolonged Ca^{2+} influxes that support normal endocytosis. Finally, also the coupling of Ca$_V$1.2 and Ca$_V$1.3 to calmodulin, the Ca^{2+} sensor of different forms of endocytosis,[81] could be a further molecular target of differential regulation of the endocytic response. These issues will be answered using specific L-type KO animal models.[82]

Conclusions

LTTCs play multiple roles in adrenal chromaffin cells function. They set the frequency of spontaneous AP firing and condition the AP shape through their coupling to BK channels. These are critical parameters that may set the basal release of catecholamine and the response of adrenal gland to changing levels of blood hormones (histamine, angiotensin II, aldosterone). LTCCs also sustain catecholamine secretion and vesicle retrieval. Secretion occurs without any preferential Ca^{2+} channel co-localization to secretory vesicle while endocytosis requires a preferential coupling to LTCCs. Spontaneous AP firing, low-threshold exocytosis and compensatory/excess endocytosis are likely to be directly controlled by Ca$_V$1.3, which then turns out to be an important molecular gateway for controlling catecholamine release and regulate the "fight-or-flight" response under stress condition. Since Ca$_V$1.3 is also critical for the control of vital functions such as: heart beating,[4] hearing[83] and dopamine release,[5,57] it is evident that the availability of new DHP compounds that selectively block Ca$_V$1.3 would be beneficial for the therapeutic treatment of Parkinson disease, cardiac arrhythmias, chronic stress and other neuro- and cardiovascular pathologies in which Ca$_V$1.3 is likely to be involved.

Acknowledgements

We wish to thank Dr. Claudio Franchino for technical assistance. This work was supported by the Marie Curie Research Training Network "Ca$_V$NET" Contract MRTN-CT-2006-035367, the Italian M.I.U.R. Grant PRIN 2007SYRBBH_001 to Emilio Carbone and the San Paolo Company Grant 2008.2191 to Andrea Marcantoni.

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