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Are Ca$_v$1.3 pacemaker channels in chromaffin cells?
Possible bias from resting cell conditions and DHP blocker usage

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ouse and rat chromaffin cells (MCCs, RCCs) fire spontaneously at rest and their activity is mainly supported by the two L-type Ca$_{v}$2 channels expressed in these cells (Ca$_v$1.2 and Ca$_v$1.3). Using Ca$_v$1.3-/- KO MCCs we have shown that Ca$_v$1.3 possess all the prerequisites for carrying subthreshold currents that sustain low frequency cell firing near resting (0.5 to 2 Hz at -50 mV): low-threshold and steep voltage dependence of activation, slow and incomplete inactivation during pulses of several hundreds of milliseconds. Ca$_v$1.2 contributes also to pacemaking MCCs and possibly even Na$^+$ channels may participate in the firing of a small percentage of cells. We now show that at potentials near resting (-50 mV), Ca$_v$1.3 carries equal amounts of Ca$^{2+}$ current to Ca$_v$1.2 but activates at 9 mV more negative potentials. MCCs express only TTX-sensitive Na$_v$1 channels that activate at 24 mV more positive potentials than Ca$_v$1.3 and are fully inactivating. Their blockade prevents the firing only in a small percentage of cells (13%). This suggests that the order of importance with regard to pacemaking MCCs is: Ca$_v$1.3, Ca$_v$1.2 and Na$_v$1. The above conclusions, however, rely on the proper use of DHPs, whose blocking potency is strongly holding potential dependent. We also show that small increases of KCl concentration steadily depolarize the MCCs causing abnormally increased firing frequencies, lowered and broadened AP waveforms and an increased facility of switching “non-firing” into “firing” cells that may lead to erroneous conclusions about the role of Ca$_v$1.3 and Ca$_v$1.2 as pacemaker channels in MCCs.

Introduction
L-type calcium channels (LTCCs, Ca$_v$1) contribute to the pacemaker current of central neurons,3,4 neuroendocrine1,7,8 and cardiac sino-atrial node cells9 generating spontaneous action potentials of low frequency (0.5 to 2 Hz) (reviewed in refs. 10 and 11). Between the two LTCCs expressed in these tissues (Ca$_v$1.2, Ca$_v$1.3), Ca$_v$1.3 appears the most suitable isoform for pacemaking in excitable cells: (1) it activates at relatively more negative potentials12-15 and (2) has faster activation but slower and less complete voltage-dependent inactivation.13,14 Using Ca$_v$1.3-/- KO mice,12 we found that these peculiarities are well preserved in mouse adrenal chromaffin cells and that Ca$_v$1.3 and Ca$_v$1.2 contribute equally to the total Ca$^{2+}$ current (-25% each).2 Given this, it appears evident that near resting conditions (-50 mV), Ca$_v$1.3 is privileged for controlling AP firings with interpulse intervals of 0.5 to 2 s (2-0.5 Hz). As suggested by the blocking action of nifedipine on Ca$_v$1.3-/- MCCs, we also concluded that Ca$_v$1.2 also contributes to pacemaking, but at a lower degree given its more positive voltage range of activation and faster inactivation. A small contribution to the pacemaker current is expected also from TTX-sensitive Na$^+$ channels, which can account for a small fraction of Ca$_v$1.3-/- MCCs that preserves their ability to fire
in the presence of nifedipine, i.e., when Cav1.2 is pharmacologically blocked and Cav1.3 is absent.¹

In apparent contrast with us, a recent paper by Pérez-Alvarez et al.² reports that Cav1.2 is likely to be the dominant pacemaking channel in MCCs and that Cav1.3 contributes only marginally to the spontaneous activity. The conclusions are based on experiments in WT and Cav1.3⁻⁻ KO-MCCs bathed in solutions with higher KCl concentration than ours (5.5 vs. 4 mM) which steadily depolarize the cells by -6 mV. Moreover, the authors evaluated the contribution of Cav1.2 and Cav1.3 by the block of DHPs at Vᵢ₋₋ = -80 mV assuming that the same is valid at -40 mV, without taking into consideration that block of LTCCs by DHPs is strongly holding potential-dependent.¹³⁻¹⁵⁻¹⁷ We show here that the findings by Pérez-Alvarez et al.² are not in contrast with ours if properly corrected for the different KCl concentration and if DHP blocking potency is suitably tested at the interspike potential of spontaneous firing (-50 mV in 4 mM KCl). Thus, with the proper corrections their data confirm our previous proposal that, near resting physiological conditions the order of importance for pacemaking is: Cav1.3, Cav1.2 and Naᵥ₁ channels.

To clarify these critical points, we show here that: (1) full block of Cav1.3 in MCCs requires high doses of nifedipine at Vᵢ₋₋ = -80 mV (-30 μM) and 10-fold lower concentrations at Vᵢ₋₋ = -50 mV, making this channel very sensitive to DHPs near resting conditions, (2) the predominance of Cav1.3 in pacemaking derives from its steep voltage range of activation which is 9 and 24 mV more negative than Cav1.2 and Naᵥ₁ channels, respectively, (3) MCCs express only fast and fully inactivating TTX-sensitive Naᵥ₁ channels that can contribute little to pacemaker currents lasting 0.5 to 2 s. Saturating doses of TTX block the firing in a small fraction of MCCs (13%), (4) steady depolarizations of -6 mV using higher KCl concentrations or few pA of current injection cause a marked reduction and broadening of AP waveform, a 3-fold increase of firing frequency and an increased capability of turning “non-firing” into “firing” cells. These conditions favor the contribution of fast inactivating channels, which activate at more positive potentials (Cav1.2 and Naᵥ₁ channels) and introduce serious bias to the true estimate of Cav1.3 role in pacemaking MCCs.

Results

Cav1.3 and Cav1.2 contribute equally to the total Ca²⁺ current in WT MCCs. MCCs express both Cav1.2 and Cav1.3 and the two channels together contribute about 50% of the total Ca²⁺ currents.¹⁵ It is thus critical to establish the percentage of current carried by each of the two Cav1 channels at the potentials where cells spontaneously fire (-50 mV). We have previously shown that WT and Cav1.3⁻⁻ express equal Ca²⁺ current amplitudes (Fig. 1A top-right inset) and that the block of LTCCs by nifedipine at Vᵢ₋₋ = -50 mV saturates at about 3 μM to give maximal inhibitions of 52.8% (WT) and 22.4% (Cav1.3⁻⁻) (Fig. 1A bottom-right inset). Given the importance of this issue, we show here two examples of L-type current block with increasing doses of nifedipine in WT and KO MCCs held at -50 mV (Fig. 1A). Since WT currents are carried by Cav1.3 and Cav1.2, and KO currents by Cav1.2 alone, it is evident that the DHP effectively blocks both Cav1.2 and Cav1.3 and that Cav1.3 contributes to the total L-type current equally to Cav1.2. The same is not valid when Vᵢ₋₋ is lowered to -80 mV, where LTCCs sensitivity to DHPs decreases,¹⁶,¹⁷ requiring higher doses of nifedipine to fully block the channels. Figure 1B shows that after blocking N-, P/Q- and R-type channels with ω-CTX-GVIA (3.2 μM), ω-CTX-MVIIC (10 μM) and SNX-482 (0.4 μM), full block of the remaining LTCCs requires nifedipine concentrations >10 μM. Comparison of the dose response curve of nifedipine action on LTCCs at Vᵢ₋₋ = -80,-50 and -40 mV shows that nifedipine concentrations that mediate complete block of L-type currents at -50 mV or -40 mV can only block a small fraction of current at -80 mV (Fig. 1C). This suggests that using DHP concentrations tested on whole cell Ca²⁺ currents at -80 mV is not an appropriate basis for arguing on their action on AP firing at interspike potentials near -50 mV or -40 mV, as in the case of reference 2. This procedure would lead to an obvious underestimation of Cav1.3 with respect to Cav1.2.

Cav1.3 activates at more negative potentials than Cav1.2 and Naᵥ₁ channels. Given that Cav1.3 and Cav1.2 contribute equally to L-type currents, our next issue was to determine the true voltage-dependence of Cav1.3 activation using the conductance vs. voltage curves of LTCCs in WT and KO MCCs previously determined (Fig. 2B in ref. 1). We assumed that the WT conductance curve is a linear combination of the Cav1.3 and Cav1.2 curves with equal weight (50%) and that the KO curve is representative of Cav1.2. The resulting Cav1.3 curve is shown in Figure 2A (dashed line) and appears shifted by about -9 mV from Cav1.2 curve (see legend). This finding together with the observation that Cav1.3 and Cav1.2 contribute equally to the total Ca²⁺ current and that Cav1.3 inactivates slowly and incompletely during long depolarizations suggest that Cav1.3 outweighs Cav1.2 in pacemaking MCCs.

Once we established the voltage-dependence of Cav1.3 and Cav1.2 we next studied the gating properties and the range of activation of Naᵥ₁ channels that support the upstroke of APs and may contribute to the pacemaker current in MCCs. We found that MCCs express mainly Na⁺ TTX-sensitive channels that activate at -24 mV more positive potentials than Cav1.3 (Fig. 2A) and inactivate fully within tens of milliseconds at all potentials (maximal peak current -635 ± 46 pA at +10 mV, n = 12; Fig. 2B). In twelve MCCs we found no evidence of slowly inactivating “persistent” Na⁺ channels, which are able to support neuronal pacemaker currents.¹⁸ This confirms that Naᵥ₁ channels contribute little to the pacemaker current in MCCs (Fig. 3C in ref. 11). Interestingly, when applied to spontaneously firing cells, saturating doses of TTX (300 nM) caused full block only in two out of 17 cells (13%; Fig. 2A). In the remaining 15 cells (87%), TTX caused a 5–6 mV depolarization followed by a persistent firing of smaller APs, often characterized by unusually rapid bursts of three to five spikes (Fig. 2D and legend). In conclusion, fast inactivating Naᵥ₁ channels activate at much more positive potentials than Cav1.2 (+15 mV) and Cav1.3 (+24 mV) and
Contribute little to pacemaking MCCs near resting physiological conditions.

Higher KCl solutions or steady depolarizations increase the firing frequency and lowers the AP size. For instance, ~6 mV positive resting potentials overestimate the firing threshold of activation (Cav1.2 and Na v1 channels). Since Cav1.3 are still able to fire and nifedipine is able to reduce or block the spontaneous firing in most, but not all the KO MCCs.1 This does not exclude the possibility that Cav1.2 also contributes to pacemaking in slow firing MCCs. Finally, Cav1.3 contributes to about half of the pacemaker currents in slow firing MCCs.13,15 It is evident that Cav1.3 is the most suitable channel for carrying most of the subthreshold pacemaker currents in slow firing MCCs. This may explain the larger number of firing Cav1.3−/− KO-MCCs estimated by reference 2 (57%) with respect to us (30%)1 and suggests that most of the different results can be simply explained by methodological differences rather than postulating pathological states of KO mice (see Discussion in ref. 2).

Discussion and Conclusions

The present study extends the work of Marcantoni et al.1 concerning the role of Cav1.3 as pacemaker channel in MCCs. Here we establish unequivocally that Cav1.3 contributes to about half of the L-type current which sustains AP firings near -50 mV and that Cav1.3 activates at 9 mV and 24 mV more negative potentials than Cav1.2 and Na1 channels. Since TTX-sensitive Na1 channels are fully inactivating within tens of milliseconds and Cav1.2 inactivates faster and more completely than Cav1.3,13,15 it is evident that Cav1.3 is the most suitable channel for carrying most of the subthreshold pacemaker currents in slow firing MCCs. This does not exclude the possibility that Cav1.2 also contributes to pacemaking these cells. Indeed, KO MCCs lacking Cav1.3 are still able to fire and nifedipine is able to reduce or block the spontaneous firing in most, but not all the KO MCCs.1
This also suggests that besides Ca v1.3 and Ca v1.2, Na v1 or other ion channels carrying subthreshold inward currents may also contribute to the pacemaker current in a small fraction of cells. Figure 2C clearly shows that this occurs in 13% of cells in which TTX causes a slight hyperpolarization and a block of MCCs firing.

The current study gives also a partial explanation to the contrasting results reported by Pérez-Alvarez et al.2 regarding the marginal role of Ca v1.3 as pacemaker channel in MCCs. We showed that this might derive from two anomalous methodological approaches. The first one is the improper use of DHPs that, when tested at V h -80 mV, underestimate the true contribution of Ca v1.3 channels near resting potentials (-50 mV). DHPs have very low-affinity for Ca v1.3 at very negative holding potentials.13-16 Regarding this issue, the use of 0.3 μM nifedipine to identify the contribution of Ca v1.2 on AP firing, as proposed by reference 2, is unjustified because this DHP concentration blocks equal fractions of Ca v1.2 currents in WT and KO-MCCs at -50 mV (see the dose-response curves of Fig. 1C in ref. 1). If 0.3 μM nifedipine inhibits equally Ca v1.2 and Ca v1.3 a similar block potency of WT and KO firing cells by this DHP concentration cannot be taken as proof that Ca v1.2 is the only LTCC pacemaking MCCs. The second one is related to the use of KCl solutions, which depolarize the cell by 5–6 mV, increases the firing and changes the AP shape with marked alteration of cell functioning. As shown in Figure 3, the fraction of “firing” vs. “non-firing” cells can be significantly increased at more depolarized resting potentials. We also cannot exclude that cell firing at higher frequencies (2–3 Hz or 5 Hz as in the case of ref. 2) will increase Ca v1.2 entry and stimulate Ca v1.2-dependent pathways.
that upregulate LTCCs openings\textsuperscript{19,20} and further contributes to switch "non-firing" into "firing" cells.

In conclusion, to answer the question raised in the title, Ca\textsubscript{1,3} is unequivocally a pacemaker channel in MCCs, not secondary to Ca\textsubscript{1,2}. Most likely its role is not limited to chromaffin cells, dopaminergic neurons\textsuperscript{6} and cardiac sinoatrial node cells.\textsuperscript{9} Ca\textsubscript{1,3} may even soon result a potential pacemaker channel in other neurons and neuroendocrine cells (reviewed in ref. 10). We also recall that Ca\textsubscript{1,3} is particularly coupled to fast inactivating BK channels\textsuperscript{1} and drives a considerable fraction of SK channels at resting potentials (Vandael DHF, Marcantoni A and Carbone E, unpublished results). Thus, their loss in Ca\textsubscript{1,3}+ MCCs is not expected to necessarily reduce the firing frequency as postulated by reference 2, but depending on their coupling to Ca\textsuperscript{2+} activated K+ channels\textsuperscript{21,22} it could paradoxically increase it.\textsuperscript{1}

Finally, we would underline the new frontiers on Ca\textsubscript{1,3} gating properties associated with the existence of the newly identified short splice variant Ca\textsubscript{1,3,3.2A}\textsuperscript{23} whose degree of expression and role in different tissues still needs to be clarified. An important clarification is still expected about the role of Ca\textsubscript{1,2} and Ca\textsubscript{1,3} on catecholamine secretion and vesicles retrieval in which LTCCs are shown to play a critical role (reviewed in ref. 11). Recent data suggest that Ca\textsubscript{1,3} contributes more to the exocytosis at low membrane potentials,\textsuperscript{2,11} in a way resembling T-type Ca\textsubscript{3,2} channels.\textsuperscript{24}

Materials and Methods

Isolation and culture of WT and Ca\textsubscript{1,3}− mouse adrenal medulla chromaffin cells.

All experiments were performed in accordance with the guidelines established by the National Council on Animal Care and were approved by the local Animal Care Committee of Turin University. Chromaffin cells were obtained from young (13 months) male C57BL/6N
CdCl₂. Action potentials (APs) were measured following the method described previously. 8 chromaffin cells were isolated and cultured following the method described previously.8

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