**Activity-dependent regulation of T-type calcium channels by submembrane calcium ions**

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Voltage-gated Ca2+ channels are involved in numerous physiological functions and various mechanisms finely tune their activity, including the Ca2+ ion itself. This is well exemplified by the Ca2+-dependent inactivation of L-type Ca2+ channels, whose alteration contributes to the dramatic disease Timothy Syndrome. For T-type Ca2+ channels, a long-held view is that they are not regulated by intracellular Ca2+. Here we challenge this notion by using dedicated electrophysiological protocols on both native and expressed T-type Ca2+ channels. We demonstrate that a rise in submembrane Ca2+ induces a large decrease in T-type current amplitude due to a hyperpolarizing shift in the steady-state inactivation. Activation of most representative Ca2+-permeable ionotropic receptors similarly regulate T-type current properties. Altogether, our data clearly establish that Ca2+ entry exerts a feedback control on T-type channel activity, by modulating the channel availability, a mechanism that critically links cellular properties of T-type Ca2+ channels to their physiological roles.

Voltage-gated Ca2+ channels (VGCCs) are unique among voltage-gated ion channels because the permeant Ca2+ ion also acts as an intracellular second messenger, triggering diverse cellular functions (Berridge et al., 2003). VGCCs are therefore involved in neuronal and cardiac excitability as well as in muscle contraction, neurotransmitter release, hormone secretion and gene expression (Berridge et al., 2003, Mangoni and Nargeot, 2008, Catterall, 2011, Simms and Zamponi, 2014, Zamponi et al., 2015). Consequently the modulation of VGCC activity plays a pivotal role in the regulation of cardiac and brain activities and this modulation is controlled by a variety of processes, including intracellular Ca2+ itself, which provides an important Ca2+-driven feedback control (Eckert and Chad, 1984, Zuhlke et al., 1999, Peterson et al., 1999, Liang et al., 2003, Green et al., 2007, Tsuruta et al., 2009, Oliveria et al., 2012, Hall et al., 2013, Zamponi et al., 2015).

VGCCs comprise 3 distinct subfamilies classified with respect to their biophysical and pharmacological (type), and molecular (Cav) entities: the L-type / Cav1, the N-, P/Q-, R-type / Cav2 and the T-type / Cav3 channels (Ertel et al., 2000). It was well demonstrated that both Cav1 and Cav2 channels are modulated by intracellular Ca2+ (Liang et al., 2003, Dick et al., 2008). For the Cav1 / L-type VGCCs, this Ca2+ feedback mechanism has been extensively studied in a wide spectrum of biological contexts and a rise in submembrane Ca2+ concentration induces complex effects depending on both the Ca2+ concentration and the duration of the Ca2+ entry (Eckert and Chad, 1984, Zuhlke et al., 1999, Peterson et al., 1999, Liang et al., 2003, Green et al., 2007, Tsuruta et al., 2009, Oliveria et al., 2012, Hall et al., 2013). At the millisecond time scale, the Ca2+ entry via L-type channels induces a Ca2+-dependent inactivation (CDI) characterized by an acceleration of their inactivation kinetics (Eckert and Chad, 1984, Zuhlke et al., 1999, Peterson et al., 1999, Liang et al., 2003, Hall et al., 2013). For several seconds to few minutes of stimulation, the cumulative Ca2+ entry induces a decrease of the L-type current amplitude, which is reversible if stimulation ceases for several minutes (Eckert and Chad, 1984, Oliveria et al., 2007, Oliveria et al., 2012, Hall et al., 2013). For longer period of stimulation, or activation of the ionotropic NMDA receptors, L-type channels are internalized, potentially degraded in lysosomes or recycled to the plasma membrane depending of the amount and the duration of the Ca2+ entry (Green et al., 2007, Tsuruta et al., 2009, Hall et al., 2013). This precise Ca2+-dependent regulation of the L-type channel activity has a strong physiological role in avoiding cytotoxicity arising from Ca2+ overload (Lee et al., 1999, Berridge et al., 2003, Green et al., 2007, Tsuruta et al., 2009, Hall et al., 2013). Consequently, alteration of the Ca2+-dependent regulation of the L-type channels is deleterious and has important pathophysiological consequences as observed in the Timothy syndrome (Splawski et al., 2004, Barrett and Tsien, 2008, Blaich et al., 2012, Limpitikul et al., 2014, Dick et al., 2016).

Contrasting with this well-established Ca2+-dependent regulation of the L-type channels, it is presently unknown whether a change in intracellular Ca2+ concentration is involved in regulating T-type Ca2+ channel activity. The low-voltage-activated, T-type/Cav3 channels are specifically activated by small membrane depolarization below the threshold of classical sodium action potentials, producing a Ca2+ entry near the resting membrane potential and low-threshold Ca2+ spikes (Huguenard, 1996, Huguenard, 1998, Perez-Reyes, 2003, Zamponi, 2016). Importantly, availability of Cav3 channels is critically regulated by the resting membrane potential to control T-type channel activity. Because of the negative range of their steady-state inactivation (V0.5 near -70 mV), Cav3 channels are partially inactivated (reduced availability) in the range of the resting membrane potential of most neurons and a membrane hyperpolarization (usually triggered by inhibitory postsynaptic events) is needed to allow their recovery from inactivation (de-inactivation) and their subsequent opening (Huguenard, 1996, Perez-Reyes, 2003, Zamponi, 2016). This behavior is of particular importance in many types of neurons, in which Cav3 channels mediate rebound burst firing, especially in the thalamo-cortical circuit, where Cav3 channels control transition between awake and sleep states (Huguenard, 1996, Perez-Reyes, 2003, Beenhakker and Huguenard, 2009, Zamponi, 2016, Tatsuki et al., 2016). Overall, Cav3 channels are involved in the control of the Ca2+ homeostasis (Chemin et al., 2000, Bijlenga et al., 2000, Perez-Reyes, 2003), in Ca2+-dependent differentiation of neuronal, muscular and neuroendocrine cells (Bijlenga et al., 2000, Mariot et al., 2002, Chemin et al., 2002b), as well as in Ca2+ overload toxicity in ischemia (Nikonenko et al., 2005, Bancila et al., 2011, Gouriou et al., 2013). Importantly, an increase in the Cav3 channel activity has been implicated in several diseases including epilepsy, chronic pain, autism and primary aldosteronism (Beenhakker and Huguenard, 2009, Zamponi, 2016). Although it is evident that a tight control of Cav3 channel activity is necessary to maintain Ca2+ homeostasis, there is no evidence yet that Cav3 channels are regulated by intracellular Ca2+ ions and/or by Ca2+ entry.

In this study, we have designed complementary electrophysiological experiments to explore whether the T-type/Cav3 channels are modulated by intracellular Ca2+ concentration. We document a feedback control mechanism that relies on Ca2+ entry via activated Cav3 channels or nearby Ca2+-permeable receptors. We provide evidence that dynamic changes and localized increase in the intracellular Ca2+ concentration at the vicinity Cav3 channels control availability of these channels, which underlies this novel regulation.

**Results**

***Inhibition of the native T-type and the recombinant Cav3 currents by ionomycin.***

In order to evaluate whether T-type channels would be regulated by a rise in intracellular Ca2+ ([Ca2+]INT), we first used the Ca2+ ionophore ionomycin. In D-hair mechanoreceptor sensory neurons, which specifically express a high density of T-type channels (Shin et al., 2003, Dubreuil et al., 2004, Voisin et al., 2016), extracellular perfusion of 10 µM ionomycin induced a potent decrease of the T-type current (Fig. 1a). This current inhibition (~ 85% in average) occurred in the minute range (Fig. 1b) and was associated with a large hyperpolarizing shift in the steady-state inactivation curve (~22 mV, *p<*0.001, Fig. 1c). Similar findings were obtained with the three cloned Cav3 channels transiently expressed in tsA-201 cells. Ionomycin potently inhibited the Cav3.1, Cav3.2 and Cav3.3 currents by about 80 % (Fig. 1d). This effect was combined with an acceleration of the inactivation kinetics (~3 times, Fig. 1e) and a hyperpolarizing shift in the steady-state inactivation curve (~23 mV, *p<*0.001, Fig. 1f).

***Inhibition of Cav3.3 current at high frequency of stimulation is caused by the Ca2+ entry.***

Considering that recombinant Cav3 channels, especially Cav3.3 channels, can generate large entry of Ca2+ during fast stimulation protocols (Huguenard, 1998, Kozlov et al., 1999, Chemin et al., 2002a, Perez-Reyes, 2003), we have investigated whether these channels might be modulated by their own activity. Cav3 currents were recorded in the presence of 2 mM extracellular Ca2+ using fast test-pulse (TP) stimulation (1 Hz), which allows a cumulative Ca2+ entry. Experiments were performed after dialyzing the cell with 10 mM EGTA, which delimits the change in Ca2+ concentration only at the vicinity of the channel (Marty and Neher, 1985, Roberts, 1993, Deisseroth et al., 1996). The Cav3.3 current amplitude recorded using fast TP stimulation (1 Hz) progressively decreased to ~50 % of the control value (Fig. 2a). In average, the current decrease was maximal and stable after ~ 40 s with an average half-time of 18 s (Fig. 2a and 2f). The current decrease was also associated with a marked acceleration of the inactivation kinetics (Fig. 2a), ~3 times after 40 s stimulation (Fig. 2e). This effect was fully reversible and activity-dependent since the stopping of the TP stimulation for only 5 s already induced ~30 % recovery of the current. Recovery was complete after ~2 min without TP stimulation with an average half-time of 20 s (Fig. 2f).

In contrast with the data obtained for a TP at -30 mV (Fig. 2a), the Cav3.3 current was unchanged when the TP was set at +100 mV (a membrane potential value above the reversal potential of Cav3 current, leading to an outward current, Fig. 2b). These experiments clearly indicate that the decrease in current amplitude observed for a fast TP stimulation at -30 mV does not involve a voltage-dependent inactivation process that would occur at high frequency of stimulation, but is rather related to the Ca2+ entry via Cav3.3 channels. Also, in the absence of extracellular Ca2+, the sodium inward current through Cav3.3 channels remained unchanged during the time of the fast TP protocol at -30 mV (Fig. 2c). In addition, no change in Cav3.3 current properties were obtained in the presence of 2 mM extracellular Ca2 when cells were dialyzed with an intracellular medium containing BAPTA instead of EGTA (Fig. 2d, 2e). This difference in susceptibility to BAPTA and EGTA is characteristic of a process driven by a localized rise in submembrane Ca2+, without the need for a global Ca2+ increase (Marty and Neher, 1985, Roberts, 1993, Deisseroth et al., 1996).

We further investigated whether Ca2+ ions could affect the Cav3.3 current in cell-free inside-out patches (Fig.2-figure supplement 1). Cav3.3 currents were recorded by voltage-ramps in the presence of 100 mM external Ba2+ whereas Ca2+-containing solutions were applied to the internal side of the membrane in the inside-out patch configuration (Fig.2-figure supplement 1a). Because of the surface charge effect due to the use of 100 mM Ba2+, voltage ramps were applied from a HP -50 mV to match whole-cell experiments (see also Fig. 4). In this configuration, the application of 1, 10 or 100 µM Ca2+ during more than 60 seconds (Fig.2-figure supplement 1b) did not induce a significant inhibition of the Cav3.3 current as compared to a control solution containing 1 mM EGTA / 0 mM Ca2+ (Fig.2-figure supplement 1c), suggesting that Ca2+-induced Cav3.3 inhibition requires some additional components preserved in the whole-cell configuration. However, our data do not exclude a possible direct effect of Ca2+ ions at higher (mM) concentrations.

The decrease in Cav3.3 current gradually developed with the increase in TP frequency. While no decrease was observed at low frequency of TP (0.033 Hz), the decrease in Cav3.3 current became significant at 0.2 Hz and further increased at 0.5 and 1 Hz (Fig. 2g). Similar experiments were conducted with the Cav3.1 and Cav3.2 T-type channels. The amplitude of Cav3.1 current decreased modestly and only at the TP frequency of 1 Hz (Fig. 2g and Fig.2-figure supplement 2a-b). Contrasting with the results described above, the Cav3.2 current showed no inhibition but rather a small increase in amplitude at fast stimulation (Fig. 2g and Fig.2-figure supplement 2d-e). For Cav3.1 channels, the decrease in current amplitude was associated with faster inactivation kinetics (Fig.2-figure supplement 2c), while inactivation kinetics of the Cav3.2 current was unchanged (Fig.2-figure supplement 2f). Similar to that described for the Cav3.3 current, Cav3.1 current amplitude and inactivation kinetics were unchanged after dialyzing the cells with BAPTA (Fig. 2g).

***Ca2+*** ***entry during fast TP stimulation induces a negative shift in the steady-state inactivation.***

We next investigated the biophysical mechanism underlying the Cav3.3 current decrease. We first hypothesized that the recovery from inactivation of the Cav3.3 current might be affected during a fast stimulation protocol in a Ca2+-sensitive manner. A paired-pulse protocol with increasing inter-pulse durations (100, 400 or 1000 ms) was designed to analyze the kinetics of recovery from the first (Stim 1) to the fortieth (Stim 40) paired-pulse stimulation, as exemplified for an inter-pulse duration of 100 ms in Fig. 3a. These experiments revealed that the recovery kinetics of the Cav3.3 current was unaffected at the three inter-pulse durations tested (Fig. 3b). In contrast, we found that the steady-state inactivation of Cav3.3 current was strongly modified using fast stimulation protocols. This was evidenced using a paired-pulse protocol with variable inter-pulse potentials ranging from -40 to -110 mV (as exemplified for an inter-pulse potential of -70 mV in Fig. 3c). This fast stimulation protocol produced a ~10 mV hyperpolarizing shift in the *V*0.5 value of the steady-state inactivation curve (from -72.4 mV for Stim 1 to -81.1 mV for the Stim 40, *p<*0.001), without any change in the slope of the inactivation curve (Fig. 3d). Importantly, this effect was lost in the presence of intracellular BAPTA (Fig. 3d). In addition, we found that the fast stimulation of Cav3.3 current also induced a small but significant leftward shift in the steady-state activation (from -52.6 to -55.9 mV, *p<*0.01) as well as an increase in the slope of the activation curve (from 4.7 to 5.6 mV, *p<*0.05, Fig. 3e-f).

Similar findings were obtained with Cav3.1 channels since these paired-pulse protocols revealed a ~5 mV hyperpolarizing shift of the steady-state inactivation curve (*p<*0.001, Fig.3-figure supplement 1a-b) with no significant effect on the recovery kinetics (Fig.3-figure supplement 1c) and the steady-state activation curve of the Cav3.1 current (Fig.3-figure supplement 1d).

***Ca2+-dependent inhibition of T-type channels is higher at physiological resting membrane potentials.***

These latter findings strongly suggest that the decrease in Cav3.3 current observed in fast stimulation protocols might be more important in the range of physiological resting membrane potentials ~ -70 / -80 mV, for which Cav3.3 channels are partially inactivated. In order to test this possibility, the Cav3.3 current properties before and after fast stimulation were modeled using the Hodgkin-Huxley formalism as previously described for the native T-type current in thalamic neuron (Huguenard and Prince, 1992, Destexhe et al., 1996). We used the NEURON simulation environment (Hines and Carnevale, 1997) as modified previously in order to perform voltage-clamp experiments (Destexhe et al., 1996). In this simulation, the model indicated that the Cav3.3 current elicited from HP -70 mV would decrease ~75 % (Fig. 4a) according to the shift in the steady-state inactivation observed in Fig. 3d. To validate experimentally these data, we performed voltage-clamp experiments at different HPs. Using 0.2 Hz frequency of TP stimulation (to allow current recovery at more depolarized HPs), the decrease in Cav3.3 current was below 20 % at HP -110 mV whereas the current decrease was more prominent (~40 %, *p<*0.01) at HP -80 mV (Fig. 4b). Interestingly, the decrease in Cav3.3 current was also reduced at HPs above -70 mV and was less than 10 % at HP -55 mV (Fig. 4c). This U-shaped relationship (Fig. 4c) could be explained by two interlinked mechanisms: i) at negative membrane potentials (below -90 mV) the shift in the steady-state inactivation curve has little impact on the decrease in current amplitude (see Fig. 3d) ; ii) the small decrease observed at HPs above -70 mV would be related to the reduced Ca2+ entry at depolarized membrane potentials.

To directly test this latter hypothesis, the Cav3.3 current was recorded using fast TP stimulation (1 Hz) at HP -100 mV and then immediately at HP -70 mV (Fig. 5a). At HP -100 mV during 40 s, the fast TP stimulation induced a large Ca2+ entry as evidenced by the decrease in Cav3.3 current amplitude (Fig. 5b, left panel). Then, when the HP was immediately switched to -70 mV (Fig. 5b, right panel), we observed a significant increase in the current amplitude, i.e. recovery, that reached a steady-state after ~100 s (Fig. 5b). The Cav3.3 current increased furthermore after the stopping of the simulation for 2 min (Fig. 5b). The average current amplitude increase during these experiments was ~400 % when using a 0.2 Hz TP stimulation, and up to 600 % at 1 Hz (Fig. 5d). Similarly, large effects on the Cav3.1 current were also found in these experiments (Fig. 5c). Whereas the Cav3.1 current decreased only modestly at HP -100 mV (~10 %, see also Fig. 2g), the increase in the Cav3.1 current following the switch to HP -70 mV reached ~600 % (Fig. 5c and 5d) as described for the Cav3.3 current. This strong recovery of Cav3.3 and Cav3.1 currents at depolarized HPs clearly indicate that the shift in the steady-state inactivation is a dynamic and reversible mechanism. Importantly, no increase in Cav3.3 and Cav3.1 current was obtained in cells dialyzed with BAPTA (Fig. 5d), further confirming a local Ca2+-dependent feedback mechanism.

Interestingly, we did not observe any variation in the Cav3.2 current in these experiments (Fig. 5d and Fig.5-figure supplement 1b). This finding suggests that because of its biophysical properties, i.e. rapid inactivation kinetics (as compared to Cav3.3) and its slow recovery from inactivation (as compared to Cav3.1 and Cav3.3) (Klockner et al., 1999, Kozlov et al., 1999, Satin and Cribbs, 2000, Chemin et al., 2002a, Perez-Reyes, 2003), the Cav3.2 current generated in fast TP stimulation does not allow sufficient Ca2+ entry to induce the Ca2+-dependent regulation observed for Cav3.1 and Cav3.3 channels. To directly test this hypothesis we have studied a Cav3.2 gain of function mutant at Met1549, recently identified in patients with hypertension due to primary aldosteronism (Scholl et al., 2015, Daniil et al., 2016). The Met1549Ile Cav3.2 mutant presents slower inactivation and deactivation kinetics and is expected to induce much larger Ca2+ entry than the wild-type channel (Daniil et al., 2016). We found that the Met1549Ile Cav3.2 current decreased by ~15 % during 1 Hz stimulation at HP -100 mV, whereas the current progressively increased when switched at HP -80 mV to reach a ~300 % increase (Fig.5-figure supplement 1c). Altogether, these results demonstrate a common Ca2+-dependent modulation mechanism for the three Cav3 currents, which depends mainly on the amount of the Ca2+ entry and on Cav3 biophysical properties.

***Ca2+-dependent inhibition of Cav3.3 current during action potential (AP) clamp stimulation.***

In order to investigate whether the Ca2+-dependent modulation of the T-type current occurred during more physiological paradigms, we recorded Cav3.3 current duringa voltage-clamp protocol mimicking thalamic neuronal activities, which was previously described in details (Chemin et al., 2002a). In these experiments, we found that the Cav3.3 current progressively increased during the first burst of spikes and then progressively decreased during the time course of the stimulation (Fig. 6a). In addition, a “rebound” in the Cav3.3 current was clearly associated with the depolarization after potential (DAP) transition, as previously described (Chemin et al., 2002a). We estimated the current increase as the ratio of the Cav3.3 current obtained at the fourth spike to the first one (Fig. 6b), whereas the decrease of the current was estimated as the ratio of the Cav3.3 current obtained at the thirteenth spike to the first one (Fig. 6c). Interestingly, the Cav3.3 current increase was similar when cells were dialyzed with an intracellular medium containing either EGTA or BAPTA and reached in average ~300 %, suggesting that the current increase is not dependent of intracellular Ca2+ (*p>*0.05, Fig. 6b). In contrast, the decrease in the Cav3.3 current was bigger in cells dialyzed with EGTA as compared to BAPTA-dialyzed cells (*p<*0.001, Fig. 6c). Importantly, these results were obtained during the first stimulation of the Cav3.3 current suggesting that the Ca2+-dependent modulation of the Cav3.3 current could have a strong neuronal impact. To further investigate the behavior of the Cav3.3 current during AP clamp experiments, we performed this stimulation several times at a frequency of 1 Hz whereas the cells were clamped at HP -100 mV between each stimulation to allow Cav3.3 current recovery (Fig. 6a). We found that the Cav3.3 current recorded during an AP as well as the “rebound” in the Cav3.3 current associated with the DAP progressively decreased when the protocol was repeated 40 times in cells dialyzed with EGTA (Fig. 1a). To account for the total Cav3.3 current variation, we calculated the integral of the Cav3.3 current at each stimulation (Fig. 6d). This analysis revealed that the total Cav3.3 current decrease was ~80 % in cells dialyzed with EGTA whereas the current decrease was less than 15 % in cells dialyzed with BAPTA (*p<*0.001, Fig. 6d), indicating further the robust Ca2+-dependent modulation of Cav3.3 current during AP-clamp stimulation.

***Activation of Ca2+-permeable ionotropic receptors also induces Cav3 current decrease.***

We next investigated whether activation of a Ca2+-permeable ionotropic receptor might also induce Cav3 current inhibition, including Cav3.2. In cells co-expressing the purinergic P2X4 receptor and Cav3.1, the Cav3.1 current was strongly decreased after extracellular perfusion of a solution containing the purinergic agonist ATP, which generated an inward current of several seconds (Fig. 7a). Similar results were obtained for Cav3.2 and Cav3.3 currents, and ATP caused in average ~80 % inhibition of the three Cav3 current (Fig. 7b). The decrease in Cav3 current induced by P2X4 activation was coupled to an acceleration of the current inactivation kinetics (Fig. 7c) and a negative shift in the steady-state inactivation (Fig. 7d). Importantly, these effects were absent when similar experiments were performed in the absence of extracellular Ca2+ (Fig. 7b). Because the current decrease could involve a change in the cell surface density of the channels, we have investigated whether the membrane expression of Cav3.3 channels would be modulated by P2X4 activation (Fig.7-figure supplement 1). To this end, we used a Cav3.3 channel construct containing an extracellular HA tag (Baumgart et al., 2008), which allows the measure of its cell surface expression by enzyme-linked immunosorbent assay/luminometry (Fig.7-figure supplement 1a). We found that the ATP treatment did not induce significant change in membrane expression of Cav3.3-HA channels in cells co-transfected with either the P2X4 receptor or the pcDNA3 plasmid (Fig.7-figure supplement 1b), suggesting that the current decrease did not involve a change in the cell surface density of the channels. To extend these findings to other classes of physiologically relevant ionotropic receptors, electrophysiological experiments were performed with the Ca2+-permeable NMDA, 5-HT3, TRPA1 and TRPV1 receptors (Fig. 7e). According to the results obtained with the P2X4 receptors, activation of all these receptors produced a 60 to 90 % decrease of Cav3.1, Cav3.2 and Cav3.3 currents and this effect was only observed in the presence of extracellular Ca2+ (*p<*0.001, Fig. 7e). This set of experiments further demonstrate that Ca2+ entry into cells controls a Ca2+-dependent modulation of Cav3 availability.

**Discussion**

This study reveals that T-type / Cav3 channels are dynamically regulated by changes in intracellular Ca2+ concentration. This novel regulation involves a Ca2+-dependent modulation of Cav3 availability. It was unraveled by demonstrating that a rise in submembrane Ca2+ at the vicinity of the Cav3 channels can cause a hyperpolarizing shift in the steady-state inactivation, leading to a strong Cav3 current decrease at physiological resting membrane potentials. This localized increase in intracellular Ca2+ can be generated by the Cav3 channel activity itself, especially for Cav3.3 channels, or by other routes of Ca2+ entry through the plasma membrane as obtained following activation of various Ca2+-permeable ionotropic receptors, all inducing a potent inhibition of Cav3.1, Cav3.2 and Cav3.3 currents. Importantly, all the effects described here were observed in 2 mM Ca2+-containing saline, which corresponds to the physiological range of extracellular Ca2+ concentration in native tissues (Jones and Keep, 1988), and were lost in the presence of intracellular BAPTA.

The Ca2+-dependent modulation revealed here was best evidenced with Cav3.3, which allows the largest Ca2+ entry among Cav3 channel family (Klockner et al., 1999, Kozlov et al., 1999, Chemin et al., 2002a, Perez-Reyes, 2003). The decrease in Cav3.3 current amplitude was well correlated with the increase in the frequency of the TP stimulation. The reduction in Cav3.3 current amplitude was ~50 % at 1 Hz frequency of stimulation and reached ~80 % during neuronal activities in action potential clamp, suggesting that intracellular Ca2+ could be an important physiological regulator of Cav3 current. Importantly, this effect was very dynamic both in its induction (T0.5 ~18 s) and in its recovery (T0.5 ~20 s). Altogether our results demonstrate that the Cav3 current decrease is linked to the cumulative Ca2+ entry through Cav3 channels. This Ca2+-dependent inhibition of Cav3 channels was observed only in the presence of external Ca2+ indicating that a voltage-dependent component in the mechanism is unlikely to contribute to the reported effect. Importantly, this Cav3 current inhibition induced by the Ca2+ entry is observed using intracellular EGTA but not anymore after the cell dialysis with BAPTA. These data reveal that this Ca2+-dependent modulation of Cav3 channels involves a localized increase in submembrane Ca2+ at the vicinity of the Cav3 channels without the need for a global Ca2+ increase (Marty and Neher, 1985, Roberts, 1993, Deisseroth et al., 1996). Interestingly, in the inside-out patch configuration, the direct application of Ca2+-containing solutions up to 100 µM to the internal side of the membrane did not produce the current inhibition, suggesting that this phenomenon requires some additional components preserved in the whole-cell configuration.

A key finding of this study is that the current inhibition is closely linked to the resting membrane potential (Vm; see the schematic representation in Fig. 7). On one hand, an increase in submembrane Ca2+ promotes a strong negative shift in the steady-state inactivation properties of T-type channels, leading to a more prominent inhibition of the Cav3 current at physiological Vm (~ -65/-85 mV; Fig. 7). On the other hand, the Ca2+ entry via T-type channels is maximal at negative Vm (~ -100 mV), but the consequence of the shift in the steady-state inactivation is minimized at negative Vm (Fig. 7). Consequently, the inhibition of the Cav3 current is maximal at physiological resting membrane potentials after stimulation at negative HP. Using this paradigm our data revealed an important finding: a wide range of modulation (~600 %) of both Cav3.3 and Cav3.1 currents, but not of Cav3.2 current which was resistant to this modulation.

The lack of Ca2+-dependent modulation of Cav3.2 channels using fast TP stimulation protocols could be explained by the specific electrophysiological properties of this Cav3 isoform. It is expected that the Cav3.2 current would generate little cumulative Ca2+ entry because of its rapid inactivation kinetics combined with its slow recovery from inactivation (Klockner et al., 1999, Kozlov et al., 1999, Satin and Cribbs, 2000, Chemin et al., 2002a, Perez-Reyes, 2003). Interestingly, the Met1549Ile gain-of-function mutant of the Cav3.2 channel that displays very slow inactivation and deactivation kinetics (Scholl et al., 2015, Daniil et al., 2016), exhibits a significant Ca2+-dependent inhibition and a wide range of modulation (~300 %). Collectively, our data indicate that the Ca2+-dependent mechanism described here can modulate all three Cav3 isoforms. Overall, the amplitude of the modulation (Cav3.3 > Cav3.1 >> Cav3.2 ~ 0) critically depends on the amount of the Ca2+ entry and therefore relies on the biophysical properties of each Cav3 isoform.

The large Ca2+ entry generated by the activation of ionotropic receptors induces a strong inhibition (~85 %) of all three Cav3 currents, including wild-type Cav3.2. We observed this effect with a variety of Ca2+-permeable ionotropic receptors, including the purinergic P2X4, the glutamatergic NMDA, the serotoninergic 5-HT3 and the TRP (TRPV1 and TRPA1) receptors. Notably, no inhibition of the T-type current is observed when these experiments are performed in the absence of extracellular Ca2+, demonstrating the critical role played by the Ca2+ entry in this mechanism of Cav3 inhibition. It is noteworthy that the tsA-201 cells, which derived from HEK-293 cells, endogenously express another class of purinergic receptors, P2Y, which are Gq-coupled receptors and induce a global increase in intracellular Ca2+ in response to ATP (Chemin et al., 2000, Fischer et al., 2005). Interestingly, we observed no change Cav3 current properties in pcDNA3 transfected cells (Fig. 6b) indicating that Cav3 currents are not inhibited in P2X untransfected cells following ATP application. These data suggest that activation of P2Y receptors is unable to mediate a Ca2+-dependent modulation of Cav3 currents further supporting a membrane-delimited mechanism for the Ca2+-dependent modulation of Cav3 channels.

Since the initial discovery of T-type currents, it was admitted that these channels were not regulated by intracellular Ca2+ ions or by changes in intracellular Ca2+ concentration because they do not present CDI (Carbone and Lux, 1984, Fedulova et al., 1985, Bean, 1985, Bossu et al., 1985, Bossu and Feltz, 1986, Dupont et al., 1986). More recently, these findings were confirmed using cloned T-type channels (Staes et al., 2001, McRory et al., 2001, Perez-Reyes, 2003) but see (Lacinova et al., 2006). Indeed, our data showing that the inactivation kinetics are similar at the beginning of the stimulation obtained in external Ca2+ and in external sodium (Fig. 2e) also support the lack of CDI for Cav3 channels. Accordingly, the important structural motifs for CDI present on L-type channels, as the Ca2+ (EF Hand) and the calmodulin (IQ motif) binding sites are absent on the C-terminus of Cav3 channels (Staes et al., 2001, McRory et al., 2001, Perez-Reyes, 2003). Therefore, CDI was considered in pioneer studies as a hallmark to distinguish between L-type and T-type Ca2+ currents (Carbone and Lux, 1984, Fedulova et al., 1985, Bean, 1985, Bossu et al., 1985, Bossu and Feltz, 1986, Dupont et al., 1986). Increasing the intracellular Ca2+ concentration [Ca2+]INT from 10-10 to 10-7 M or even to 10-6 M was classically used to isolate a "pure" T-type current, which presented no change in its inactivation kinetics, whereas the L-type current disappeared because of the acceleration of its "run-down" (Bossu et al., 1985, Bossu and Feltz, 1986, Dupont et al., 1986). Interestingly, in these seminal studies, the native T-type current was mostly related to Cav3.2 (nickel-sensitive) channels (Carbone and Lux, 1984, Fedulova et al., 1985, Bean, 1985, Bossu et al., 1985, Bossu and Feltz, 1986, Dupont et al., 1986), for which we show that the intrinsic electrophysiological properties do not allow the triggering of the Ca2+-dependent current inhibition, contrary to Cav3.1 and Cav3.3 channels. However, and consistent with our present findings, these early studies performed in the presence of an increasing amount of [Ca2+]INT have reported important features of the T-type current: (1) at 10-8 M, the T-type current was stable during 60 minutes whereas at 10-7 M the T-type current decreased and was suppressed after 10-15 minutes (Bossu et al., 1985); (2) at 10-7 M, an hyperpolarizing shift (~10 mV) of the steady-state inactivation occurred (Bossu and Feltz, 1986); and (3) at 10-6 M no T-type current was recorded at HPs above -100 mV (Dupont et al., 1986). Altogether, these historical results and our present data are in favor of a ‘minute scale’ Ca2+-dependent modulation of the T-type current, which induces a hyperpolarizing shift in the steady-state inactivation and consequently a decrease in T-type current amplitude (see Fig. 7).

It is also important to depict our results in the light of recent findings obtained on L-type channels using high frequency of stimulation (Oliveria et al., 2007, Oliveria et al., 2012). In 15 mM external Ca2+, the Cav1.2 current dropped to 40 % of the control amplitude during 1 Hz stimulation and this inhibition was abolished in the presence of intracellular BAPTA (Oliveria et al., 2007, Oliveria et al., 2012). The Cav1.2 current decrease was stable after 3-5 minutes, similar to that obtained with 40-50 seconds stimulation for Cav3.3 in the presence of the physiological 2 mM Ca2+ concentration, and the recovery was almost total after 4-5 minutes. However, contrary to Cav3 currents, the decrease in the Cav1.2 current stimulated at 1 Hz did not involve a shift in its steady-state inactivation (Oliveria et al., 2007, Oliveria et al., 2012), suggesting distinct mechanisms for the L-type and the T-type current modulation. In addition, a prolonged stimulation of Cav1.2 current (in 10 mM external Ca2+ saline) induced channel endocytosis (Green et al., 2007, Tsuruta et al., 2009, Hall et al., 2013) and, in this case, the recovery of the L-type current took approximatively 30 minutes (Green et al., 2007). This Ca2+-dependent modulation of L-type channels appears distinct of that described here for Cav3 channels, both regarding the time of the recovery and the shift in the steady-state inactivation. In addition, we found that ATP treatment in cells expressing both the P2X4 receptors and the Cav3.3 channels did not induce significant changes in membrane expression of Cav3.3, suggesting that the current decrease did not involve an endocytosis mechanism. Therefore, although the L- and the T-type channel regulation share apparent similar properties, modulation of the T-type current by cumulative Ca2+ entry has unique features, depending mostly on the shift of the steady-state inactivation, i.e. the modulation of Cav3 availability.

The discovery of a Ca2+-driven feedback regulation of T-type channels may have important physiological and pathophysiological implications. Indeed, an increase in the activity of T-type channels have been implicated in several diseases linked to altered Ca2+ signaling (Orestes et al., 2013, Jagodic et al., 2007, Scholl et al., 2015, Zamponi, 2016, Daniil et al., 2016) and T-type channel activity is also linked to Ca2+ overload toxicity occurring in ischemia (Nikonenko et al., 2005, Bancila et al., 2011, Gouriou et al., 2013). Also, our study reveals that activation of Ca2+-permeable ionotropic receptors could also markedly inhibit T-type channel activity, and interestingly, cross-talk between these receptors and T-type channels have been recently observed (Comunanza et al., 2011, Kerckhove et al., 2014, Wang et al., 2015, Tatsuki et al., 2016).

In summary, we have identified a novel regulation pathway for T-type Ca2+ channels. By demonstrating that Ca2+ entry exerts a feedback control on T-type channel activity, our study opens up new horizons towards deciphering how this local and dynamic Ca2+-dependent modulation of Cav3 channels can impact the cellular and physiological roles of T-type channels in normal and disease states.

**Methods.**

*Cell culture and transfection protocols.*

tsA-201 cells (RRID:CVCL\_2737) were obtained from the European Collection of Authenticated Cell Cultures (ECACC 96121229). The identity of tsA201 has been confirmed by STR profiling and the cells have been eradicated from mycoplasma at ECACC. We routinely tested the cells for the absence of the mycoplasma contamination. Cells were cultivated in DMEM supplemented with GlutaMax, 10 % fetal bovine serum and 1% penicillin / streptomycin (Invitrogen). Transfections were performed using jet-PEI (QBiogen) with a DNA mix (1.5 µg total) containing 0.5 % of a GFP encoding plasmid and 99.5 % of either of the plasmids ( pcDNA3.1) that code for the human Cav3.1a, Cav3.2, Cav3.3 and Met1549Ile Cav3.2 constructs. In experiments with ionotropic receptors, 1 µg of either of the plasmid constructs that code for human P2X4, mouse 5-HT3, human TRPV1, mouse TRPA1 and rat NMDA receptor (NR1A and NR2A (0.5 µg each)) were added to the DNA mix. Two days after transfection, tsA-201 cells were dissociated with Versene (Invitrogen) and plated at a density of ~35×103 cells per 35 mm Petri dish for electrophysiological recordings, which were performed the following day.

*DRG neurons*

All animal use procedures were done in accordance with the directives of the French Ministry of Agriculture (A 34-172-41). Dorsal root ganglion (DRG) neurons were prepared as described earlier (Voisin et al., 2016). Briefly, adult male C57BL/6J mice were anaesthetized with pentobarbital injection and transcardially perfused with HBSS (pH 7.4, 4°C). Lumbar DRGs with attached roots were dissected and collected in cold HBSS supplemented with 5 mM HEPES, 10 mM D-glucose and 1 % penicillin/streptomycin. DRGs were treated with 2 mg/ml collagenase II and 5 mg/ml dispase for 40 minutes at 37°C, washed in HBSS and resuspended in 1 ml of neurobasal A medium supplemented with B27, 2 mM L-glutamine and 1 % penicillin/streptomycin (Invitrogen). Single-cell suspensions were obtained by 5 passages through 3 needle tips of decreasing diameter (gauge 18, 21, and 26). Cells were plated onto polyornithine/laminin-coated dishes. After 2 hours, the medium was removed and replaced with neurobasal B27 supplemented with 10 ng/ml neurotrophin 4 (NT4) and 2 ng/ml glial derived neurotrophic factor (GDNF). Patch clamp recordings were performed 6-24h after plating on neurons with a ‘‘rosette’’ morphology corresponding to D-hair neurons that express large density of T-type current (Dubreuil et al., 2004, Voisin et al., 2016).

*Electrophysiological recordings*

Macroscopic currents were recorded at room temperature using an Axopatch 200B amplifier (Molecular Devices). Borosilicate glass pipettes had a resistance of 1.5–2.5 MOhm when filled with an internal solution containing (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 3 Mg-ATP, 0.6 GTPNa, and 3 CaCl2 (pH adjusted to 7.25 with KOH, ~315 mOsm, ~100 nM free Ca2+ using the MaxChelator software, http://maxchelator.stanford.edu/). Similar results were obtained using either 10 mM or 20 mM EGTA. In some experiments, BAPTA (20 mM) was substituted with EGTA. The extracellular solution contained (in mM): 135 NaCl, 20 TEACl, 2 CaCl2, 1 MgCl2, and 10 HEPES (pH adjusted to 7.25 with KOH, ~330 mOsm). To avoid inhibition of 5-HT3 and NMDA receptors, NaCl was substituted with TEACl and MgCl2 was also omitted in NMDA experiments. In the cell-free inside-out patch experiments the intrapipette solution contained 100 mM BaCl2 and 10 mM HEPES (pH adjusted to 7.25 with NaOH, ~310 mOsm) and the bath solution contained (in mM): 145 KCl, 10 HEPES and 1 MgCl2 (pH adjusted to 7.25 with KOH, ~305 mOsm). In the inside-out configuration the patch was perfused with the bath solution supplemented with either 1 mM EGTA or increasing concentration of CaCl2 (1, 10 and 100 µM). For D-hair neuron recordings, the bath solution contained (in mM): 140 NaCl, 10 HEPES, 5 KCl, 2 CaCl2, 1 MgCl2 and 10 glucose (pH adjusted to 7.25 with NaOH, ~330 mOsm) and cells were perfused with an extracellular solution containing (in mM): 140 TEACl, 10 HEPES, 5 KCl, 2 NaCl, 2 CaCl2, 1 MgCl2 and 10 glucose (pH adjusted to 7.25 with TEAOH, ~330 mOsm). Recordings were filtered at 2 kHz. Steady-state inactivation curves were fitted using the Boltzmann equation where I/I max=1/(1+exp((Vm−V0.5)/slope factor)). Data were analyzed using pCLAMP9 (Molecular Devices) and GraphPad Prism (GraphPad) softwares. Results are presented as the mean ± SEM, and n is the number of cells. Statistical analysis were performed with the Student *t*-test or with one-way ANOVA combined with a Tukey post-test for multiple comparisons (\* *p*<0.05, \*\* *p*<0.01, \*\*\* *p*<0.001).

*Modelling.*

Modelling was performed using the NEURON simulation environment (Hines and Carnevale, 1997). The model was modified to simulate voltage-clamp experiments in thalamic reticular neurons (Destexhe et al., 1996) (as available from the model database at Yale University (https://senselab.med.yale.edu/modeldb/). The electrophysiological properties of the Cav3.3 channels were modelled using Hodgkin-Huxley equations as described previously (Huguenard and Prince, 1992, Destexhe et al., 1996). The values obtained for Cav3.3 were substituted for the corresponding values of native T-channels in thalamic reticular neurons (Huguenard and Prince, 1992), as previously described (Chemin et al., 2002a). To match the voltage clamp data, the modelling experiments were performed at 24 °C.

The equations to model the Cav3.3 current properties at rest were:

m\_inf = 1/(1+exp(-(v+52.6)/4.7))

h\_inf = 1/(1+exp((v+72.4)/5.7))

tau\_m = (1.377+1.512/(exp((v+12.52)/14.38)+exp(-(v+81.59)/5)))

tau\_h = (65.34+1/( exp((v+41.04)/4.01)+exp(-(v+333.1)/46.86)))

The equations to model the Cav3.3 current properties after 40 s stimulation at 1 Hz frequency were:

m\_inf = 1/(1+exp(-(v+55.9)/5.6))

h\_inf = 1/(1+exp((v+81.1)/5))

tau\_m = (1.141+0.9592/( exp((v+14.95)/13.97)+exp(-(v+81.53)/5)))

tau\_h = (26.55+0.66/( exp((v+32.42)/6.4)+exp(-(v+225)/22.21)))

*Luminometric Analysis of HA-tagged Cav3.3 Channels*.

The membrane expression of the Cav3.3 channel was quantified as previously described (Chemin et al., 2007). The tsA-201 cells were cultured in 24-well plates and co-transfected with a Cav3.3-HA construct (Baumgart et al., 2008) and either the P2X4 receptor or the pcDNA3 plasmid (ratio 1:1). Two days after transfection, ATP treatments were performed as in the electrophysiological experiments. The cells were washed with the electrophysiological extracellular solution containing 3 µM ivermectin and afterward 10 µM ATP was applied for 30-45 seconds at room temperature. Then the cells were directly fixed for 5 min in PBS containing 4% paraformaldehyde. After three PBS washes, the cells were incubated for 30 min in blocking solution (PBS plus 1% fetal bovine serum). The Cav3.3-HA protein was detected using a rat anti-HA monoclonal antibody (1:1000 dilution; clone 3F10, Roche Diagnostics) after incubation for 1 hour at room temperature. After four washes with PBS plus 1% fetal bovine serum for 10 min, cells were incubated for 30 min with horseradish peroxidase-conjugated goat anti-rat secondary antibody (1:1000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were rinsed four times with PBS for 10 min before addition of SuperSignal enzyme-linked immunosorbent assay Femto maximum sensitivity substrate (Pierce). Luminescence was measured using a VICTOR2 luminometer (PerkinElmer Life Sciences), and the protein amount in each well was then measured using the BCA assay (Pierce) to normalize the measurements. All data were normalized to the level of signal obtained in P2X4 transfected cells without the ATP treatment. Each experiment was performed in quadruplicate and three independent sets of transfection experiments were performed under each condition. The results are presented as the mean ± SEM.

*Chemical reagents*

Compounds were purchased from Sigma. To activate ionotropic receptors, we used 10 µM ATP in the presence of 3 µM ivermectin for P2X4, 100 µM glutamate in the presence of 100 µM glycine for NMDA, 10 µM serotonin for 5-HT3, 0.5 µM capsaicin for TRPV1 and 100 µM allyl isothiocyanate for TRPA1. Drugs were applied using a gravity-driven homemade perfusion device and control experiments were performed similarly using the vehicle alone.

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