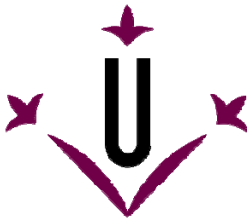

TITLE:
The hard-to-close window of T-type calcium channels

SHORT TITLE: Targeting T-type channels

Keywords: T-type calcium channels, pharmacological block, window currents, cancer cells

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ABSTRACT

T-type Calcium channels (TTCCs) are key regulators of membrane excitability, reason why TTCC pharmacology is subject to intensive research in the neurological and cardiovascular fields. TTCC also play a role in cancer physiology, and pharmacological blockers such as tetralols and dihydroquinazolines reduce the viability of cancer cells *in vitro* and slow tumor growth in murine xenografts. However, available compounds are better suited to block TTCC in excitable membranes rather than TTCC contributing *window currents* at steady potentials. Consistently, tetralols and dihydroquinazolines exhibit cytostatic/cytotoxic activities at higher concentrations than those required for TTCC block, which may involve off-target effects. Gene silencing experiments highlight the targetability of TTCC, but further pharmacological research is required for TTCC block to become a chemotherapeutic option.

Physiological and pathophysiological roles of TTCC

Voltage-gated Ca^{2+} channels (**Ca_v** channels, see Glossary) constitute the only biological transducers of electrical into biochemical signals (cytosolic Ca^{2+} elevations) that initiate multiple physiological events. There are 10 members of the Ca_v family in mammals, that are classified into 3 subfamilies based on sequence homology: Ca_v1, Ca_v2 and Ca_v3 [1]. In a simplified view, Ca_v channels can adopt 3 conformational states: resting-deactivated (closed and sensitive to depolarizing stimuli), activated (open) and inactivated (non-conducting and refractory to depolarizing stimuli) [2]. Forward/backward transitions between these states (called **gating**) are time- and voltage- or state-dependent **(Figure 1A, B).** By virtue of their distinctive

biophysical properties, i.e. activation near resting membrane potentials (low voltage-activation, **LVA**) and slow deactivation kinetics, the 3 members of the Ca_v3 subfamily (T-type Ca^{2+} channels, TTCC) are suited for amplifying small depolarizations of the membrane and mediating Ca^{2+} oscillations [3][4][5]. Indeed, the expression of TTCC was initially related to membrane excitability, such as neuron oscillatory firing [6][7] and cardiac pacemaker potentials [8]. More recently, abnormalities in TTCC function or expression levels in neurons have been linked to tremor [9], absence seizures [10], spinocerebellar ataxia [11][12][13], different types of epilepsies [14] and visceral or neuropathic pain pathophysiology [15][16][17][18][19]. Correspondingly, pharmacological targeting of TTCC has shown effectiveness in preclinical models of pain (reviewed in [20]) and epilepsy (reviewed in [14]), by reducing hyperexcitability. Nonetheless, TTCC provide a key pathway for Ca^{2+} entry near the resting membrane potential, and display an augmented expression during the G1-S transition in non-excitable cells [21][22][23][24][25][26][27]. During this period both the availability of TTCC and the driving force for Ca^{2+} entry are also expected to increase, because the plasma membrane is hyperpolarized in correspondence with the overexpression of different types of K^+ channels [28]. The existence of a sustained Ca^{2+} influx through voltage-gated channels relies on the overlap between **steady-state activation** and **inactivation**, a phenomenon known as **window currents (Figure 1C)**. Window currents are estimated to be contributed by ~1 % of TTCC present in the membrane, involving a low-level Ca^{2+} entry which may be physiologically relevant because TTCC are strategically located in microdomains and associated with signaling complexes [29][30]. Cancer cells viability depends on TTCC function [31][32][33][34], reason why TTCC pharmacological block is a tentative chemotherapeutic strategy (see Clinician Corner). TTCC "hyperactivation" has been attempted as an alternative approach to dysregulate Ca^{2+} homeostasis/signaling in cancer cells. The green tea polyphenol epigallocathechin-3-gallate (EGCG) proved cytotoxic to cultured mesothelioma [35] and breast cancer [36] cells by a mechanism involving reactive oxygen species (ROS) production and subsequent activation of $Ca_v3.2$ channels. However, at the concentrations used in these studies EGCG is known to target several proteins involved in tumor progression [37], so that currently TTCC block remains the strategy of choice from the viewpoint of specificity.

Block of voltage-gated channels is often state-dependent

Different electrophysiological protocols have been developed to study the pharmacological interaction and blocking mechanism of several **Na_v** and Ca_v channel blockers, which are determinant for their therapeutic profiles. The term **use-dependent block** refers to the positive correlation between frequency of stimulation (i.e. membrane depolarizing stimuli) and degree of block, thus it is suggestive of drug binding to open

and/or inactivated channels. The desired selectivity of local anesthetics, antiarrhythmic and anticonvulsant drugs for excitable membranes is based on their preferential interaction with Na_v (or Ca_v) channels in these gating states, which are promoted by repetitive firing of action potentials [38]. On the contrary, *tonic block* is experimentally assessed by low-frequency stimulation and is attributable to binding to resting channels. Additional criteria have been proposed to evaluate the **state-dependence** of block: increased blocking potency at depolarized **holding potentials**, slowing of recovery from inactivation, and hyperpolarizing shift of the steady-state inactivation curve, have been interpreted as preferential binding and selective stabilization of the inactivated states [39]. Based on these and other electrophysiological procedures, most tested Na_v/Ca_v blockers have shown to display a higher affinity for channels in their resting or inactivated states from the perspective of the **modulated receptor theory** [40][41]. This feature is likely to hinder the block of the fraction of channels contributing window currents, which are permanently open.

The issue of selectivity

Na^+ and Ca^{2+} channel inhibitors have long been applied in clinical practice to tackle diverse cardiovascular and neurological conditions. A number of dihydropyridines, phenylalkylamines and benzodiazepines are used as antihypertensives and antiarrythmics because they promote vasodilation and/or negative inotropic or chronotropic effects. Albeit initially these compounds were regarded as selective for Na_v or L-type Ca_v channels (**LTCC**), eventually interactions with different ion channels including TTCC were described for many of them [42]. Similarly, diphenylbutylpiperidines, diphenylpiperazines, succinimides and trimethiadones, originally used as neuroleptics/antiepileptics based on dopamine receptor or Na_v antagonism, were later reported to exert TTCC block [43]. Arachnid neurotoxins [44][45] and endogenous modulators such as endocannabinoids were also found to target TTCC among other channels [46]. In the last 10 years the arsenal of TTCC modulators has been extended with the development of new agents pursuing TTCC selective block, mainly in the context of pain research [47][48][49]. However, all voltage-gated channels are traced back a common ancestor and display structural homology, and the finding of *bona fide* TTCC (or TTCC-isoform) selective blockers is challenging.

TTCC blockers are cytotoxic to cancer cells at supramaximal concentrations

Most widely used TTCC blockers in cancer research are membrane-permeable benzimidazolyl-substituted tetraline derivatives of phenylalkylamines (tetralols), dihydroquinazoline derivatives (DHQ) and diphenylbutylpiperidine pimozide. Some of these compounds have been tested in preclinical *in vivo* models, in which have shown to slow tumor growth (see [50] for a recent review).

Mibefradil was released in 1997 as a long-acting Ca^{2+} channel blocker for the management of ischemia, angina and cardiac arrhythmias. Its protective actions and lack of negative inotropic effects were attributed to a selectivity for TTCC over LTCC [51][52][53][54]. Analogues of mibefradil have been synthetized by replacing a methoxyacetyl esther with more lipophilic groups, in order to limit cytoplasmic hydrolysis, and NNC-55-0396 was distinguished by its potent and selective TTCC blocking properties [24][55].

Both mibefradil and NNC-55-0396 exert a poorly reversible block consistent with a high affinity binding to the inactivated state, and it is suggested that the drug binding site/s reside within transmembrane domains [56][57][58][59][60]. A slow onset seems to be behind notable discrepancies for reported blocking potencies [55]. Other reasons for discrepant inhibitory concentration 50 (**IC_{50}**) values are the variable voltage protocols and recording conditions used for measuring TTCC currents, such that blockade is enhanced at depolarized holding potentials and low Ca^{2+} solutions [58]. Potency is also affected by temperature, such that the IC_{50} for mibefradil on $Ca_v3.2$ channels expressed in HEK293 cells increased up to 5-fold at 35°C compared to room temperature, from 139 to 792 nM [58]. Assuming this variability, significantly lower IC_{50} values on TTCC compared to the effective/growth inhibition concentrations (**EC_{50}/GI_{50}**) against cancer cells, can still be observed for tetralols **(Table 1)**. In excitable membranes, gating of TTCC occurs with action potential firing, and TTCC experience all possible conformational states, so that high-affinity drug binding to the non-conducting states will prevent channel opening most efficiently. However, at steady, depolarized potentials typical of cancer cells [61], the only TTCC achieving the open state are a small fraction of inactivation-resistant channels contributing window currents, for which drug potency is reduced **(Figure 1)**. In line with this rationale, phasic use-dependent block of $Ca_v3.2$ channels by mibefradil was considerably more potent than tonic block [60][62]. Similarly, block of $Ca_v3.1$ by NNC-55-0396 demonstrated partial relief at hyperpolarized potentials and was enhanced by increasing the frequency of stimulation [60]. In fact, the influence of tetralols on the magnitude of window currents is enigmatic. Gomora et al. showed that in adrenocortical cells, mibefradil shifted the voltage-dependence of steady-state inactivation of TTCC by -5.7 mV, without changing the voltage-dependence of channel opening [63], an effect that would minimize window currents. Nonetheless, the steady-state inactivation of TTCC currents recorded in DRG neurons [64] and in HEK293 expressing $Ca_v3.2$ channels [55][65] was flattened by mibefradil and NNC-55-0386, respectively, increasing the area of overlap with activation voltage ranges. Calculation of channel availability as the product of steady-state inactivation and activation curves, shows that increasing the slope factor for steady-state inactivation may counteract a blocking effect in the voltage range for window currents **(Box 1)**.

In 2004 Lee et al. evaluated the blocking properties of a series of 3,4-DHQ against recombinant $Ca_v3.1$ and $Ca_v3.2$ channels, and one of the compounds was disclosed as equipotent and more selective when compared with mibefradil [66]. This work was followed by structure-activity relationship studies that led to the synthesis of other DHQ able to block TTCC, which demonstrated acute toxicity on cultured cancer cells at supramaximal concentrations [67][68][69]. In the latter work, KYS05090 had an additional analgesic effect in acetic acid-induced writhing test, that was further proved in mouse models of neuropathic and neuroinflammatory pain [70]. These studies were further extended with the testing of new DHQ in ovarian cancer [71] and lung adenocarcinoma cells [72]. Again, the concentrations required for channel inhibition and cytotoxic effects were discrepant, with IC_{50} values 4-30 fold smaller than GI_{50} values **(Table 1)**. The scant information available for DHQ points nonetheless to a use-dependent block of TTCC, similarly to tetralols. KYS0590 and a series of fluoro-substituted DHQ shifted the voltage-dependence of inactivation of recombinant $Ca_v3.2$ channels toward negative potentials, suggesting the interaction with the inactivated states [70][73].

Anti-psychotic drug pimozide has shown tumoricidal activity in a wide rage of cancer cells (at μM concentrations) and in mouse xenografts, but its actions are principally attributed to inhibition of dopamine, serotonin, epinephrine and sigma receptors (reviewed in [74]). With regards to TTCC, pimozide and analogs exert a potent state-dependent block on the three isoforms (at nM concentrations), indicative of a preferential binding to the inactivated states [43], similarly to tetralols and DHQ.

On-target vs off-target effects of TTCC blockers on cancer cells viability

From the perspective of TTCC contribution to G1-S progression, it would be expected that TTCC block would reduce cancer cell viability by decreasing cytosolic Ca^{2+} oscillations/concentration. However, anticancer effects of tetralols and DHQ include dysregulation of key homeostatic processes and induction of caspase-dependent apoptosis **(Table 2)**, for which on-target effects are not obvious.

Analysis of cytotoxic effects becomes *de facto* complex when considering that higher drug concentrations associate to a higher risk of off-targeting. Notorious examples are the reported membrane potential shifts, hardly owed to TTCC block. Nilius et al. described that mibefradil at 10 μM hyperpolarized the membrane potential of calf pulmonary artery endothelial cells by ~13 mV, an effect that was attributed to blockade of Ca^{2+}-dependent Cl^- channels [75]. In contrast, 10 μM mibefradil depolarized the plasma membrane of smooth muscle cells [76], human lens epithelial cells [77] and glioma initiating cells [78]. At μM concentrations mibefradil has been shown to block different types of voltage-gated Ca^{2+} channels [79][80][60] and K^+ channels [81][82][83][84][85][86][87], including oncogenic $\mathbf{K_v}10.1$ channels [88], and to

activate K(Ca) channels [83] and TRPM7 channels [89]. Of note, K^+ channels block or cation-permeable TRPM7 channels activation would depolarize membrane potential, whereas activation of K(Ca) channels would exert a hyperpolarizing effect. We can envisage two different scenarios for Ca^{2+} entry depending on tetralol influence on membrane potential: (1) hyperpolarization would involve increased TTCC window currents in most cancer cells (holding membrane potentials > -40 mV), and increased driving force for Ca^{2+} entry; (2) depolarization would result in reduction of TTCC window currents, but also increased Ca^{2+} entry through activation of high voltage-activated (**HVA**) Ca^{2+} channels **(Box 2)**. HVA Ca_v channels, shown to contribute window currents in neurons [90], are widely expressed in cancer cells [91]. Membrane depolarization may also negatively affect the electrochemical gradient necessary to extrude Ca^{2+} by the Na^+/Ca^{2+} antiporter, thereby limiting Ca^{2+} export [78]. Indeed, a clear picture is emerging that the biochemical pathways and cellular processes triggered by exposure to tetralols are consistent with elevations of cytosolic Ca^{2+} **(Table 2)**. Other off-target effects point to this direction: in the early years after mibefradil synthesis, Eberhard et al. described that at concentrations above 10 μM mibefradil mobilized Ca^{2+} from inositol-3-phosphate-sensitive intracellular Ca^{2+} stores in rat cardiac fibroblasts and human platelets [92]. More recently, mibefradil or NNC-55-0396 exposure increased intracellular Ca^{2+} levels by inducing ER calcium release in Jurkat leukaemic T cells [93]. It should be noted that Ca^{2+}-dependent pathways may promote cell proliferation and/or survival, instead of cell death [94][95][96].

Targets other than TTCC have not been thoroughly investigated for DHQ, beyond reports of a generally less potent inhibitory action on N-type $Ca_v2.2$ channels exerted by some derivatives [72][73][97], although the mechanisms involved in the reduction of cell viability appear to be different from tetralols **(Table 2)**. Rim et al. concluded that supramaximal concentrations of KYS05047 induced G1 phase cell cycle arrest by decreasing intracellular Ca^{2+} levels [98]. In contrast, KYS05090 led to autophagy and apoptosis of A549 cells through generation of ROS, by inhibiting glucose uptake [99]. Events upstream of oxidative stress were not investigated but, intriguingly, the authors observed that, although KYS05090 progressively decreased cytosolic Ca^{2+} levels in a time span of 150 min, this feat was not directly related with KYS05090-induced cell death.

Effects of gene knockout/knockdown

Gene silencing has demonstrated the specificity of TTCC pharmacological block in mice models of neurological disorders. Consistently, amide TTA-A2 promoted slow-wave sleep in wild-type mice but not in mice lacking $Ca_v3.1$ and $Ca_v3.3$ [100], did not prevent tonic seizures in $Ca_v3.1$ knockout (KO) mice [101], and its antinociceptive effect on formalin-induced mechanical hypersensitivity was lost in $Ca_v3.2$ KO mice [102].

Similarly, the analgesic action of DHQ KYS05090 was ablated in $Ca_v3.2$ null-mice [70], whereas the administration of antisense oligonucleotides against $Ca_v3.2$ abrogated the antihyperalgesic effects of piperidine TTA-P2 [48] and of mibefradil [55] in diabetic rats, in tune with a prominent role for this isoform in nociception.

Similar approaches have not been attempted in cancer research. Sparse data indicates that *in vitro* knockdown of TTCC leads to cell cycle arrest and/or apoptosis of cancer cells, although the molecular pathways linking TTCC downregulation and viability are ill-defined [103][104][105]. Nevertheless, gene overexpression/silencing experiments point to the existence of functional differences between Ca_v3 isoforms [106], already inferred from the observation of their differential (in some cases complementary) expression in various cells/tissues [50].

Concluding Remarks

TTCC pharmacological targeting is being intensively investigated in the neurological and cancer fields. Most blockers target preferentially TTCC in their non-conducting states, and thus appear well-suited to tackle TTCC contribution towards (hyper)excitability. In contrast, in cancer cells, TTCC currents are contributed by a small population of non-inactivating TTCC, for which currently used blockers display little affinity. Off-target effects are the price to be paid for low potency block, and they should be thoroughly investigated in order to dissect the fundamental functions of TTCC in cancer (see Outstanding Questions). Mibefradil, for which clinical trials have been conducted in Glioblastoma Multiforme (**GBM**) patients, induces membrane potential shifts liable to affect Ca^{2+} fluxes through voltage-dependent and independent channels. Reported off-target effects also include Ca^{2+} mobilization, bound to deregulate multifaceted cellular processes like the *unfolded protein response* or macroautophagy [107]. Available data suggests that mibefradil may, in fact, induce a paradoxical activation of Ca^{2+}-dependent pathways/cell processes that have negative consequences for the viability of cancer cells *in vitro* and in murine xenografts, but with unknown effects on tumor progression in human patients. Thus, we propose the cytotoxic evaluation of potent/highly selective state-dependent TTCC blockers developed in the context of neurological research, such as amides TTA-A1/TTA-A2, piperidine TTA-P2 [47] or piperazine-based Z941/Z944 [108]. Alternatively and, resting on the assertion that potent block of TTCC window currents is achieved by open-channel block, we encourage the evaluation of compounds that enhance inactivation kinetics, and/or induce a depolarizing shift of TTCC activation, such as some succinimides [109], cannabinoids [110], and gating modifier neurotoxins [45]. An additional issue and principal end point is the analysis of functional differences between TTCC isoforms,

which is beyond the resolution of current pharmacology and can only be approached by gene knockdown strategies.

Clinician's Corner

T-type Ca^{2+} channels (TTCC) play a role in membrane excitability, and increased TTCC function or expression levels have been linked to a range of disorders affecting the central and peripheral nervous systems, as well as the heart function. TTCC pharmacological targeting is currently an effervescent field of research and, notably, there are several recent developments focused in ameliorating neuropathic pain.

Nonetheless, TTCC provide a key pathway for Ca^{2+} entry at negative membrane potentials and play a pivotal role in cell proliferation. For this reason, repurposing of TTCC blockers for cancer treatment is under consideration. The role of TTCC GBM progression has received considerable attention in recent years, and cytostatic/cytotoxic effects of TTCC pharmacological blockers described *in vitro* have given way to studies on murine xenografts, and to preliminary clinical trials regarding the safety and optimal dosages of tetraline-derivative mibefradil.

Yet, currently available blockers are not able to discriminate between TTCC isoforms, a relevant distinction since they play different physiological roles. Furthermore, most compounds display a higher affinity for channels in their resting or inactivated states, and thus are better suited to block TTCC in the context of action potential firing rather than at steady membrane potentials. Low-potency block of TTCC by tetralols and dihydroquinazolines in cancer cells is likely to underlie their use at supramaximal concentrations which, in turn, increases the risk of off-targeting. Off-target effects reported for mibefradil include membrane potential shifts and Ca^{2+} mobilization, with unpredictable consequences at the clinical level. Both the pharmacological profile and the cytotoxicity of TTCC blockers recently developed in the context of neurological research need to be carefully studied to assess their applicability as chemotherapeutics. Gene silencing experiments highlight the value of targeting TTCC against hyperalgesia/allodinia and cancer, but pharmaceutical research needs to press ahead to block them in a potent and selective manner, particularly in the latter case.

Box 1. How channel availability depends on steady-state inactivation parameters

Steady-state inactivation parameters have shown to be modified by drugs targeting TTCC. A hyperpolarizing shift of the mid-point for inactivation is bound to reduce channel availability, whereas an increase of the k slope factor for inactivation will cause the opposite effect. Both effects have been reported for tetralols. Despite scarcity, data available for other blockers including dihydroquinazolines,

diphenylbutylpiperidines, diphenylpiperazines and succinimides are also indicative of a hyperpolarizing shift of inactivation. Additionally, an increased slope factor for inactivation could be seen for α-methyl-α-phenylsuccinimide on $Ca_v3.1$ and $Ca_v3.2$ channels in [109] and for KYS05090S [70], KCP10060F and KCP10067F [73] DHQ on $Ca_v3.2$ channels, although these data were not quantified.

Figure I: Top: normalized steady-state inactivation/activation curves built from data published in [111] (black curve), and after shifting the mid-point for inactivation by -5.7 mV, an effect described for mibefradil at 1 μM on TTCC currents [63] (red curve). In the former work, Boltzmann activation parameters were: V_{50} = -49 mV, slope K factor = 4.6 mV; inactivation parameters were: V_{50} = -74.2 mV, slope k factor = 5.5 mV. In the latter work, Boltzmann parameters for activation did not differ significantly in the presence of mibefradil, and the slope factor for inactivation was not reported. Bottom: the fraction of open channels enabling window currents (channel availability) is calculated as the product between steady-state inactivation and activation curves.

Figure II: Top: normalized steady-state inactivation/activation curves built from data published in [55] before (black curve) and after application of NNC-55-0396 at 8 μM (red curve). Activation parameters: V_{50} = -32.6 mV and -32.1 mV for controls and cells treated with NNC-55-0396, respectively; slope k factor = 4.2 mV in control cells and = 4.33 mV cells incubated with NNC-55-0396. Inactivation parameters: V_{50} = -59.2 mV and -62.8 mV for controls and cells incubated with NNC-55-0396, respectively; slope k values were 4.9 mV for controls and 8.0 mV for cells incubated with NNC-55-0396, respectively. Bottom: channel availability was calculated as the product between steady-state inactivation and activation curves. Note that, in the -50 mV - 0 mV range, the fraction of open channels is considerably higher in the presence of NNC-55-0396 (normalized values, left Y axis). Discontinuous blue curve represents the % block necessary to null the increase in window currents due to NNC-55-0396-mediated increase in K slope value for steady-state inactivation (right Y axis).

Box 2. Effects of tetralols on membrane potential and their influence on Ca^{2+} influx

Off-target effects associated to the use of mibefradil (and unexplored for other blockers) are either plasma membrane hyperpolarization [75] or depolarization [76][78]. In turn, membrane potential shifts are bound to affect: **(1) TTCC channel availability**, that will increase or decrease depending on membrane potential values before treatment. A majority of cancer cells display depolarized mean membrane potentials (resting between -10 and -50 mV [61]) relative to the peak of window currents measured in 2 mM Ca^{2+} (between -50 and -70 mV approximately for $Ca_v3.1$ and $Ca_v3.2$, see **Figure I**). Hence, membrane depolarization will exponentially reduce and, conversely, hyperpolarization will exponentially increase TTCC window currents.

(2) HVA Ca_v channels availability. By definition, HVA channels activate at higher potentials compared to LVA, but steady-state inactivation is also shifted in the positive direction. Inactivation of HVA Ca_v channels depends largely on the assembly of β and $\alpha 2\delta$ accessory subunits with the main pore-forming subunits [112][113]. A comparison between $Ca_v3.1$ (black curve) and $Ca_v2.3$ (+β1b + $\alpha 2\delta$1) channels (blue curve) expressed in HEK293 cells and recorded in 10 mM Ba^{2+} accounted for +21.2 /+18.2 mV shifts in V_{50} for activation/inactivation [114]. In this example, $Ca_v2.3$ window currents will increase exponentially by depolarization in the range between -50 and-30 mV. Available data for $Ca_v2.1$ channels co-expressed with auxiliary β4a and $\alpha 2\delta 1$ subunits in COS-7 cells, and recorded in 10 mM Ca^{2+}, places midpoints for activation and inactivation further depolarized at -1.6 and -21 mV, respectively [115]. Here, membrane depolarization will increase $Ca_v2.1$ window currents exponentially in the -40 to -10 mV range (green curve). **(3) Driving force for Ca^{2+} entry**, decreasing linearly by depolarization according to Ohm's law ($I_{Ca} = (V_m-V_{Ca})^* g_{Ca}$. Where I_{Ca} is Ca^{2+} currents, V_m membrane potential, V_{Ca} the equilibrium potential for Ca^{2+} and g_{Ca} the Ca^{2+} conductance, (V_m-V_{Ca}) the driving force for Ca^{2+} entry). These putative actions at multiple levels make difficult to predict a net effect of tetralols on Ca^{2+} homeostasis/signaling.

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Figure 1: Gating of TTCC and steady-state (window) currents.

A. Transitions between activated-open (O), closed-deactivated (C) and inactivated (I) states are time- and voltage- or state-dependent. Activation is a fast process compared to inactivation. The inactivated state is thermodynamically stable and can be achieved from both open and closed states.

B. TTCC currents elicited by stepping from -100 mV (HP) to -30 mV (TP). Inactivation is evidenced by the progressive current decline during TP. Deactivation is observed by the presence of tail currents after TPs.

C. Voltage protocols for measuring steady-state inactivation and activation, and the respective curves. For activation, a range of increasingly depolarizing 50-100 ms TPs is applied from a fixed, negative HP (-70 to -120 mV), and the amplitude of deactivating tail currents is measured after TPs. For steady-state inactivation, long depolarizing prepulses (PP, >1 s) are applied prior to a depolarizing test pulse. Steady-

state activation/inactivation curves for normalized currents are calculated by fitting data to first-order Boltzmann functions. Activation: $I = Imax\ /[1 + \frac{\exp(V50-V)}{K}]$. Inactivation: $I = Imax\ /[1 + \frac{\exp(V-V50)}{K}]$, where I is current amplitude, I_{max} is maximal current amplitude (can be substituted by G_{max} or maximal conductance), V is membrane potential, V_{50} is mid-potential for activation/inactivation (at which half of the channels are open/inactivated) and K is slope factor (mV/e-fold change). Window currents are defined by the voltage range of overlap between the inactivation and activation curves. Panel A, B was reproduced from Karmažínová and Lacinová, Physiol. Res. 59 (Suppl. 1): S1-S7, 2010, with permission. Panel C was adapted from Trends Pharmacol Sci. 30(1):32-40. Iftinca and Zamponi, 2009, Copyright Elsevier.

Figure 2. Scheme illustrating the conclusions attained in this article

The state-dependence of most currently available TTCC blockers implies a low potency block of inactivation-resistant, permanently-open TTCC expressed in cancer cells. This is evidenced by higher EC_{50} values on cancer cells viability, compared to IC_{50} values on TTCC currents elicited by depolarizing voltage steps. The application of high drug concentrations is associated to increased off-target effects, which are evidenced by membrane potential shifts and intracellular Ca^{2+} mobilization upon application of tetralols, and that are unexplored for other TTCC blockers. Subsequently, activation of Ca^{2+}-dependent pathways/cell processes may lead to reduced cell proliferation or increased cell death observed *in vitro*, but it could be also associated to increased proliferation, migration, survival or chemoresistance with unknown consequences for human tumor progression. Membrane depolarization has shown to promote cancer cell proliferation and migration independently of Ca^{2+} levels.

Glossary

Ca_v channels: voltage-gated Ca^{2+} channels, activated by membrane depolarization. A large family including Ca_v1 (4 isoforms, also called L-type channels), Ca_v2 (3 isoforms, including P/Q-type, N-type and R-type Ca^{2+} channels) and Ca_v3 channels (3 isoforms, also called T-type Ca^{2+} channels, TTCC).

EC_{50}: half maximal effective concentration, the drug concentration at which 50% of the maximum effect (on viability) is produced**.** This term is equivalent to **GI_{50}**, as the drug concentration at which 50% of growth inhibition is produced.

Gating: refers to the conformational changes experienced by ion channels leading to opening (activation), closing (deactivation) or to a non-conductive, refractory state (inactivation).

GBM: Glioblastoma Multiforme, a brain cancer characterized by high cell proliferation, invasivity and tumor cell infiltration into the surrounding brain parenchyma and angiogenesis.

HVA: high voltage-activated. Channels activated by strong depolarization.

Holding potential: membrane potential before test pulses, whose values are fixed by the voltage-clamp technique.

IC_{50}: half maximal inhibitory concentration, the drug concentration at which 50% of the maximum inhibition/block (on Ca^{2+} channels in this article) is produced

K_v channels: voltage-gated K^+ channels, activated by membrane depolarization.

LTCC: L-type Ca^{2+} channels. Belong to the Ca_v channels family and are activated by strong membrane depolarization.

LVA: Low voltage-activated. Channels activated by weak depolarization, such as T-type Ca^{2+} channels.

Modulated receptor theory: explains state-dependent block by proposing that a drug can bind to the drug receptor regardless of the state of the targeted ion channel, but its binding affinity in each state is different. An alternative explanation is provided by the is the **guarded receptor theory**, suggesting that the affinity of drug binding to ion channels is state-independent, but is the access of the drug to the binding site that depends on channel conformation

Na_v channels: voltage-gated Na^+ channels, activated by membrane depolarization.

State-dependence: when drug affinity/access depends on the gating state of the channel (resting/activated/inactivated).

Steady-state activation/inactivation curves: curves illustrating the voltage dependence for channel activation and inactivation under stationary-state conditions (devoid of time-dependence). Steady-state activation is achieved by applying sufficiently long (typically > 50 ms) depolarizing test pulses (by the voltage clamp technique) for the channels to reach the open state. Steady-state inactivation is that achieved from the closed states through long-lasting prepulses (voltage pulses applied prior to the test pulse applied with the voltage-clamp technique, typically > 1 s) at subthreshold potentials. Steady-state activation/inactivation curves are fit by first-order Boltzmann functions.

Use-dependence: when drug affinity/access correlates positively with the frequency of stimulation (i.e. membrane depolarizing stimuli), thus it is suggestive of drug binding to open and/or inactivated channels.

Window currents: currents resulting from channels permanently open at steady potentials (resting membrane potentials in excitable cells), due to the overlap between steady-state inactivation and activation curves.

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Table 1: potencies of TTCC block and cancer cell viability reduction of tetralol and 3,4 dihydroquinazoline derivatives

Compound	IC_{50} or Kd (μM)	GI_{50} on cancer cells (μM) , 24-72 h
Mibefradil	0.2-1.3 ($Ca_v3.1$ expressed in HEK293) [71] [72] [116] [117] 0.1-0.9 ($Ca_v3.2$ expressed in HEK 293) [58] [62] [118] 0.1 (native TTCC of rat Purkinje neurons)[119] 0.6 (native TTCC of dorsal root ganglion neurons [120] 1.0 (native TTCC of bovine adrenal fasciculata cells) [63] 1.2 (native TTCC of pulmonary microvascular endothelial cells) [121]	3.3 (HCT116 colon carcinoma) [104] 3.5 (U251 GBM) [105] 20.5-24.3 (ovarian cancer cell lines) [71] 24.8 (A549 lung adenocarcinoma) [72] 31.4 (A549 lung adenocarcinoma) [116]
NNC-55-0396	< 1 (30-60 min)-10.1 (3 min) ($Ca_v3.1$ expressed in HEK293) [55]	2.1-2.9 (MCF-7 and MDA-MB-231 breast cancer cells) [32] 4.8/5.3 (A2780/HO8910 ovarian carcinoma/adenocarcinoma) [33]
KYS05090	0.3 ($Ca_v3.1$ expressed in HEK293)[72]	4.1 (A549 lung adenocarcinoma) [72]
KYS05090S, KCP10043F	0.5, 0.9 ($Ca_v3.1$ expressed in HEK293) [116]	4.7, 7.3 (A549 lung adenocarcinoma) [116]
5b, 6b,6c, BK10040, 8	0.5-1.8 ($Ca_v3.1$ expressed in HEK293) [72]	2.3-20.7 (A549 lung adenocarcinoma) [72]
KYS05042, KYS05043, KYS05046, KYS05047, KYS05048, KYS05055, KYS05056, KYS05057, KYS05065, KYS05080, KYS05085, KYS05089, KYS05090	0.04-1.0 ($Ca_v3.1$ expressed in HEK293) [68]	0.17->100 μM (A549 lung adenocarcinoma) [68]
KYS05090, 6a, 6c, 6d, 6f, 6g, 6h	0.3-0.6 ($Ca_v3.1$ expressed in HEK293) [71]	2.3-9.8 (SK-OV-3 epithelial ovarian cancer) [71]

Table 2: Effects of tetralols and 3,4-dihydroquinazolines on cell pathways/processes

Drug	Effects on membrane potential	Effects on intracellular Ca^{2+}	Effects on intracellular pathways/processes
Mibefradil	hyperpolarization [75] depolarization [78] [76] [77]	triggered Ca^{2+} release from ER and increased Ca^{2+} cytosolic concentration [92][93]	increase of P38-MAPK signaling [104] reduction of Akt/PkB signaling [105] induction of caspase3/7-dependent apoptosis [104][105] induction of caspase 3/9-dependent apoptosis [103][78] mTOR inhibition [105][122] induction of the unfolded protein response [103] deregulation of macroautophagy [103][122] upregulation of p27(KIP1) and BAX [122]
NNC-55-0396	N.D.	triggered Ca^{2+}-release from ER and increased Ca^{2+} cytosolic concentration [93]	N.D.
KYS05047	N.D.	decreased cytosolic Ca^{2+} concentration [98]	upregulation of p27(KIP1) [98]
KYS05090	N.D.	decreased cytosolic Ca^{2+} concentration [99]	generation of ROS and induction of macroautophagy [99] induction of caspase-3 dependent apoptosis [99]

N.D.: Not Determined.

Highlights

- T-type Ca^{2+} channels (TTCC) play a role in membrane excitability, and the development of selective TTCC pharmacological blockers for neurological conditions such as pain, epilepsy, tremor, ataxia and insomnia is an active area of research.
- TTCC also enable sustained Ca^{2+} entry (known as *window currents*) which facilitates G1-S transition and appears necessary for survival of proliferating cells. Pharmacological targeting of TTCC is currently explored as a chemotherapeutic option, and different compounds with TTCC blocking ability have shown to reduce cancer cell viability *in vitro*.
- Two classes of TTCC blockers, tetralols and dihydroquinazolines (DHQ), have shown to slow Glioblastoma progression in mouse models, and clinical trials are performed to assess the optimal dosing and tolerance of tetralol mibefradil in Glioblastoma Multiforme patients.
- A majority of TTCC blockers (including tetralols and DHQ) display a state-dependent affinity which implies a low-potency block on permanently open TTCC contributing window currents, and are cytotoxic to cancer cells at higher concentrations than those required for channel block. Reported off-target effects of tetralols, including membrane potential shifts and Ca^{2+} mobilization from the endoplasmic reticulum, are largely unexplored for other TTCC blockers.
- Gene silencing experiments highlight the value of targeting TTCC in neurological diseases and cancer, and can be used to evaluate the pharmacological specificity of curren and novel compounds.

Outstanding Questions

- Available TTCC blockers exhibit increased affinity for the closed and/or inactivated states; to what extent does it imply a low-potency block of window currents?
- Are there any cytostatic and/or cytotoxic effects on cancer cells of tetralols and DHQ at concentrations compatible with their reported IC_{50}s?
- Are cytostatic/cytotoxic effects of TTCC blockers used at supramaximal concentrations due to off-target effects?
- Can state-dependent, highly-potent TTCC blockers -developed in the context of neurological research- be repurposed for cancer treatment?
- Gene silencing experiments show that depletion of $Ca_v3.1$ or $Ca_v3.2$ affects negatively the viability of cancer cells *in vitro*; to what extent the cellular pathways modulated by channel knockdown coincide with those triggered by pharmacological blockers?
- What are the effects of the selective knockdown of TTCC isoforms in preclinical *in vivo* models?

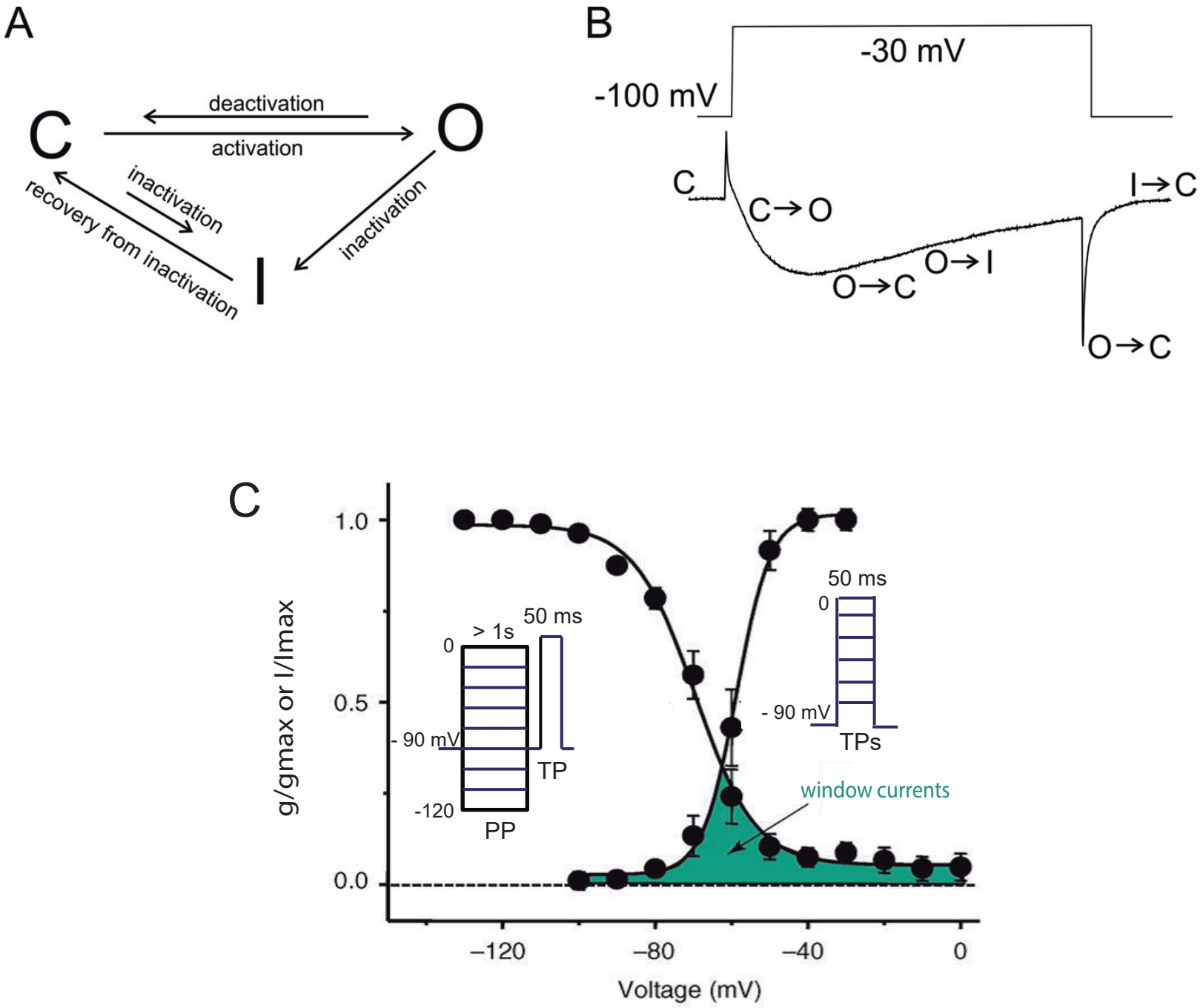

A
C
O
I
deactivation
activation
inactivation
recovery from inactivation
inactivation
B
-30 mV
-100 mV
C
C→O
O→I
O→C
I→C
O→C
C
g/gmax or I/Imax
1.0
0.5
0.0
> 1s
50 ms
0
- 90 mV
-120
PP
TP
50 ms
0
- 90 mV
TPs
window currents
–120
–80
–40
0
Voltage (mV)

Figure 1

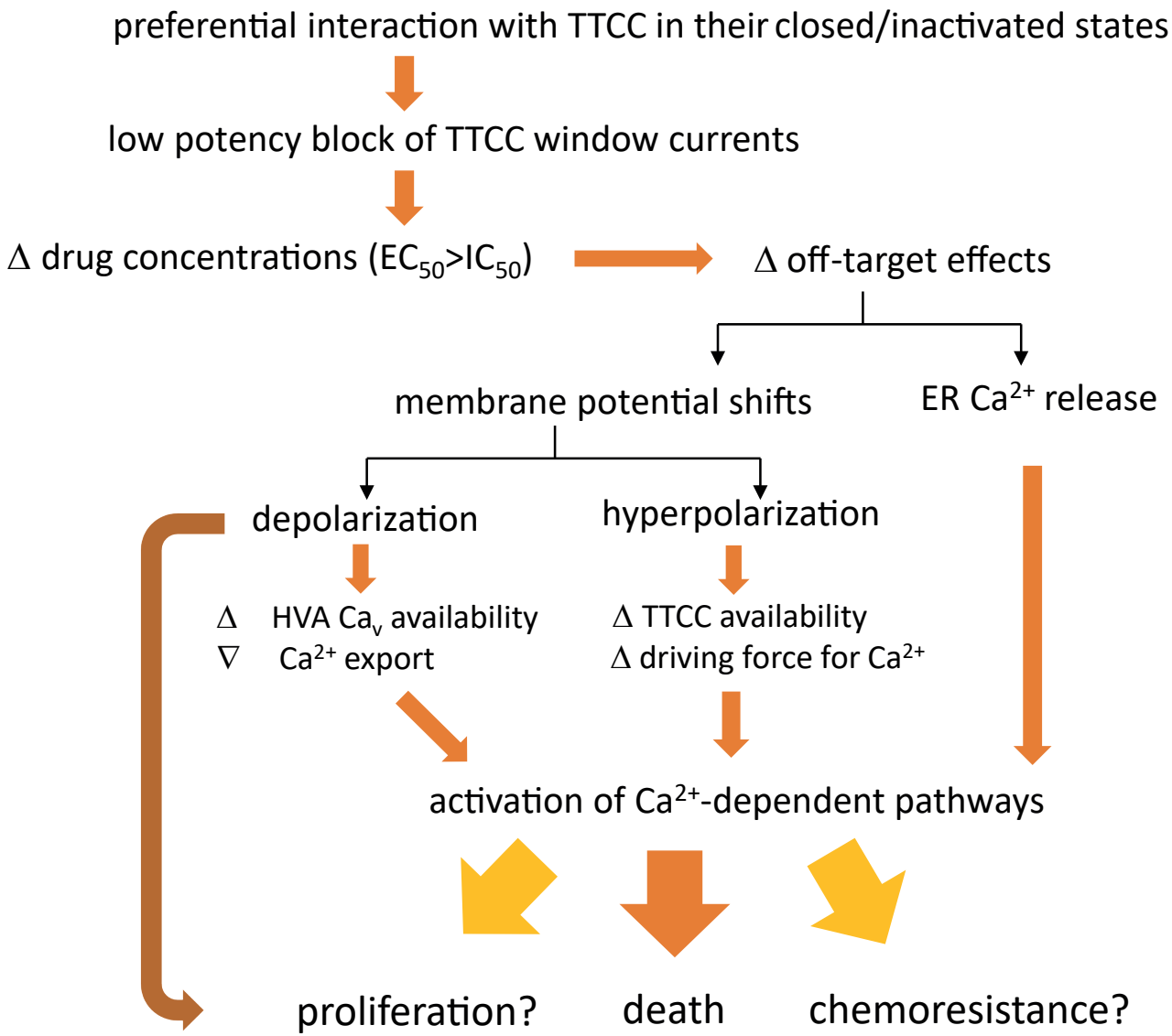
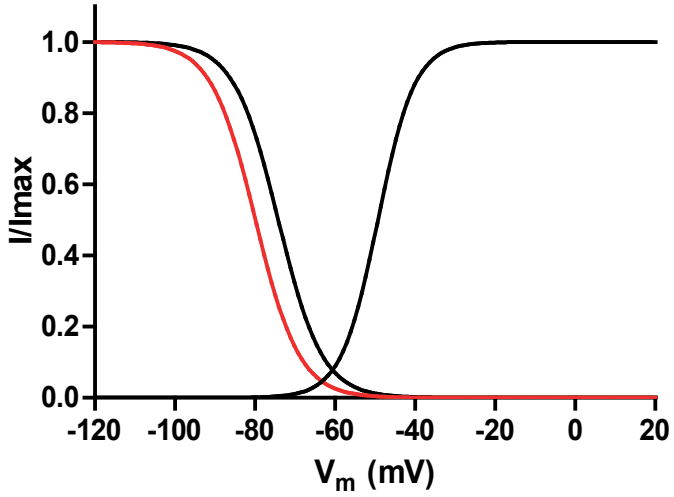

preferential interaction with TTCC in their closed/inactivated states
low potency block of TTCC window currents
Δ drug concentrations (EC_{50}>IC_{50})
Δ off-target effects
membrane potential shifts
ER Ca^{2+} release
depolarization
hyperpolarization
Δ HVA Ca_v availability
∇ Ca^{2+} export
Δ TTCC availability
Δ driving force for Ca^{2+}
activation of Ca^{2+}-dependent pathways
proliferation?
death
chemoresistance?

Figure 2

Figure I

hyperpolarizing shift of steady-state inactivation



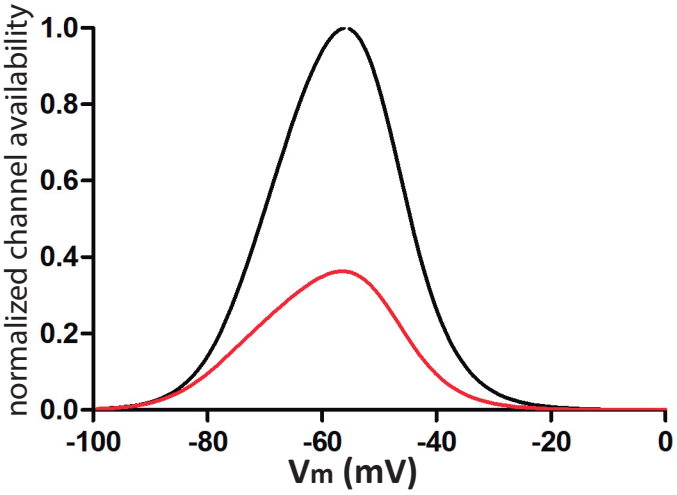
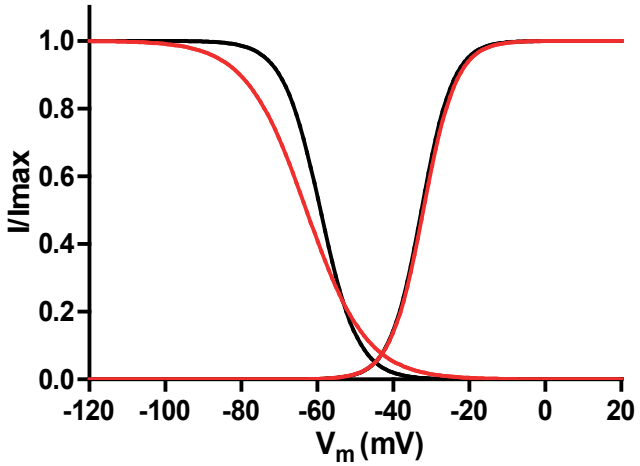
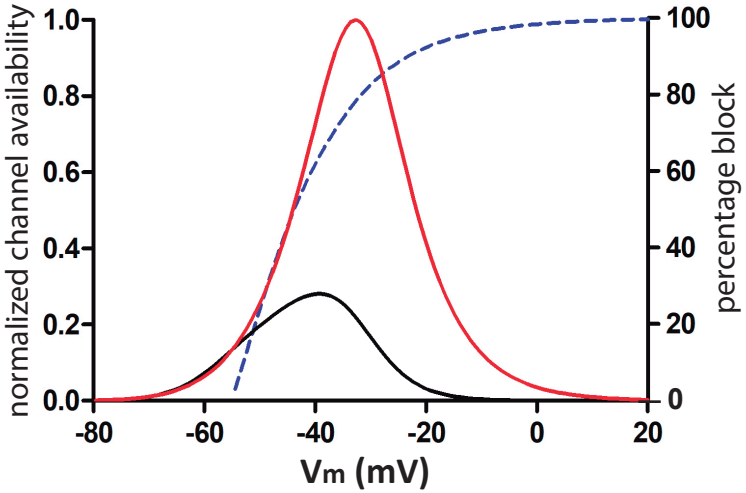


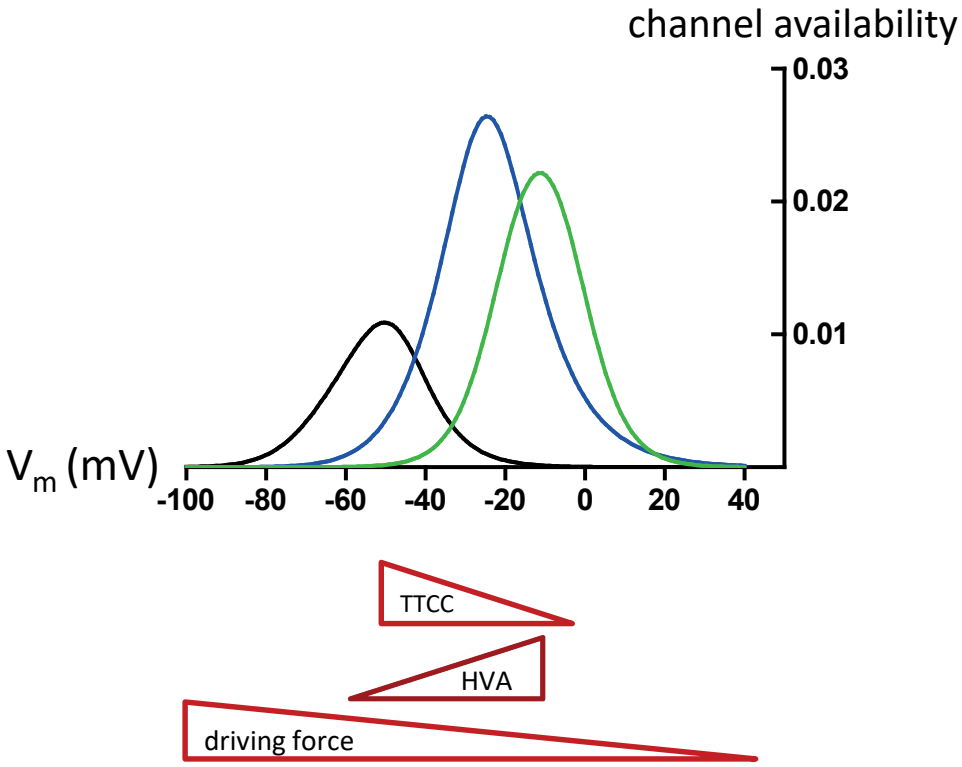
Figure II

reduced slope of steady-state inactivation





Box 1


channel availability
0.03
0.02
0.01
V_m (mV)
-100
-80
-60
-40
-20
0
20
40
TTCC
HVA
driving force

Box 2