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Use-dependent blockade of $Ca_v2.2$ voltage-gated calcium channels for neuropathic pain

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Abstract

The translocation of extracellular calcium (Ca^{2+}) via voltage-gated Ca^{2+} channels (VGCCs) in neurons is involved in triggering multiple physiological cell functions but also the abnormal, pathophysiological responses that develop as a consequence of injury. In conditions of neuropathic pain, VGCCs are involved in supplying the signal Ca^{2+} important for the sustained neuronal firing and neurotransmitter release characteristic of these syndromes. Preclinical data have identified N-type VGCCs ($Ca_v2.2$) as key participants in contributing to these Ca^{2+} signaling events and clinical data with the peptide blocker Prialt™ have now validated $Ca_v2.2$ as a bona fide target for future drug discovery efforts to identify new and novel therapeutics for neuropathic pain. Imperative for the success of such an endeavor will be the ability to identify compounds selective for $Ca_v2.2$, versus other VGCCs, but also compounds which demonstrate effective blockade during the pathophysiological states of neuropathic pain without compromising channel activity associated with sustaining normal housekeeping cellular functions. An approach to obtain this research target profile is to identify compounds, which are more potent in blocking $Ca_v2.2$ during higher frequencies of firing as compared to the slower more physiologically-relevant frequencies. This may be achieved by identifying compounds with enhanced potency for the inactivated state of $Ca_v2.2$. This commentary explores the rationale and options for engineering a use-dependent blocker of $Ca_v2.2$. It is anticipated that this use-dependent profile of channel blockade will result in new chemical entities with an improved therapeutic ratio for neuropathic pain.

Keywords: Neuropathic pain; Voltage-gated calcium channels; Use-dependent block

1. Introduction

Calcium (Ca^{2+}) entry via voltage-gated Ca^{2+} channels (VGCCs) in neurons is critical to initiate and/or sustain general cellular functions as well as uniquely regulating neurotransmitter/neurohormone release and synaptic plasticity [1–3]. As the Ca^{2+} entry gateways, VGCCs supply an intracellular Ca^{2+} ''trigger'' which is instrumental for the transmission of sensory information by nociceptive neurons, the specialized detectors of potentially painful and harmful environmental stimuli, to spinal relay systems for further central processing and/or reflex motor responses. In chronic pain, sustained conditions of sensory neuron firing, resulting from such noxious stimuli as fulminating inflammation or tissue destruction, leads to a prolonged increase in the excitability of central nociceptive neurons (e.g., central sensitization). VGCCs are important for supplying the Ca^{2+} necessary for the increased sensory nerve excitability as well as the complex patterns of neurotransmitter release associated with chronic pain syndromes. Both preclinical, and now clinical data, have identified N-type VGCCs ($Ca_v2.2$) as particularly instrumental in instigating the increased excitability and neurotransmitter release in severe chronic and neuropathic pain syndromes. The data indicate that reduction of $Ca_v2.2$-mediated Ca^{2+} entry in these circuits result in pain amelioration. This recognition of the role of $Ca_v2.2$ in pain has resulted in the vigorous pursuit in the pharmaceutical industry of an orally active blocker of these channels. However, the structural homology amongst the VGCCs, as well as the importance of

Abbreviations: VGCC, voltage-gated calcium channel; Ct, conotoxin; V_{Rest}, resting membrane potential; $V_{Threshold}$, threshold membrane potential for activation

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$Ca_v2.2$ in subserving normal functions in neurons poses challenges for the widespread and safe use of a $Ca_v2.2$ blocker in chronic pain syndromes. Indeed, Prialt™, the recently approved peptide blocker of $Ca_v2.2$ for severe, intractable pain, can be associated with marked side effects associated with sympatholysis when administered systemically [4].

In order to achieve an acceptable efficacy side-effect window for an orally active therapeutic, the drug would need to block $Ca_v2.2$ under conditions of increased neuronal firing/excitability whilst having little to no effect on channel activity during normal physiological functions. In this commentary we will explore the rationale and the possible options for engineering a use-dependent blocker of $Ca_v2.2$. As a backdrop we will briefly review the salient features of the members of the VGCCs and the evidence implicating $Ca_v2.2$ in pain syndromes.

2. The family of VGCCs

2.1. Voltage dependent activation and inactivation

This review will use the standard nomenclature derived from the International Union of Pharmacology compendium of VGCC [5]. The IUPHAR reference is an excellent source for relating this current nomenclature with the alphabetical nomenclature originally developed for the distinct classes of calcium currents. The VGCCs were initially characterized by the degree of membrane depolarization necessary to induce an inward Ca^{2+} current. Ca_v2 and Ca_v1 VGCCs (includes the N, P/Q, R and L-type VGCCs) are ''high-threshold'' channels that are activated by a relatively large depolarization; Ca_v3 VGCCs (T-type VGCCs) are ''low-threshold'' since they are activated by smaller ranges of membrane depolarization [2,6]. Differences also exist in the relative rates of channel activation and inactivation. Inactivation is a condition during which the channel is no longer responsive to depolarizations, which are supra-threshold for activation. It is an important determinant of the duration of channel response to a stimulus, as well as the proportion of channels available for activation. Ca_v1 VGCCs exhibit slow, voltage-dependent inactivation, Ca_v3 VGCCs inactivate rapidly and Ca_v2 VGCCs are intermediate in inactivation rate [6,7].

$Ca_v2.2$ channel availability is also modulated by autacoids acting via G protein-coupled receptors (GPCRs; 2, 6, 8). This mechanism provides a negative-feedback signal to prevent Ca^{2+} overload in neurons and regulates VGCC-mediated neurotransmitter release, changing the channel activation phenotype from a ''willing'' to a ''reluctant'' mode [6], due to a shift in gating properties resulting in a slower onset of activation and less current flow at moderate membrane voltages [6,8].

These kinetic properties of VGCCs, together with their cellular localization, are critical factors in determining their roles in triggering specific cellular events. For example, transient, localized Ca^{2+} influx in nerve terminals through Ca_v2 VGCCs is important for the release of neurotransmitters [2] whilst prolonged, more global elevations of Ca^{2+} through Ca_v1 VGCCs support many functions including gene expression [9]. These channels also function as a source of depolarizing current, which directly contributes to neuronal excitability, and the properties of specific channels help determine the characteristics of these membrane currents.

2.2. The α_1 subunit of VGCCs

The VGCCs are differentiated based on the molecular structure of the central α_1 subunit which forms the common core unit and constitutes the pore, voltage sensor, activation and inactivation gate of the channel [10]. There are at least 10 discrete α_1 genes identified, divided into three major subgroups based on structural as well as functional and pharmacological properties, with at least 70% homology amongst individual members within a subgroup. The α_1 subunit is composed of four repeated domains, I–IV, with each domain containing six transmembrane α-helices (segments S1–S6; Fig. 1). Resolution of the crystal structure of a bacterial K^+-selective ion channel (KcsA) has implicated, by analogy, two transmembrane helices in each channel domain, corresponding to the S5 and S6 segments of VGCCs, that are important for the control of ion permeation [11]. Segments S5 and S6 of each domain are connected via an external loop, which reinserts into the membrane, and together provide the outer vestibule and ion-conducting pore lining of the channel. The pore-lining loops (p loops) in the extracellular aspects of the pore contain highly conserved negatively charged glutamic acid residues which were identified as critical for defining the Ca^{2+} selectivity of VGCCs [12]. Selectivity is also guided by an EF-hand motif (i.e., glycine flanked by three negatively charged residues), homologous to the Ca^{2+} binding domains resident in Ca^{2+} binding proteins, in the outer vestibule adjacent to the p-loop in domain III S5–S6 [13]. The intracellular loops (''linkers'') connecting domains I–II and domains II–III are important sites for channel modulation via the binding of auxiliary subunits and/or phosphorylation by kinases [10]. For example, the domain I–II cytoplasmic loop in $Ca_v2.2\alpha_1$ is critical for the upregulation of $Ca_v2.2$ by protein kinase C (PKC [14]). PKC-dependent phosphorylation of sites contained in this loop can reverse the ability of G proteins to inhibit $Ca_v2.2$ function [15].

The S4 segment of each homologous domain contains positively charged lysines or arginines which renders this segment sensitive to changes in voltage. Membrane depolarization appears to translocate S4 outward with respect to the rest of the protein [16] triggering further conformational changes in channel structure that result in the opening of the ion pore [17]. The membrane-spanning S6

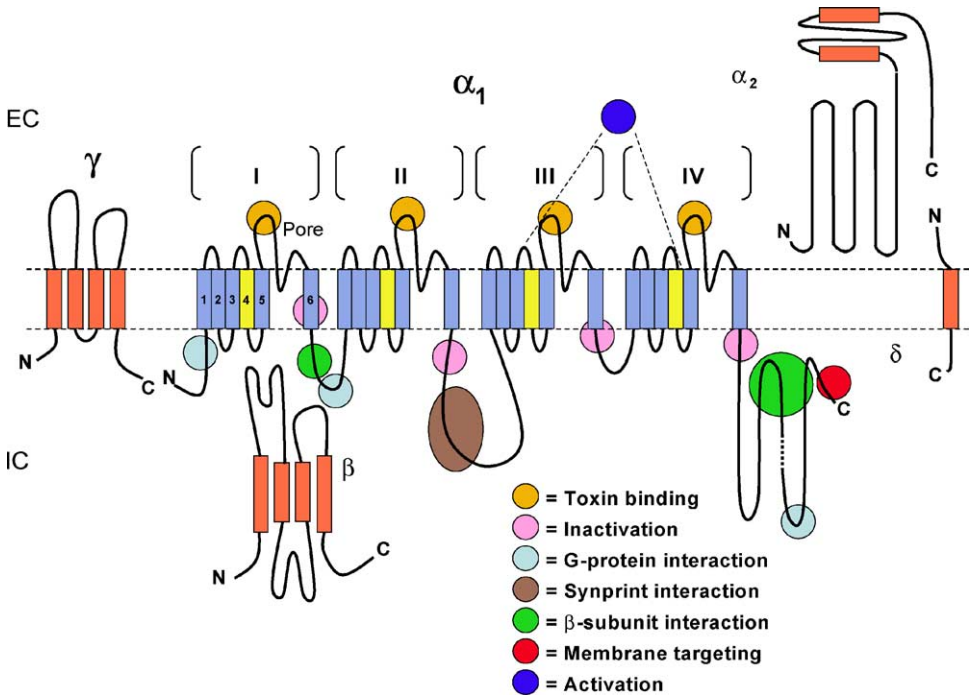

Fig. 1. Schematic diagram of VGCC α_1 and accessory subunits. The relative two-dimensional membrane position of each α_1 subunit domain (I–IV; in brackets), and the accessory β, γ, α_2, and δ subunits are shown, although the exact membrane localization and folded structure has not yet been described. The topology of each six-transmembrane segment α_1 subunit domain is illustrated using domain 1. The relative positions of some known important functional domains, discussed in the text, are indicated for the α_1 subunit, and the color code is provided in the legend.

regions have been implicated in affecting either the rate (e.g., domain I) or voltage-dependence (domain III) of VGCC inactivation [18,19].

The intracellular loop linking domains I and II may be important for effecting voltage-dependent inactivation, as it may physically block the channel pore by docking to the ends of the S6 segments [20]. This mechanism underlying voltage-dependent inactivation may be similar to that described for sodium and potassium channels, which is pore blockade by a cytoplasmic region that exerts a voltage-dependent movement into the channel [20]. Several factors could influence this particular mechanism for voltage-dependent inactivation since, as stated above, the intracellular loop contains binding sites for the auxiliary subunits as well as modulatory proteins such as G proteins. Thus, voltage-dependent inactivation is influenced by several sites and multiple modulatory factors impinging on the α_1 subunit, which are likely to underlie the concept of multiple states of channel inactivation [7]. This plasticity in channel inactivation is likely to add to the regional differences in VGCC function in normal cells/tissues throughout the mammalian nervous system but also to altered channel function during pathophysiological states.

2.3. The heter-oligomeric structure of VGCCs

The VGCCs contain a β subunit for which four genes have been identified, although splice variants and tissue-restricted expression adds to the diversity of hetero-oligomer expression amongst tissues [21]. The β subunits are located intracellularly (Fig. 1), where they affect the function of VGCCs primarily by increasing the level of channel expression at the plasma membrane [21]. The close proximity of the bound β subunits to areas such as the I-S6 and the I–II linker region may underlie their ability to modulate channel function by altering gating kinetics, e.g., shifting the activation voltage to more hyperpolarized voltages [21].

The channel complex contains an α_2 and a δ subunit existing as a heterodimer, with the externally located α_2 anchored to the membrane via a disulfide bond to the embedded δ subunit (Fig. 1 [22]). The $\alpha_2\delta$ heterodimer is formed from a single gene product, by posttranslational proteolytic processing, and four different such genes have been described [10,23]. The $\alpha_2\delta$ subunit modifies the expression pattern as well as the biophysical and pharmacological properties of the channel complex [24]. This particular subunit has generated intense interest for drug discovery programs due to its identification as the high-affinity binding site of gabapentin (Neurotin^{TM}) as discussed below.

VGCCs from skeletal muscle were found to contain a γ subunit (γ_1) which has four transmembrane domains and acts to minimize VGCC activity [25]. Functional studies suggest that the neuronal γ subunits have an inhibitory effect on both the high-threshold (Ca_v1 and Ca_v2) and low-threshold (Ca_v3) VGCC activity [26,27]. There are

presently at least eight isoforms of the γ subunit that have been identified.

2.4. Pharmacology of VGCCs

The VGCCs can also be differentiated pharmacologically. Most extensively studied are the three main classes of Ca_v1 VGCC antagonists which have spawned successful therapeutic agents and provided validation for VGCCs as targets in drug discovery efforts.

Small molecule blockers/inhibitors of other VGCCs are only recently the subject of research efforts. Peptides derived from the venom of the marine cone snails (genus *Conus*) have been powerful tools in identifying and characterizing the function of $Ca_v2.2$ (see below) and $Ca_v2.1$ VGCCs in neurons [28]. Moreover, the $Ca_v2.2$ blocking peptide ω-conotoxin MVIIA (ct-MVIIA, SNX-111, ziconotide, Prialt^{TM}) is the first therapeutic agent to specifically target these channels.

Both ct-MVIIA and ω-conotoxin GVIA (ct-GVIA) appear to bind to the same critical residues in the p-loop region of the channel pore (Fig. 1) but also in the EF hand motif of the outer vestibule of $Ca_v2.2$, consistent with a direct pore-blocking mechanism [29,30]. ct-MVIIA does not show frequency-dependent block of $Ca_v2.2$ which is consistent with the toxin accessing the outer vestibule/extracellular aspects of the channel [31]. The lack of frequency dependent block may, however, also reflect a relatively slow off-rate for these toxins. These ''pore-blocking'' peptides block the release of neurotransmitter from both central neurons and peripheral sympathetic efferents [28,32].

ω-Conotoxin CVID (ct-CVID, AM336), isolated from *Conus catus*, has a similar affinity as ct-GVIA and ct-MVIIA in displacing ^{125}I-ct-GVIA from rat brain membranes (IC_{50} values of 70, 38 and 55 pM, respectively [33]). Compared to ct-MVIIA, ct-CVID was found to be 10–100-fold more selective for $Ca_v2.2\alpha_1$ versus $Ca_v2.1\alpha_1$. Both ct-CVID and ct-MVIIA potently (20–60 nM) inhibited substance P release in rat spinal cord slices and were antinociceptive following intrathecal administration in a rat model of adjuvant arthritis [34]. ct-CVID, but not ct-MVIIA, inhibited neurotransmitter release from preganglionic cholinergic nerves which suggests that ct-CVID may differ from ct-MVIIA in blocking a variant form of $Ca_v2.2$, or a closely related VGCC, in some tissues [35].

3. $Ca_v2.2$: pivotal role in pain

The lure of some VGCCs as novel pain targets lies in their critical location in nerve terminals and important roles in the fast synaptic release of neurotransmitters. Ca_v2 VGCCs are the main Ca^{2+} channels involved in release of neurotransmitters at the majority of mammalian synapses [10]. $Ca_v2.2$ has been demonstrated to physically interact with members of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family of synaptic proteins [10,36]. The site of interaction is the large cytoplasmic loop connecting domains II and III (termed 'synprint') of the $Ca_v2.2\alpha_1$ core subunit (Fig. 1 [36]). The sustained firing of sensory afferents during pain syndromes leads to increases in cytosolic Ca^{2+} in the nerves' terminals found in the dorsal horn of the spinal cord. These terminals contain $Ca_v2.2$ as determined by autoradiography using a radiolabeled, $Ca_v2.2$ specific ω-conotoxin [37]. $Ca_v2.2$ co-localizes with neurotransmitters associated with pain syndromes (e.g., substance-P) in the dorsal horn [38]. $Ca_v2.2$ channels are also instrumental in mediating a component of the rise in intracellular Ca^{2+} in the nociceptive-processing neurons resident in the dorsal horn which receive synaptic input from the sensory afferents [39].

Validation of the role of $Ca_v2.2$ in pain is also found in the analysis of channel expression in animal models of neuropathic pain. In the chronic constrictive injury model of neuropathic pain in rats (i.e., Bennett model), the expression of the α_1 subunit of $Ca_v2.2$, using immunocytochemistry, was upregulated in the dorsal horn concurrently with the development of both mechanical and hot/cold allodynia [40]. A pivotal role of $Ca_v2.2$ in pain is also suggested from studying mice genetically deficient in the expression of the channel's $Ca_v2.2\alpha_1$ protein. The $Ca_v2.2\alpha_1{}^{-/-}$ mice exhibited a diminished late phase (i.e., persistent phase) of overt behaviors attributed to pain (e.g., flinching or biting/licking) following subcutaneous or intraplantar injection of formalin and an increased threshold for acute thermal nociception [41–43]. The $Ca_v2.2\alpha_1{}^{-/-}$ mice were also shown to develop significantly less mechanical allodynia and thermal hyperalgesia following spinal nerve ligation [41]. In all of these evaluations heterozygous mice showed responses similar to wild-type mice. The $Ca_v2.2\alpha_1{}^{-/-}$ mice presented with an abnormal baroreflex response, and atria isolated from these mice had a diminished inotropic response to electrical stimulation, indicative of the role of $Ca_v2.2$ in the release of transmitter from sympathetic nerve terminals important for modulation of cardiovascular function [44].

Blockade of $Ca_v2.2$ by selective antagonists, such as with the ω-conotoxins, has been shown to be effective in several preclinical models of pain syndromes. These studies have typically used intrathecal administration in order to assure adequate exposure of these peptide blockers in the central nervous system. Administration of ziconotide or ct-GVIA inhibited the mechanical allodynia and hyperalgesia in rat models of acute (e.g., postoperative [45]) and neuropathic pain [46–48]. Spinal blockade of $Ca_v2.2$ with either ct-GVIA, ct-MVIIA or ct-CVID effectively inhibited mechanical allodynia following spinal nerve ligation in rats [49] and (with ct-GVIA) inhibited evoked neuronal responses in the dorsal horn of spinal cord in this model [47]. The $Ca_v2.2$ blocker conotoxin SNX-239 relieved the mechanical allodynia which develops with streptozotocin-

induced diabetes [50]. Blockade of $Ca_v2.1$ (with ct-IGA) was less effective.

As mentioned above, these experiments were conducted with intrathecal administration of the peptide blockers. Local administration (i.e., perfusion over injured nerves via chronically implanted cannula) of ct-GVIA or ct-MVIIA has been found to reduce heat hyperalgesia and mechanical allodynia in the Bennett model of neuropathic pain [51]. Therefore, peripheral blockade of $Ca_v2.2$ may diminish the harmful sequelae following nerve damage. However, as already noted, blockade or decreased expression of $Ca_v2.2$ can affect the sympathetic control of organ function. Both ct-GVIA and ct-MVIIA inhibited the inotropic response to stimulation of the sympathetic nerves in cardiac atria (e.g., [52]). Administration of ct-GVIA in vivo inhibited sympathetic control of cardiovascular function, lowered blood pressure and was associated with orthostatic hypotension [53]. Therefore, when targeting $Ca_v2.2$ for developing novel therapeutics for pain, the strategy must take into account the importance of this VGCC in the sympathetic control of physiological functions.

3.1. Clinical studies

Data from human trials is, of course, the most validating of all target data. Prialt™ has become one of the most extensively studied pain drugs in the clinic prior to its recent approval. It has been shown to be efficacious following intrathecal administration in various acute and chronic pain syndromes including postoperative pain [54], chronic neuropathic pain [55], and cancer and HIV-associated neuropathy [56]. Nonetheless, systemic and even intrathecal administration of Prialt™ has been associated with dose-limiting side effects [4,57]. This would be anticipated given the importance of $Ca_v2.2$ with ''housekeeping'' functions and the ''pore-blocker'' mechanism of action for Prialt™.

4. Concept of use-dependent blockade

An acceptable therapeutic window for a next-generation blocker of $Ca_v2.2$ will depend on the compound's ability to spare functions, such as neurotransmitter release, that are required for normal central processing and peripheral sympathetic control of cardiac and smooth muscle. The goal would be to design a molecule with the right combination of state-dependent affinity and interaction rates such that blockade would be robust only during the high-frequency firing operant during pain syndromes. This use-dependence, and in particular frequency-dependence, may best be achieved by targeting the inactivation mechanisms which have evolved to regulate the open probability of $Ca_v2.2$ in neurons.

Voltage-gated ion channels can be thought to exist in one of three general conformational states; these are the closed state (resting state), the open or conducting (activated) state, and an inactivated state (Fig. 2A). In the simplest of models, inactivation is analogous to receptor desensitization, wherein the continued influence of an activating stimulus, in this case depolarizing transmembrane voltage sufficient to cause channel opening, loses its ability to sustain the channel in the open state. That this inactivated state involves a different channel conformation than the closed or open states is supported by many lines of evidence [18,20,58]. Once channels enter the inactivated state, a characteristic interval must pass before the channel can be re-activated. In general, VGCCs can inactivate by multiple mechanisms, but most relevant for a drug discovery discussion is voltage-dependent inactivation. Voltage-dependent inactivation, in turn, can be accessed directly from the open or activated state (current-dependent inactivation), or can be accessed from the closed or resting state of the channel. The latter form of inactivation, closed-state inactivation (CSI), involves direct entry into the inactivated state by depolarizations that are sub-threshold to channel opening. CSI is also important for regulating the dynamic availability of VGCCs as a function of changes in membrane potential.

This is a particularly important mechanism in neurons. Current models of VGCC states and gating, including CSI, incorporate the notion of multiple intermediate states between the closed and open state (Fig. 2A). While the exact physical representation and the number of these states are unknown, these can be thought of as representing fractional positions of the voltage-sensor at subthreshold (non-current inducing) depolarizations. Patil et al. [59] demonstrated that Ca_v2 VGCCs, which inactivated slowly during sustained suprathreshold depolarizations, inactivated much more readily under conditions which emulated repetitive neuronal action potentials. The Ca^{2+} currents produced by trains of depolarizations mimicking high-frequency action potential bursts were shown to be reduced under these conditions. Jones et al. [60] demonstrated, using comparative gating charge and current inactivation analyses, that $Ca_v2.2$ appears to preferentially inactivate via a CSI route. The implications for neuronal Ca^{2+} current regulation are quite profound, given the brief nature of depolarizations associated with action potentials and the dynamic variability of neuronal membrane potentials. Access to the inactivated channel conformation occurs during channel activation and deactivation, implying a greater probability of channel inactivation when channels are cycled through repetitive brief periods of activation/deactivation. Some degree of cumulative inactivation of these channels would occur within any train of action potentials, but more readily during high-frequency bursts in damaged neurons. The degree of cumulative inactivation is dependent on burst frequency and duration, as well as specific channel characteristics such as alternative splicing that control the kinetics of state transition [61]. This process is an important contributor to short-term plasticity of neurotransmitter release at central synapses and can be

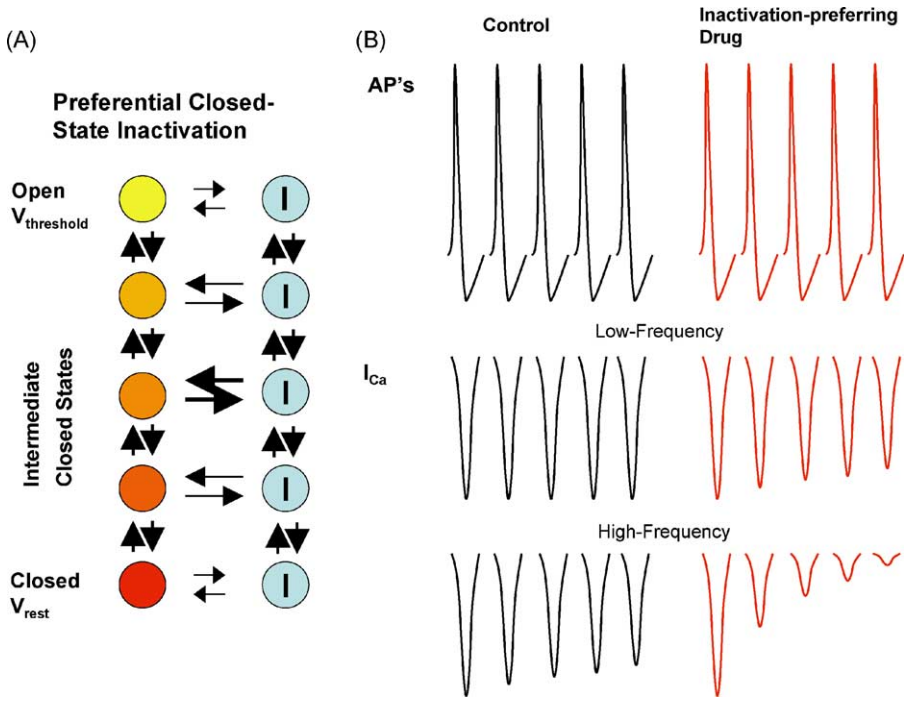

Fig. 2. (A) State-diagram of the $Ca_v2.2$ VGCC illustrating the principle of preferential closed-state inactivation. In this scheme, entry into the inactivated state occurs preferentially in these channels from voltage-dependent intermediate closed states, and provides for the frequency-dependent inactivation observed with neuronal VGCCs, including $Ca_v2.2$ channels, during periods of activation insufficient to produce current-dependent inactivation. Bursts of short-duration events such as action potentials would provide the activating depolarizations resulting in repetitive transitions from the closed to the open state. The degree of cumulative inactivation observed at the end of such a burst is dependent on the number of state transitions, their frequency, and the particular configuration of the channel. The particular $Ca_v2.2\alpha_1$ subunit, accessory (particularly β) subunit configuration, and other factors such as phosphorylation state will affect how readily particular $Ca_v2.2$ channels inactivate (the voltage for preferential inactivation). (B) Illustration of the effect of an inactivation preferring $Ca_v2.2$ VGCC inhibitor/blocker on action potentials (upper traces) and terminal $Ca_v2.2$ currents evoked by a noxious stimulus in a dorsal root ganglionic neuron. In control conditions some degree of auto-regulation of $Ca_v2.2$ current is observed at high-frequency (lower black traces). This situation is exaggerated in the presence of an inactivation-preferring blocker of these channels. Unlike state-independent block, at low blocker concentrations an inactivation-preferring $Ca_v2.2$ inhibitor, due to its inactivation-dependent shift in channel affinity, would spare most $Ca_v2.2$ function at low-frequency when cumulative inactivation is minimal (middle red traces). The drug would preferentially exert its influence to reduce $Ca_v2.2$ current at higher frequencies (lower red traces). While this may not produce cessation of the action potential burst in the primary neuron responding to a painful stimulus, reduction of $Ca_v2.2$-dependent neurotransmitter release in the terminals of these neurons, and at more central second and greater order components of the pain circuits, would reduce the response of more central neurons to the stimulus. This differential effect would be reduced at high drug concentration, and at concentrations approaching the IC_{50} for blocking the non-inactivated state of the channel the advantage of such an approach would disappear.

viewed as a mechanism that neurons use to autoregulate Ca^{2+} entry during periods of repetitive and high-frequency discharge.

The goal of drug discovery efforts directed toward use-dependent small molecule blockers of $Ca_v2.2$ is to target this auto-regulatory pathway with compounds, which have higher apparent affinity for the inactivated state. There are several hypotheses to explain a state-dependent change in the potency of a drug, based in part on data from blockers of other voltage-gated ion channels serendipitously found to possess a degree of use-dependence (e.g., antiarrhythmic drugs [62]). In the guarded receptor hypothesis, a state-dependent conformational change reveals a previously unavailable receptor to which the drug binds at high affinity [63]. In the modulated receptor hypothesis, the affinity of a drug for its receptor site in the channel complex changes as a function of channel state, but the access of the drug for its receptor is thought to be non state-dependent [64]. Finally, both the allosteric effector [65] and the modulated receptor [66] hypotheses explain shifts in inactivation by drugs via a drug-induced stabilization of the inactivated state. Each of these hypotheses explains some but not all of the observations concerning state-dependent drug action.

Whatever the mechanism, a use-dependent $Ca_v2.2$ blocker would likely be more effective during the enhanced activation patterns in pain syndromes (high-frequency) and far less effective during the firing patterns associated with normal nociceptive functioning (lower-frequency). The compound could become more efficacious in blocking channels during pathophysiological conditions with minimal side effects [67]. An illustration of such an effect is provided in Fig. 2B. This approach is currently being pursued by a number of pharmaceutical companies for the development of pain therapeutics.

It is important to note that the concept of use-dependent blockade underlies the therapeutic benefit of certain voltage-gated sodium channel blockers such as lidocaine, amitriptyline and mexiletine in hyperexcitability syndromes including neuropathic pain [67]. These compounds

exhibit use-dependent blockade of voltage-gated sodium channels and can inhibit the rapid, abnormal repetitive neuronal firing associated with pain syndromes whilst not appreciably affecting the lower-frequency neuronal activity subserving normal nociceptive surveillance. However, the therapeutic benefit of these drugs can be restricted by dose-limiting toxicities including nausea, vertigo, somnolence, convulsions and cardiac arrhythmias [67–70], which may appear inconsistent with the touted benefit of use-dependent blockade. Upon closer examination, these side effects may reflect separate issues such as accumulation of the compounds into the CNS, which over time would dissipate the functional benefit of use-dependent blockade, and/or the drugs having additional modes of action [67–69]. In the heart, use-dependent voltage-gated sodium channel blockade may be associated with proarrhythmic activity by facilitating ventricular reentry conduction events resulting in premature ventricular contractions [70]. Clearly, the development of novel compounds targeting $Ca_v2.2$ will be of value in defining whether use-dependent blockade of this channel precipitates any tissue- or organ-specific dysfunction.

5. Targets for use-dependent blockade

Identification of use-dependent blockade requires the incorporation of functional, cell-based assays. This strategy complicates elucidation of the exact molecular mechanism of drug action but has the obvious advantages of assessing use-dependent effects in relevant cell types. Opportunities exist for the development of compounds selective for particular splice variants, channel auxiliary subunits and/or regulatory pathways, which may provide effective use-dependent blockers of $Ca_v2.2$.

5.1. Splice variants

In a different sense of the term, ''functional'' use-dependent blockade can, in theory, be realized by inhibiting a particular splice variant of $Ca_v2.2$ which is only expressed during the increased excitability of nociceptive neurons found in chronic pain syndromes.

A $Ca_v\alpha_1$ subunit gene typically consists of >50 exons, spans >250 kb in the human genome and undergoes extensive alternative splicing [71,72]. The $Ca_v2.2\alpha_1$ gene is subject to considerable alternative splicing which impacts on the localization and the gating kinetics of $Ca_v2.2$ [72,73]. Tissue-specific location of a splice variant, such as reported for a C terminus variant (e.g., exon 37a [74]) in nociceptive versus non-nociceptive neurons, could provide a novel target with an acceptable therapeutic window. Sympathetic ganglia have multiple splice variants associated with different regions of the $Ca_v2.2\alpha_1$ subunit believed important for gating kinetics (e.g., the S4 voltage sensor, the extracellular loops between III-S3 and III-S4 and between IV-S3 and IV-S4 [72]). These variants are reciprocally expressed in brain and ganglia with the ganglia-dominant variant having slower activation kinetics versus the brain channel. Inclusion of exon 31a in the IV-S3-IV-S4 region is a variant found primarily in peripheral ganglia and is characterized by slowed kinetics of activation and inactivation [73]. PCR analysis of a human brain cDNA library identified two $Ca_v2.2\alpha_1$ variants, Delta-1 and Delta-2, which have deletions in the domain II–III linker region [75]. Both are extensively expressed in the human CNS and have a shift toward more depolarized potentials in the voltage-dependence of inactivation. Interestingly, there is a different sensitivity to pharmacological blockade with the Delta-1 variant being less sensitive to either ct-GVIA or ct-MVIIA than either Delta-2 or the wild type channel [75]. Therefore, the possibility exists that selective blockers of these particular splice variants may be beneficial in inhibiting either regionally important $Ca_v2.2$ channels and/or particular $Ca_v2.2$ channels associated with remodeling events in chronic pain syndromes. Site-specific small molecule ligands would be valuable to assess whether regional differences in altering $Ca_v2.2$ function translates into a specific functional blockade of pain. A 21 amino-acid variant in the domain II–III cytoplasmic loop (exon 18a), which constitutes the predominant form of $Ca_v2.2$ expressed in sympathetic ganglia, affects voltage-dependent inactivation (i.e., shifted to more depolarized potentials) in a β subunit-specific manner, without affecting voltage-dependent activation [76]. The presence of exon 18a prevents CSI [61], suggesting that a ligand specific for sites in exon 18a may shift the channel conformational towards the inactivated state. However, it is currently unknown whether small molecule modulators can distinguish between splice isoforms to an extent sufficient to result in significant functional distinction. Moreover, it is also not clear at present which particular splice variant and/or variants are operational in the various neuropathic pain syndromes.

5.2. Subunit structure

The hetero-oligomeric structure of $Ca_v2.2$ provides multiple potential substrates for targeting a particular component to affect channel function. Inhibition of β subunit binding to the α_1 core has been probed [77], but the inhibitors will likely result in a global decrease in functional expression of $Ca_v2.2$. Similarly, inhibition of channel function by enhancing $Ca_v2.2$ β subunit and G protein interaction (i.e., Gβγ binding) would be anticipated to occur at all stimulation frequencies. Inhibition of the binding of specific β splice variants, which may be upregulated during chronic pain, may theoretically lead to effective and safe therapeutics. There is no clear evidence that targeting the binding of the β subunit will result in use-dependent inhibition of channel function.

The $\alpha_2\delta$ subunit heterodimer is upregulated in animal models of neuropathic pain and is the likely target for the

GABA analogs gabapentin and pregabalin [78]. Both gabapentin (Neurontin™) and pregabalin (Lyrica™) represent important new therapeutic entities for the treatment of neuropathic pain (e.g., [79]). There has been some discrepancy regarding the ability of gabapentin to directly inhibit $Ca_v2.2$ activity in cellular or tissue preparations [80,81]. This may be due, in part, to different culture conditions. Gabapentin may owe its mechanism of action to decreasing channel expression [82] or to exerting a conformational change in the channel complex [83]. Gabapentin has been shown to exhibit a use-dependent profile by being more effective in blocking sustained versus transient nociceptive responses [84]. The specificity for $Ca_v2.2$ would loom as a possible issue for each new compound targeting $\alpha_2\delta$, as this mechanism of action would be expected to affect several VGCCs. Such a profile may, however, be tolerated as recordings from mouse spinal cord suggest that gabapentin, which exhibits a good safety profile in humans (Neurontin™, [79]), inhibits $Ca_v2.1$ [85].

5.3. Development of small molecule antagonists

One strategy for the development of small molecule antagonists has been to mimic key structural features of $Ca_v2.2$-selective conotoxins. Schroeder et al. [86] designed and synthesized cyclic pentapeptides based on structural studies with ct-CVID. The compounds had modest potency (approximately 20 μM) in blocking $Ca_v2.2$ but, importantly, were inactive at $Ca_v2.1$. ct-CVID has a better efficacy side-effect profile versus ziconotide in animals (e.g., less postural hypotension [32]) which may be a consequence of greater selectivity versus other VGCCs and/or due to a different binding site on $Ca_v2.2$ which results in greater use-dependent blockade. Baell et al. [87] reported on a series of non-peptide, benzothiazole compounds modeled after the pharmacophore(s) of ct-GVIA believed important for binding to $Ca_v2.2$. The compounds exhibited low micromolar potency for binding to $Ca_v2.2$ with an approximate 20-fold selectivity ratio versus $Ca_v2.1$. Another approach has involved classical medicinal chemistry and functional screening. Several publications have reported synthetic efforts to identify novel scaffolds as blockers of $Ca_v2.2$, but no clear evidence of use-dependent blockade has been presented [88–91]. Recently, independent presentations by the companies NeuroMed Technologies [92] and Scion Pharmaceuticals [93–95] have disclosed compounds which exhibit strong use-dependent blockade of $Ca_v2.2$ and efficacy in models of neuropathic pain. One such compound, SPI-860, exhibited an approximate five-fold increase in potency at the fifth versus first pulse in a train of depolarizing pulses at 5 Hz in Xenopus oocytes which expressed $Ca_v2.2$. Increasing the frequency to 20 Hz further enhanced the "P5/P1" ratio [95]. Similar results were obtained in transfected mammalian (HEK) cells. These compounds will likely have nuances with respect to mechanism of $Ca_v2.2$ blockade which will be of tremendous value in (a) understanding the functional consequences of different modulation events and (b) elucidating improved approaches for designing effective therapeutics in pain syndromes.

As stated above, reliable determination of use-dependent blockade necessitates evaluation of channel activity in cell-based assays by direct electrophysiological recordings. However, conventional voltage clamp and patch clamp electrophysiology techniques do not allow sufficient throughput for the timely evaluation of the large scale chemical libraries typically found in pharmaceutical companies. Recent technological advances have spawned higher-throughput approaches including parallel oocyte recording systems and microchip-based, multiwell patch clamp systems allowing whole cell recordings from hundreds to thousands of cells per day [95–97]. These new technologies are providing the necessary throughput, and definitive evaluation, for the screening of chemical libraries for new use-dependent blockers of $Ca_v2.2$.

6. Conclusions

The ion channel modulators currently on the market were developed without knowledge of the molecular structure of the channel protein, nor an understanding of the mechanism of blockade. Many compounds were discovered to be ion channel modulators post hoc. An exception is the preclinical and clinical development of Prialt™ for severe, intractable pain, which has contributed to our knowledge of $Ca_v2.2$ function and involvement in pain syndromes. This elegant work has validated $Ca_v2.2$ as an important target for alleviation of pain while also demonstrating the need for an improved safety profile for the next generation product. The progress in the biophysics and biochemical pharmacology of $Ca_v2.2$ has produced important insights into strategies for developing use-dependent blockers of this channel. New data on expression profiles in disease together with information gleaned from experiments to determine structure–activity relationships against $Ca_v2.2$ will aid drug discovery efforts to develop novel, specific therapeutics.

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