

Review

How “Pharmacoresistant” is $\text{Ca}_v2.3$, the Major Component of Voltage-Gated R-type Ca^{2+} Channels?

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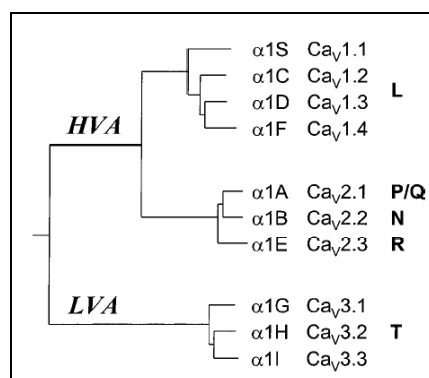
Abstract: Membrane-bound voltage-gated Ca^{2+} channels (VGCCs) are targets for specific signaling complexes, which regulate important processes like gene expression, neurotransmitter release and neuronal excitability. It is becoming increasingly evident that the so called “resistant” (R-type) VGCC $\text{Ca}_v2.3$ is critical in several physiologic and pathophysiologic processes in the central nervous system, vascular system and in endocrine systems. However its eponymous attribute of pharmacologic inertness initially made in depth investigation of the channel difficult. Although the identification of SNX-482 as a fairly specific inhibitor of $\text{Ca}_v2.3$ in the nanomolar range has enabled insights into the channels properties, availability of other pharmacologic modulators of $\text{Ca}_v2.3$ with different chemical, physical and biological properties are of great importance for future investigations. Therefore the literature was screened systematically for molecules that modulate $\text{Ca}_v2.3$ VGCCs.

Keywords: drug sensitivity; anticonvulsive drugs; experimentally induced epilepsy

1. The Ca_v2.3 Voltage-Gated Ca²⁺ Channel

Ca_v2.3 belongs to the family of voltage-gated Ca²⁺ channels which comprises ten different genes for ion conducting pore proteins (Figure 1). The ion conducting pore protein of the Ca_v2.3 VGCCs was initially cloned from a rabbit brain cDNA library [1]. After functional expression of the rat Ca_v2.3 clone, it was initially speculated that it may represent the low voltage-activated T-type Ca²⁺ channel, which was not yet structurally identified at that time [2]. However, consecutive cloning and expression of human Ca_v2.3 splice variants in *X. laevis* oocytes or HEK-293 cells revealed a VGCC with properties closer resembling a high-voltage-gated Ca²⁺ channel [3,4].

Figure 1. Evolutionary tree of voltage-gated Ca²⁺ channels (modified according to [5]). The cDNA of the putative membrane-spanning regions including the pore loops of the human sequences were aligned.



Although the structure of Ca_v2.3 deduced from sequencing of cDNA has now been known for several years [6,7], its physiological and pathophysiological roles are far from fully understood [8–10]. Evolutionarily, Ca_v2.3 may have developed very early [5,11], which may underline its great significance *in vivo*. The total quaternary structure of a Ca_v2.3-containing native VGCC is still unknown, but may contain additional subunits including the well known auxiliary β-subunits, which have been shown to modulate Ca_v2.3-mediated inward currents in heterologous expression systems [12,13]. Molecular properties of Ca_v2.3 have been characterized on the amino acid level for functional protein-protein interaction [14–16] however to date, Ca_v2.3 VGCCs have yet to be purified as has been done for L-type Ca²⁺ channels from rabbit skeletal muscle [17–20], and bovine heart [21] and for the neuronal N-type Ca²⁺ channels [22,23].

Sequence comparison of the deduced primary sequence revealed the well known intramolecular homology pattern, which is known for all voltage-gated Ca²⁺ as well as for voltage-gated Na⁺ channels. It contains four internal repeats, which have been termed domains I, II, III, and IV. Secondary structure analysis predicts 6 transmembrane segments including a random coiled short part between transmembrane segment 5 and 6, the pore forming segment (P-loop) [24]. Many of these structure predictions resemble the confirmed structural elements in the bacterial and rat voltage-gated K⁺-channel [25,26] and a bacterial Na⁺-channel [27,28].

Additional elements may contribute to the kinetic properties of Ca_v2.3-mediated inward currents as reported for structurally similar ion channels. The segments S6 participate in gating the ion channels [29–32], and the P-loops form essential parts of the selectivity filters, thereby also

influencing the speed of the ion flux through the pore [33–40]. The segment S4 acts mainly as the voltage sensor [41,42], and its detailed orientation to the pore region has been elucidated in crystals from the bacterial K^+ channel to a great extent [43].

Only segments of the cytosolic loops from $Ca_v1.2$ L-type VGCCs have been co-crystallized with functionally auxiliary subunits [44] or functionally interacting calmodulin [45–48]. Few protein interactions of $Ca_v2.3$ have been reported such as with a β -subunit [15,16] or with novel partners in heterologous expression systems [49–52], however, they have yet to be investigated by crystallization. The β -subunit interaction site with $Ca_v1.1$ and $Ca_v1.2$ is located in a conserved region between domain I and II [53,54], which also contains the interaction site of $Ca_v2.3$ with β -subunits [14–16].

The II-III linker harbors a unique site located within the arginine-rich stretch, which is responsible for Ca^{2+} -mediated modulation of the $Ca_v2.3$ voltage-gated Ca^{2+} channel [55]. It may be involved in the protein kinase C (PKC)-mediated signaling to $Ca_v2.3$ [56], linking $Ca_v2.3$ signaling to muscarinic receptor activation [57–61] and perhaps also to muscarinic enhancement of the “toxin-resistant” R-type Ca^{2+} current in hippocampal CA1 pyramidal neurons [62]. $Ca_v2.3$ also contains the better known, carboxyterminal Ca^{2+} /calmodulin interaction site, which was not only found for the members of the Ca_v2 /non-L-type but also for members of the classical L-type Ca^{2+} channel subfamily [63].

Structurally, a broad set of splice variants can be predicted from the different cloning approaches (Table 1), which result from alternate use of exon 19 encoded arginine-rich segment in the II-III loop, as well as from the alternate use of exon 45 in the carboxyterminal region [7]. $Ca_v2.3d$ was originally cloned as a fetal splice variant from human brain [4]. Interestingly, the major splice variants (Table 2) deduced from RT-PCR studies differ between brain regions [64] in mice. Splice variants of $Ca_v2.3$ from different species (see also Tables 1 and 2) as well as auxiliary subunits are tissue-specifically expressed [9]. In addition to expression in neuronal [65–69] and endocrine tissues [70–85], $Ca_v2.3$ transcripts have also been detected in mammalian heart [86–88], kidney [70,86,89], sperm [90–93], spleen [3], and retina [94–97]. Furthermore, the subcellular distribution of $Ca_v2.3$ has been investigated revealing both somatodendritic and presynaptic expression [98] with additional functional specificities [99].

Table 1. Splice variants of voltage-gated $Ca_v2.3$ R-type Ca^{2+} channels. Exon 19 is encoding an arginine-rich segment of the cytosolic loop between domain II and III, which is responsible for a transient positive Ca^{2+} feedback, when cytosolic Ca^{2+} is increasing by Ca^{2+} influx through the channel itself. Exon 45 is encoding a carboxyterminal insertion of unknown function. Details of exon 20 sequence are found in [7].

Nomenclature, splice variant		Structure related to alternate exons expressed (+)			Expression (tissue and species)	Ref.
Novel terms	Old terms	Exon 19 (57 nts)	Segment (21 nts) in exon 20	Exon 45 (129 nts)		
$Ca_v2.3a$	alpha1E-1	-	+	-	Rat cerebellum	[100]
$Ca_v2.3b$	alpha1E-2	+	-	-	Less important in CNS	[3]
$Ca_v2.3c$	alpha1E-3	+	+	-	Dominant in CNS	[3]
$Ca_v2.3d$	alpha1Ed	+	+	+	Human fetal brain	[4]
$Ca_v2.3e$	alpha1Ee	-	+	+	Pancreas, kidney, heart	[70,101]
$Ca_v2.3f$	alpha1Ef	+	-	+	Rat cerebellum	[100]

Table 2. Transcripts of major splice variants of voltage-gated $\text{Ca}_v2.3$ R-type Ca^{2+} channels expressed in different brain regions [64].

Brain region (mouse)	Major splice variant	Miscellaneous
Neocortex	$\text{Ca}_v2.3c$	Minor amounts of $\text{Ca}_v2.3e$
Hippocampus	$\text{Ca}_v2.3c$	Minor amounts of $\text{Ca}_v2.3e$
Thalamus	$\text{Ca}_v2.3c$	Substantial amounts of $\text{Ca}_v2.3e$ and $\text{Ca}_v2.3f$
Cerebellum, mesencephalon, medulla oblongata	$\text{Ca}_v2.3e$	minor amounts of $\text{Ca}_v2.3a$

In heterologous expression systems, $\text{Ca}_v2.3c$ [3] and $\text{Ca}_v2.3d$ [4,102] inward currents are activated at test potentials of about -30 mV. The single channel conductance is about 14 pS [103], and the channel kinetics measured by patch-clamp recordings reveal a fast activating and inactivating channel type with transient inward current characteristics [7,55], similar but not as fast as observed for T-type voltage-gated Ca^{2+} channels [13].

2. Selective and Non-Selective Antagonists of $\text{Ca}_v2.3$

The first “pharmacoresistant” Ca^{2+} current in vivo was recorded and published in 1993 [104,105], which means it occurred between the years 1987 (the first cloning of a VGCC subunit [106]) and 1994 (final cloning of the remaining high-voltage gated Ca^{2+} channels). Doe-1, cloned from *Drosophila ommata*, represented a novel Ca^{2+} channel type, which was insensitive towards dihydropyridines, but was antagonized rather than activated by $5 \mu\text{M}$ Bay K. This channel type was only slightly and readily reversibly inhibited by $5 \mu\text{M}$ ω -conotoxin-MVIIC, was insensitive towards ω -agatoxin-IVA, and fully reversibly blocked by ω -conotoxin-GVIA, an irreversible antagonist of N-type Ca^{2+} channels [104]. Interestingly, the same group identified a similar Ca^{2+} current component in rat cerebellar granule neurons and called the doe-1-like component “R-type current” [105].

The peptide antagonist SNX-482, which was initially purified from the venom of the tarantula *Hysterocallis gigas* [107] blocks $\text{Ca}_v2.3$ with an IC_{50} value of 15–30 nM and was the first and still is the only $\text{Ca}_v2.3$ -prevalent antagonist. At concentrations higher than 500 nM SNX-482 also inhibits N-type Ca^{2+} currents [107], whereas L-type Ca^{2+} currents are inhibited by about 25% at concentrations of 200 nM SNX-482 [108]. Therefore, it only can be regarded as $\text{Ca}_v2.3$ -prevalent, but not as $\text{Ca}_v2.3$ -specific or -selective.

In cerebellar granule cells, two $\text{Ca}_v2.3$ isoforms could be distinguished from each other by their varying SNX-482 IC_{50} values of 6 nM and 81 nM, and a third R-type Ca^{2+} current component by its insensitivity to SNX-482 [109].

The first gene inactivation of $\text{Ca}_v2.3$ led to knock-out mice, which in cerebellar granule cells and in DRG neurons still expressed a drug insensitive Ba^{2+} current. The peak inward current (I_{Ba}) was even larger than in cultured mouse neurons from control mice (knock-out I_{Ba} 113 ± 27 pA ($n = 5$); control 85 ± 21 pA ($n = 9$)) [110]. Only the wild type cultured neurons were inhibited by SNX-482, but not the neurons from $\text{Ca}_v2.3$ -deficient mice, leading to the conclusion that a non- $\text{Ca}_v2.3$ -dependent R-type current may exist.

In murine hippocampal and neocortical neurons, $\text{Ca}_v2.3$ contributes not only to the SNX-482-sensitive component of the R-type Ca^{2+} current, which was recorded in the presence of combination of Ca^{2+} channel antagonists (ω -conotoxin-GVIA, $2 \mu\text{M}$; ω -conotoxin-MVIIC, $3 \mu\text{M}$; ω -agatoxin-IVA, 200 nM;

nifedipine, 10 μM), but also to the SNX-482-insensitive part [66]. Interestingly, the voltage of half-maximal activation ($V_{1/2, \text{act}}$) was shifted to more positive voltages in all three cell types investigated (dissociated CA1 pyramidal cells, dentate gyrus cells, neocortical neurons), specially in the neocortex, where it was reduced from -68 ± 2 mV to -58 ± 7 mV [66]. Overall, it may be useful to keep in mind that the R-type Ca^{2+} current may be more than only the $\text{Ca}_v2.3$ -gene encoded Ca^{2+} channel in neuronal tissues [8,111,112].

Divalent and trivalent heavy metal cations were often used to antagonize either all voltage-gated Ca^{2+} inward currents (Cd^{2+} , La^{3+}) or to specifically inhibit some T-type and the R-type Ca^{2+} current (Ni^{2+}). Unfortunately, the half maximal concentrations for $\text{Ca}_v2.3$ and $\text{Ca}_v3.2$ are close to each other (10–30 μM), rendering Ni^{2+} blockade unsuitable for distinction of $\text{Ca}_v2.3$ currents in tissue in which $\text{Ca}_v3.2$ is also expressed. Physiologically, homeostasis of other divalent cations like Cu^{2+} and Zn^{2+} may play an important role [10,113,114], notably also in neurodegenerative disease [115].

Table 3 summarizes the effect of drugs and toxins on $\text{Ca}_v2.3$ reported in the literature. Most drugs in the table are non-selective, in the sense that currents through other Ca^{2+} channel $\text{Ca}_v\alpha1$ subunits are also antagonized with an IC_{50} not larger than tenfold. Many substances show inhibitory effects on $\text{Ca}_v2.3$ or on R-type Ca^{2+} currents. One set of drugs is related to anticonvulsive effects, others are used as anesthetic drugs. Even high concentrations of classical Ca^{2+} channel antagonists can inhibit $\text{Ca}_v2.3$ induced inward currents as shown for the dihydropyridines isradipine [87] and nifedipine [116]. Routinely, in order to block L-type voltage-gated Ca^{2+} channels, a dihydropyridine concentration of around 10 μM is chosen by electrophysiologists. Considering that such high concentrations of isradipine or nifedipine substantially block E-/R-type Ca^{2+} currents, lower concentrations of e.g., isradipine of 0.5 μM are more suitable, in order to observe antagonism by low concentrations of SNX-482 as shown for cardiac E-/R-type Ca^{2+} currents in murine myocytes [101]. However, one has to keep in mind that SNX-482 may block L-type Ca^{2+} current at elevated concentrations [108].

3. Physiological Functions, in Which $\text{Ca}_v2.3$ may be Involved, as Deduced from $\text{Ca}_v2.3$ -Deficient Mice

Many of the experimental results from gene-inactivated mice cannot automatically be transferred to human physiology and pathophysiology of human diseases. But some basic conclusions may be drawn from these investigations of $\text{Ca}_v2.3$ -deficient mice, which were generated and analysed in several different laboratories (for detail, see Kamp *et al.* [8]).

$\text{Ca}_v2.3$ is expressed in many regions of the CNS and also in peripheral organs and tissues, which makes it difficult to explore its full function *in vivo*. $\text{Ca}_v2.3$ triggers or participates in the release of several neurotransmitters such as dopamine in the substantia nigra [117]. In the hippocampus $\text{Ca}_v2.3$ contributes to fast glutamatergic transmission [118], where it is also involved in long term potentiation at the mossy fiber – CA3 synapses. Therefore, $\text{Ca}_v2.3$ participates in basic processes related to learning and memory formation [67,119–121]. Furthermore, $\text{Ca}_v2.3$ is an important regulator in spines: activation of $\text{Ca}_v2.3$ triggers opening of small conductance Ca^{2+} -activated K^+ -channels in CA1 hippocampal pyramidal neurons [122–124], suggesting spine-restricted local microdomains, which are important for synaptic signalling [125]. R-type Ca^{2+} currents, which were recorded as Ni^{2+} -sensitive

tail currents, are available at resting potential and contribute to after-depolarization, and thus to the initiation of burst firing in CA1 hippocampal neurons [126].

Table 3. Selected antagonists of Ca_v2.3 (modified according to: Wrubel, 2009 [127]). Recombinant Ca_v2.3 was expressed in different cell lines and was cotransfected with auxiliary subunits (β-subunits from different species). Note, trace metals must be applied under well defined conditions, which provide buffering of the cation of interest [10]. Abbreviations: n.t. = not tested.

Substance	Application	IC ₅₀ or K _d [μM]	Amount of max. Inhibition	Selectivity	Ref.
SNX-482	Peptide toxin	0.015–0.030	> 80 %	Ca _v 2.3-prevalent	[107,108,128–130]
ω-Aga-IVA	Peptide toxin	0.051	80%	non-selective	[116]
ω-Aga-IIIa	Peptide toxin	0.003–0.010	100%	non-selective	[107]
Ni ²⁺	Unphysiological	27.4/303	100%	non-selective	[3,131]
Cd ²⁺	Unphysiological	0.8	100%	non-selective	[3]
Zn ²⁺	Trace element	31.8	>90%	non-selective	[132]
Zn ²⁺ (calibrated)	Trace element	1.3	100%	non-selective	[10]
Cu ²⁺	Trace element	0.018	100%	non-selective	[10]
Topiramate	Anticonvulsive	50.9	>70%	non-selective	[133]
Lamotrigine	Anticonvulsive	>10		non-selective	[134]
Sipatrigine	Anticonvulsive	10	100%	non-selective	[134]
202W92	Anticonvulsive	56	100%		[134]
Ethosuximide	Anticonvulsive	20000	100%	non-selective	[135]
MPS (α-methyl-phenylsuccinimide)	Anticonvulsive	2300	100%		[135]
Phenytoin	Anticonvulsive	360	100%		[135]
Phenobarbital	Anticonvulsive	2700	>80%		[135]
Pentobarbital	Anticonvulsive	600	100%		[135]
Halothane	Inhalation anaesthetic				[136,137]
Isoflurane	Inhalation anaesthetic	206	100%		[136,138,139]
Fomocaine	Local anaesthetic	95	100%		[140]
Procaine	Local anaesthetic				[140]
Octanol	Organic solvent	206	100%		[135]
(+)-ACN	Steroid anaesthetic	5.3–10.2	100%		[141]
(+)-ECN	Steroid anaesthetic	9.9–16.1	>70%		[141]
Flecainide	Antiarrhythmic	320			[140]
Penfluridol	Antipsychotic	13			[140]
Verapamil	Antihypertensive	100	100%	non-selective	[142]
Diltiazem	Antihypertensive	220	100%	non-selective	[4,142]
Isradipine	Antihypertensive	9.1	100%	non-selective	[87]
Nicardipine	Antihypertensive	1	n.t.	non-selective	[116]
Mibefradil	Antihypertensive	0.4/6.5	100%	non-selective	[143]
Amiloride	Diuretic	7400	100%	non-selective	[135]
Ethoxzolamide	Carboanhydrase inhibitor/anticonvulsive	1	70%		[144]
Eugenol	Analgetic				[145]
Bisphenol A	Environmental pollutant	26	50%	non-selective	[146]

The existence of a fetal brain $\text{Ca}_v2.3$ isoform [4] and the changes in expression of $\text{Ca}_v2.3$ during neuronal development point to an important role of $\text{Ca}_v2.3$ during early prenatal stages [147–149]. At nerve terminals of the calyx of Held, N- and R-type Ca^{2+} channels are replaced by P-/Q-type Ca^{2+} channels during development [150].

$\text{Ca}_v2.3$ -deficient mice reveal altered pain response [151], and transcripts of two different splice variants of $\text{Ca}_v2.3$ could be identified in rat nociceptive neurons [152]. The major splice variant was $\text{Ca}_v2.3e$, which was also detected in the cerebellum, heart and endocrine system (Tables 1 and 2).

$\text{Ca}_v2.3$ is highly expressed in the amygdala, in which the R-type Ca^{2+} current represents the largest component of high-voltage gated Ca^{2+} currents. $\text{Ca}_v2.3$ -deficient mice exhibited signs of enhanced fear assuming that $\text{Ca}_v2.3$ -based R-type Ca^{2+} currents in the amygdala may be associated with fear [153].

$\text{Ca}_v2.3$ -deficient mice represent an important model for convulsive and non-convulsive seizures as was summarized in [9]. Based on the initial detection of $\text{Ca}_v2.3$ transcripts in the insulinoma cell line INS-1 [70,73], additional investigations were performed with $\text{Ca}_v2.3$ -deficient mice, which showed disturbance not only of glucose-induced insulin release [72,75], but also of glucose-mediated glucagon suppression [74], and more important even disturbances of glucose-mediated somatostatin-release [80].

SNX-482 sensitive R-type Ca^{2+} current was related to the release of gonadotropin-releasing hormone [81] and of oxytocin [76,77]. Overall, peptide hormone release often appears to be triggered by $\text{Ca}_v2.3$ VGCCs, possibly by producing the global increase in cytosolic Ca^{2+} required for refilling of the readily releasable pool of granules during the second phase of insulin release [75,154].

After cerebral aneurysm rupture and subarachnoid hemorrhage $\text{Ca}_v2.3$ has been shown to contribute to cerebral artery constriction *i.e.*, vasospasm [155], a devastating delayed event causing often fatal strokes. Accordingly intracisternal administration of SNX-482 reduced delayed vasospasm in a rat model of subarachnoid hemorrhage [156].

The expression of $\text{Ca}_v2.3$ in cardiomyocytes is still under discussion: $\text{Ca}_v2.3$ protein has yet to be detected reliably in murine cardiomyocytes, but transcripts could be amplified by single cell RT-PCR from microscopically identified murine cardiomyocytes [87,88]. Furthermore, $\text{Ca}_v2.3$ ablation causes cardiac arrhythmia and disturbances in autonomic cardiac control, suggesting that $\text{Ca}_v2.3$ in pacemaker cells as well as in autonomic nerve endings may participate in cardiac signalling [101].

In future, more specific $\text{Ca}_v2.3$ modulators will be a key in establishing the exact role of $\text{Ca}_v2.3$ in the physiological and pathophysiological processes, that it contributes to. Furthermore, recent evidence points to $\text{Ca}_v2.3$ as a potential pharmacologic target in therapy of epilepsy, chronic pain, endocrine disturbances and vasospasms after subarachnoid hemorrhage. In this light, non-ion selective $\text{Ca}_v2.3$ inhibitors with favourable pharmacokinetics could represent new therapeutic strategies for these disorders.

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Conflict of Interest

The authors declare no conflict of interest.

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