

Pharmacology of L-type Calcium Channels: Novel Drugs for Old Targets?

Jörg Striessnig*, Nadine J. Ortner and Alexandra Pinggera

Pharmacology and Toxicology, Institute of Pharmacy and Center of Molecular Biosciences, University of Innsbruck, Innsbruck, Austria

Abstract: Inhibition of voltage-gated L-type calcium channels by organic calcium channel blockers is a well-established pharmacodynamic concept for the treatment of hypertension and cardiac ischemia. Since decades these antihypertensives (such as the dihydropyridines amlodipine, felodipine or nifedipine) belong to the most widely prescribed drugs world-wide. Their tolerability is excellent because at therapeutic doses their pharmacological effects in humans are limited to the cardiovascular system. During the last years substantial progress has been made to reveal the physiological role of different L-type calcium channel isoforms in many other tissues, including the brain, endocrine and sensory cells. Moreover, there is accumulating evidence about their involvement in various human diseases, such as Parkinson's disease, neuropsychiatric disorders and hyperaldosteronism. In this review we discuss the pathogenetic role of L-type calcium channels, potential new indications for existing or isoform-selective compounds and strategies to minimize potential side effects.

Keywords: Calcium homeostasis, drug discovery L-type channels, neuropsychiatric disorders, pharmacology, voltage gated calcium channels.

1. INTRODUCTION

Almost 50 years ago the German physiologist Albrecht Fleckenstein discovered that organic molecules, such as verapamil, closely resembled the cardiodepressant actions of β -adrenergic receptor antagonists. However, their pharmacological effects were reversed by adrenergic agonists and thus could not be explained by binding to β -receptors. They also did not alter Na^+ -dependent action potentials but their effects could be mimicked by withdrawal of Ca^{2+} ions from the medium and weakened ("antagonized") by elevated extracellular Ca^{2+} . Instead, Fleckenstein discovered that they specifically inhibited cellular Ca^{2+} ion influx and thus termed these drugs " Ca^{2+} antagonists". Their pharmacological actions are fully explained by block of voltage-gated L-type Ca^{2+} channels (LTCCs) in the nanomolar concentration range.

In this minireview we discuss evidence that block of LTCCs outside the cardiovascular system, in particular in the brain, may also provide therapeutic benefit. The evidence for potential efficacy in new indications as well as for potential safety issues is based on our growing understanding of the physiological and pathophysiological role of these channels in the CNS and in peripheral tissues, obtained both in mice and from human genetics. Novel indications, such as neuroprotection in Parkinson's disease (PD) or neuropsychiatric disorders, may benefit from treatment with already licensed LTCC drugs although cardiovascular side effects may limit their use.

We will therefore also outline current strategies to develop drugs preferentially targeting LTCCs in the brain.

To understand novel pharmacological concepts we first briefly summarize our current knowledge about the molecular pharmacology and functional role of different LTCC isoforms in the mammalian organism.

2. STRUCTURE AND MOLECULAR PHARMACOLOGY OF LTCCs

2.1. Structural Aspects

The voltage-sensitive, Ca^{2+} -selective pores of all voltage-gated Ca^{2+} channels are comprised by $\alpha 1$ -subunits which form hetero-oligomeric complexes with modulatory accessory subunits (different β - and $\alpha 2\delta$ -subunit isoforms; [1]). Ten $\alpha 1$ -subunit isoforms are encoded by separate genes with distinct pharmacological and biophysical properties and with different tissue expression and subcellular distribution [1, 2]. Detailed reviews about the structure, function and modulation of LTCCs have been published recently [2-6]. The Cav1 family (Cav1.1-Cav1.4) of $\alpha 1$ -subunits form the L-type Ca^{2+} channel family. They are all sensitive to the main chemical classes of Ca^{2+} -channel blockers (CCBs; dihydropyridines (DHPs), phenylalkylamines, benzothiazepines) but differ with respect to tissue expression and gating characteristics [1, 2]. Cav1.1 channels are almost exclusively found in skeletal muscle where they trigger depolarization-induced Ca^{2+} release from ryanodine receptors of the sarcoplasmic reticulum [7]. Cav1.4 channel expression is largely restricted to the retina [8, 9]. In contrast, Cav1.2 and Cav1.3 are expressed in many tissues and are even present together in the same cells. However, they differ in their gating properties and protein interactions which allows them to serve different physiological functions. A major distinguishing feature is the

*Address correspondence to this author at the Department of Pharmacology and Toxicology, Institute of Pharmacy, Center for Molecular Biosciences, University of Innsbruck, A-6020 Innsbruck, Austria; Tel/Fax: +43-512-507-58800, +43-512-507-58899; E-mail: joerg.striessnig@uibk.ac.at

9-15 mV more negative activation range of Cav1.3 channels [10, 11] (Fig. 1a). This permits them to open at threshold potentials in sinoatrial node (SAN) cells and neurons and to contribute to pacemaking and stabilization of plateau potentials [2, 12-14]. They also differ with respect to their modulation by C-terminal alternative splicing (for review see [2]).

2.2. Molecular Pharmacology

Three main chemical classes of organic Ca²⁺ channel drugs can be distinguished: Dihydropyridines (prototype nifedipine), phenylalkylamines (prototype verapamil) and benzothiazepines (prototype (+)-cis-diltiazem). Despite their different structure they all bind within a single overlapping drug binding region close to the pore and to the proposed activation gate of the channel's α_1 -subunit [15-17]. They reversibly interact with this binding domain in a stereoselective manner and, in isolated membranes at zero membrane potential, with dissociation constants in the nanomolar range (0.1 - 50 nM; [16]). By binding to this site they interfere with the normal voltage-dependent cycling of the channel through its resting, open and inactivated states (modulated receptor model; [18, 19]). The uncharged DHPs primarily stabilize and induce inactivated channel states. They possess much higher affinity for the inactivated channel conformation and therefore their IC₅₀ for block of cardiovascular LTCCs is much lower at more depolarized voltages ("voltage-dependent block" [10, 18-20], Fig. 1b). Phenylalkylamines and benzothiazepines bind to open and inactivated states with high affinity. At physiological pH they primarily exist as positively charged organic cations and can access their binding site from the cytoplasmic side during channel opening [21, 22]. They stabilize inactivated channel states, thereby slowing recovery from inactivation. This results in a pronounced frequency- or use-dependent inhibition [22, 23]. Based on these state-dependent binding characteristics CCBs should be considered gating modifiers. Interference of verapamil and diltiazem with LTCC gating always reduces inward Ca²⁺ currents through LTCCs. This is in contrast to DHPs: clinically used DHPs (such as amlodipine, felodipine or isradipine) are always inhibitory; however, (-)-BayK8644 and (+)-SDZ202-791 are examples for gating modifiers that cause changes in Ca²⁺ current kinetics (increase in current amplitudes, tail currents and single channel open probability) that enhance Ca²⁺ influx during typical electrical activity patterns [20].

The state-dependent modulation by CCBs also provides these drugs with tissue-selectivity: inactivated channel states are favored in arterial smooth muscle due to their more depolarized resting membrane potential and long lasting depolarizations [18, 24]. The preferential affinity of DHPs for inactivated LTCCs can therefore explain their potent vasodilating effect without affecting cardiac inotropy at therapeutic doses. In addition to a tonic block component, verapamil and diltiazem also show pronounced use-dependent effects. By slowing the recovery of channels from inactivation the number of channels available for Ca²⁺ influx decreases when the time between depolarizations shortens. Inhibition by a given concentration therefore increases with higher heart rates. This also rationalizes the clinical use of verapamil for the treatment of tachyarrhythmias.

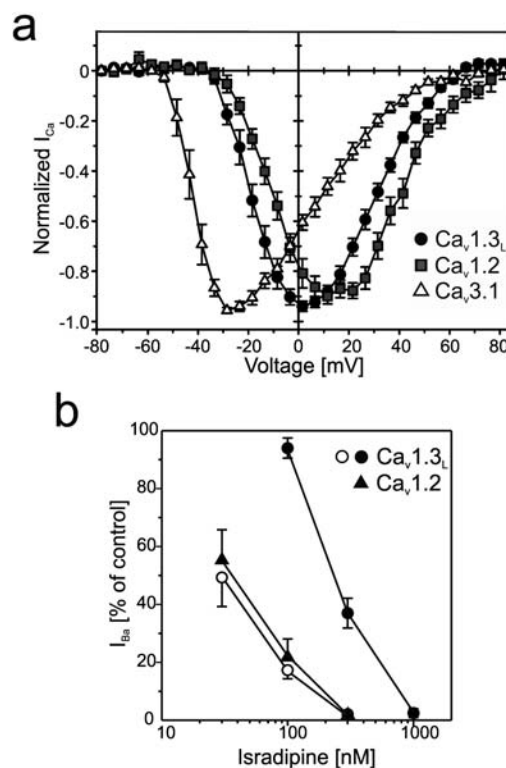


Fig. (1). Distinct biophysical and pharmacological properties of Cav1.2 and Cav1.3. a, When heterologously expressed in tsA-201 cells Cav1.3_L channels (L designating the C-terminally long splice variant) activate at lower voltages (and with a steeper voltage-dependence) than Cav1.2. This lower activation range has been confirmed for native LTCCs in tissues allowing separation of Cav1.3 and Cav1.2 current components (sinoatrial node and adrenal chromaffin cells, [25, 26]). Classical low-voltage activated T-type Ca²⁺ channels (shown for Cav3.1) activate more negative than Cav1.3. The half-maximal activation voltages strongly depend on the concentration and nature of the extracellular charge carrier and the intracellular cations replacing potassium (for details see [27]). In panel a recordings were made with 15 mM extracellular Ca²⁺ as charge carrier. Current-voltage relationships are parallel-shifted by about 15 mV to more negative voltages [11, 28] at physiological (2 mM) extracellular Ca²⁺ concentrations, placing Cav1.3 activation in a voltage-range allowing subthreshold inward current in many cells. Notice that C-terminally short Cav1.3 splice variants activate at about 7 mV more negative voltages than Cav1.3_L [27] (not illustrated). b, Concentration-response relationship of inhibition of Cav1.3 (long splice variant) and Cav1.2 by isradipine determined during 10-ms depolarizations to positive voltages from holding membrane potentials of -90 mV (filled circles) and -50 mV (open circles). Notice the strong voltage-dependence of Cav1.3 inhibition. Similarly, isradipine inhibits Cav1.2 at even lower concentrations at -50 mV holding potential (not shown). Taken from [10] and [27] with modifications.

As outlined below, Cav1.2 is the LTCC isoform in arteries and cardiac myocytes. Different Cav1.2 splice variants are expressed in these tissues which further enhance the state-dependent inhibition in smooth muscle without altering the affinity for the DHP binding pocket itself [29]. These complex pharmacodynamic aspects have to be taken into account in ongoing efforts to develop novel generations of blockers as discussed below.

3. LTCC FUNCTION AND ROLE IN HUMAN DISEASE

3.1. Cochlear and Vestibular Hair Cells

Whereas fast neurotransmitter release in neurons is tightly regulated by voltage-gated Cav2 channels (P/Q-, N- and R-type currents, [30]), LTCCs control presynaptic glutamate release in sensory cells. Cav1.3 is the major LTCC expressed in hair cells of the inner ear (inner and outer hair cells) and vestibular organ. Accordingly, Cav1.3 α 1-subunit deficient mice (Cav1.3^{-/-}) and humans (SANDD syndrome, [31]) are deaf. Its role for normal cochlear development, hearing and vestibular function has recently been reviewed [9]. In inner hair cells they are tethered to the presynaptic protein complexes forming so-called ribbon synapses. Exocytosis in inner hair cells is triggered by graded changes in membrane potential induced by sound. Channel activity and Ca²⁺ influx therefore follow the graded changes in receptor potentials which requires that these channels must be active within the negative operating range of receptor potentials (-70 – -20 mV, [32]) and inactivate slowly. Cav1.3 channels perfectly fulfill these criteria. Although Cav1.3-mediated neurotransmitter release can be completely blocked *in vitro* by high concentrations of CCBs [33, 34], no hearing impairment has yet been reported as a side effect of treatment with these drugs.

3.2. Brain

Like in the heart, Cav1.2 and Cav1.3 are the only LTCCs present in the brain [35]. Their α 1-subunits can combine with all four different β -subunit isoforms [36], exist in different alternatively spliced variants [11, 37] and, in the case of Cav1.3, are even subject to RNA-editing [38]. Cav1.2 and Cav1.3 are postsynaptic channels localized predominantly in the soma, spines and shafts of dendrites [39, 40]. They shape neuronal firing, activate Ca²⁺ signaling pathways involved in excitation transcription coupling (ETC) and thus regulate neuronal plasticity associated with learning, memory, drug addiction and neuronal development.

The physiological role of the two brain LTCC isoforms has been primarily revealed from experiments with genetically modified mice [8]. These included Cav1.3^{-/-} mice, conditional Cav1.2^{-/-} mice and a mouse model expressing fully functional but DHP-resistant Cav1.2 channels (Cav1.2DHP^{-/-}, for review [2, 8]).

3.2.1. Memory Function

Hippocampal function appears to depend more on Cav1.2 than Cav1.3. Conditional deletion of Cav1.2 α 1 in the brain reduced NMDA-receptor-independent late-phase long-term potentiation (LTP) in CA3 - CA1 synapses and caused a severe impairment of hippocampus-dependent spatial memory [41, 42]. This was accompanied by reduced activation of the mitogen-activated protein kinase (MAPK) pathway and cAMP response element (CRE)-dependent transcription in CA1 pyramidal neurons. In contrast to Cav1.2, no deficit in hippocampal LTP and spatial memory encoding in the Morris water maze was observed in Cav1.3^{-/-} mice [43]. Therefore pharmacological inhibition of Cav1.2, but not of Cav1.3, is expected to impair spatial memory in the hippo-

campus. The two LTCC isoforms seem to serve distinct functions in these neurons: Cav1.2 preferentially signals to pathways initiating ETC and mediates LTP whereas Cav1.3 couples to Ca²⁺-activated K⁺-channels and shapes the electrical properties [41, 42].

Distinct roles of the two LTCCs were also found for fear memory. Cav1.3 is not essential for acquisition and extinction of conditioned contextual fear memory [43, 44] but is required for consolidation [43, 45]. In Cav1.3-deficient mice impaired consolidation of fear memory was attributed to a significant decrease of the LTP in the basolateral amygdala synapse receiving input from the entorhinal cortex [45]. Brain-specific Cav1.2 knockout mice revealed that Cav1.2 carries essentially all of the measurable L-type current in lateral amygdala neurons. Acute pharmacological block of these channels inhibited thalamo-lateral amygdala LTP which could explain the observed inhibition of auditory cued fear memory acquisition [46].

Specific deletion of Cav1.2 channels in mouse anterior cingulate cortex impaired observational fear learning and reduced behavioral pain responses [47]. These results demonstrate a complex involvement of Cav1.2 channels in fear responses.

3.2.2. Emotional and Drug-Taking Behaviors

Cav1.2 and Cav1.3 deficiency also lead to opposite effects on anxiety- and depression-like behaviors. Reduction of Cav1.2 expression in mouse forebrain induces anxiety-like behaviors in different experimental paradigms [48]. This was not affected by shRNA-induced knockdown of Cav1.3 α 1-subunits. In contrast, Cav1.3-deficiency induces anxiolytic-like behavior (which, however, may be explained by the deaf phenotype [49]) and an antidepressant-like phenotype (not explained by deafness; [49]). Accordingly, selective activation of Cav1.3 channels (in the Cav1.2DHP^{-/-} mouse model, [50]) by the Ca²⁺ channel activator BayK8644 induces depression-like behavior [50, 51].

LTCC activity also controls signaling pathways that are involved in neuronal plasticity associated with drug-dependence. Using locomotor sensitization as a model for psychostimulant-induced long-term plasticity in Cav1.3^{-/-} and Cav1.2DHP^{-/-} mice, a distinct role for Cav1.2 and Cav1.3 LTCCs was found [52]. Whereas Cav1.3 mediates the development of sensitization, Cav1.2 is responsible for expression of the psychostimulant-induced sensitized response. Intracellular signaling pathways mediating Cav1.2 and Cav1.3 effects have been identified (for review see [2]).

Based on these experiments in mice it is tempting to speculate that long-term pharmacological inhibition of Cav1.3 channels in the brain may be of clinical value for treating major depression and drug-dependence.

3.2.3. Dopamine Neuron Physiology and Pathophysiology

The negative activation voltage range enables Cav1.3 channels to carry inward currents at threshold membrane potentials in neurons and thus shape neuronal firing patterns. In striatal medium spiny neurons they promote neuronal spiking by stabilizing glutamate-induced upstate potentials [13]. Dopamine depletion in the striatum in PD causes a

rapid and profound loss of spines and glutamatergic synapses on dopamine D2-receptor-expressing striatopallidal medium spiny neurons [13, 53]. This synaptic pruning also requires Cav1.3 [13] indicating an important role of Cav1.3 signaling for regulation of synaptic morphology.

Cav1.2 and Cav1.3 are both also present in spontaneously firing substantia nigra pars compacta (SNc) dopamine neurons, which are vulnerable to degeneration in PD [54]. At plasma concentrations corresponding to therapeutic levels in humans the DHP CCBs (isradipine, nimodipine) protect SNc neurons from degeneration in neurotoxin-based models of PD in rodents and non-human primates [55-58]. Therefore it is likely that inhibition of LTCC activity, including Cav1.3, in SNc dopamine neurons accounts for this neuroprotective effect. This is the basis for a compelling "Ca²⁺ hypothesis" [59] of high SNc neuron vulnerability: although LTCCs play no major role for pacemaking [60, 61], they mediate dendritic Ca²⁺ transients during spontaneous action potentials in these permanently active neurons which enhances oxidative stress [59]. LTCCs also enhance NMDA-receptor induced bursting [62] and promote dopamine synthesis and α -synuclein-dependent L-DOPA-induced degeneration of SNc dopamine neurons [63]. These data strongly favor a role of LTCCs (and in particular of Cav1.3) in PD pathophysiology.

Recent studies also discovered an important physiological role of Cav1.3 Ca²⁺ channels in SNc neurons [64]. Through D2-autoreceptors dendritic dopamine release tunes firing rates and dopamine release of SNc dopamine neurons in a negative feedback loop through activation of G-protein coupled K⁺-channels (GIRK2, KCNJ6) [64]. Recordings in midbrain slices revealed that this effect desensitizes quickly in juvenile neurons but is converted to a sensitized response by high dopamine states (such as a single L-DOPA injection *in vivo*). The expression of this sensitized D2-autoreceptor phenotype required Cav1.3 channel activity, intracellular Ca²⁺, and the interaction of the neuronal Ca²⁺ sensor NCS-1 with D2-autoreceptors [64]. Thus, Cav1.3 channels provide a Ca²⁺ source for a Ca²⁺-dependent NCS-1/D2-autoreceptor interaction, leading to reduced D2-autoreceptor internalization in response to prolonged dopamine exposure [64].

3.2.4. Neuronal Development and Neuropsychiatric Disorders

In addition to the above observations in medium spiny neurons on synaptic pruning, a role for Cav1.3 in synaptic development has also been described in the auditory brainstem. Cav1.3 is required for normal refinement of inhibitory synapses in projections from the medial nucleus of the trapezoid body to the lateral superior olive [65] during the first 2 postnatal weeks. In Cav1.3^{-/-} mice projections were not eliminated up to hearing onset, synaptic strengthening was strongly impaired and development of normal glycinergic transmission before hearing onset was not established [65]. Cav1.3 deficiency is also associated with a drastically reduced volume in all auditory brainstem centers (such as in the lateral superior olive), but not other selected brain regions, already before hearing onset [66]. Notably, this effect was not due to inner ear dysfunction because these histopathological changes and aberrant central auditory processing are also observed after conditional deletion of Cav1.3 only in

the auditory brainstem with preserved expression in the cochlea [67].

Human genetics strongly point to an important role of dysfunctional Cav1.2 and Cav1.3 LTCCs in the pathophysiology of neurodevelopmental and neuropsychiatric diseases, including autism spectrum disorders (ASD). Timothy syndrome is a rare multiorgan disorder resulting from Cav1.2 α 1 gain-of-function mutations (CACNA1C gene; OMIM #601005) and surviving patients often also develop autism and epilepsy [68]. Knockin mice expressing the human mutation replicate autistic behavioral traits [69]. Channels with a Timothy syndrome mutation cause activity-dependent dendrite retraction in rat and mouse neurons and in induced pluripotent stem cell-derived neurons from Timothy syndrome individuals. Like for Cav1.3, this indicates an important role also of Cav1.2 for normal neuronal development.

A role of Cav1.2 is also supported from genome-wide association studies linking intronic CACNA1C SNPs with enhanced risk for a range of psychiatric disorders of childhood and adult onset, including bipolar disease and ASD [70, 71]. Recently, also Cav1.3 α 1-subunit (CACNA1D gene) *de novo* gain-of-function mutations have been reported in two patients with sporadic autism and intellectual disability [72, 73] as well as in two patients with a severe congenital syndrome with primary aldosteronism, neurodevelopmental deficits and seizures at early age (PASNA, OMIM #615474; [74]. As discussed below, the question, if these patients would benefit from LTCC blocker therapy needs to be addressed. Moreover, these genetic data provide strong evidence that Cav1.2 and Cav1.3 channel dysfunction in the brain confer a large risk for neuropsychiatric disorders in humans. This raises the important question to which extent more subtle functional changes of these channels also contribute to overall neuropsychiatric disease risk.

3.3. (Neuro)Endocrine Cells

The differential role of Cav1.2 and Cav1.3 LTCCs has also been established in endocrine cells, in particular in pancreatic islets, adrenal chromaffin cells and aldosterone producing zona glomerulosa cells. Cav1.2 triggers fast insulin secretion in mouse pancreatic β -cells (for review see [75]) although the Ca²⁺ channel isoform mediating this effect may differ between species [76]. The important role of LTCCs for this process in humans is supported by reduced insulin secretion and hyperglycemia in patients with Ca²⁺ channel blocker overdose [77].

In adrenal chromaffin cells, LTCCs carry about 50 % of total Ca²⁺ current with about equal contributions of Cav1.2 and Cav1.3 [25]. However, due to the more negative activation voltage range of Cav1.3, this isoform also mediates inward Ca²⁺ current at subthreshold potentials. It therefore sustains spontaneous pacemaking in isolated chromaffin cells [25] and shapes their electrical activity through coupling to different Ca²⁺-sensitive K⁺-channels [25, 78].

An unexpected recent finding was the discovery of somatic gain-of-function mutations in the Cav1.3 α 1-subunit (CACNA1D) in aldosterone-producing adenomas. In human zona glomerulosa cells CACNA1D is the most abundant Ca²⁺ channel α 1-subunit [74, 79], in contrast to rat zona

glomerulosa cells in which no functional evidence for LTCC currents has so far been obtained [80]. Therefore in humans Ca^{2+} inward current through Cav1.3 channels may also be an important determinant of aldosterone secretion under physiological conditions. As mentioned above, this role of Cav1.3 also explains primary aldosteronism observed with germline gain-of-function mutations in PASNA patients [74].

3.4. Cardiovascular System

Cav1.2 is the predominant LTCC in the cardiovascular system. It is the only relevant LTCC present in arterial smooth muscle, where it controls peripheral vascular resistance [81, 82], and in heart ventricular muscle, where it determines contractility [50]. The major role of Cav1.3 is its contribution to SAN and atrio-ventricular node pacemaking which is possible due to its more negative activation voltage range allowing Ca^{2+} influx during diastolic depolarization (for a recent review see [2]). Pharmacological inhibition of Cav1.2 channels accounts for most of the therapeutically relevant antihypertensive and anti-ischemic effects, predominantly by reducing peripheral vascular resistance and negative inotropy. Bradycardic effects of CCBs must result from inhibition of both isoforms in the SAN: blocking Cav1.3-mediated pacemaker current and the Cav1.2-supported action potentials [26]. Some Cav1.3 expression is also found in atrial myocardium [26]. Cav1.3 knockout mice are bradycardic but also show enhanced susceptibility to atrial fibrillation [26, 83]. This indicates not only a prominent role in pacemaking but also in atrial conduction. When used at higher doses, as may be required for block of LTCCs in the brain (see below), the limiting side effects of currently licensed (non-selective) LTCC blockers are therefore predominantly hypotension, cardiodepression and peripheral edema (DHPs only). Instead, Cav1.3-selective blockers are expected to cause slight bradycardia but may also enhance the risk for atrial fibrillation.

4. LTCCS AS NOVEL DRUG TARGETS

Based on our detailed knowledge about the normal and pathophysiological function of the different LTCC isoforms, new therapeutic indications for LTCC blockers can be considered. The most promising are neuroprotection in PD, neuropsychiatric disorders, febrile seizures and heart failure. As outlined below, CCBs in clinical use with no relevant selectivity for Cav1.3 may be indicated for treating CNS disorders. However, an open question is if the approved doses exerting therapeutic cardiovascular effects are sufficient for efficient inhibition of neuronal LTCCs. This problem may be overcome with Cav1.3-selective blockers that are unlikely to produce major cardiovascular depression.

4.1. Targeting LTCCs in the CNS

As outlined in chapter 2.2, inhibition of LTCCs by CCBs is highly state dependent and affected by alternative splicing [84, 85]. Therefore inhibition of neuronal LTCCs by DHPs, the best characterized class of CCBs with respect to brain penetration in rodents [86], non-human primates [58] and humans [87], may require higher concentrations than inhibition of LTCCs in arterial smooth muscle. Accordingly, one study reported slow and incomplete block of recombinant

Cav1.2 channels by 5 μM concentrations of nifedipine during activity patterns mimicking rapid firing of sympathetic neurons (i.e. short action potentials at 100 Hz) when applied from a holding potential of -80 mV [88]. Complete block required long exposure to the drug and stimulation from a holding potential of -60 mV. Under the same experimental conditions inhibition of Cav1.3 was even less pronounced. Although no detailed concentration-response relationships were reported, these experiments strongly indicate that higher DHP concentrations are required to inhibit LTCCs during neuronal firing patterns than during long (“cardiac muscle-like”) step-depolarizations, for which (in case of Cav1.2) low nanomolar IC_{50} values were reported [20, 84]. Moreover, they confirm the voltage-dependence of DHP block for both channel isoforms [10, 84] and the slightly lower sensitivity of Cav1.3 for DHPs [10, 28] also under conditions of rapid neuronal firing.

Consequently, in the brain the extent of inhibition of L-type currents by DHPs in different neurons may be highly variable depending on the contribution of Cav1.2 vs. Cav1.3, the length of action potential and the resting membrane potential. Although CNS effects of clinically used CCBs have not been reported, modulatory effects of LTCC antagonists on brain function have been described in experimental clinical studies [89]. Non-invasive continuous theta burst stimulation (a repetitive transcranial magnetic stimulation protocol) has been used to unequivocally demonstrate *in vivo* effects of the CCB nimodipine on alterations of continuous theta burst stimulation - induced changes of corticospinal excitability. This was interpreted with nimodipine-induced alterations of LTP and long-term-depression (LTD) [89] and suggests that at least some populations of LTCCs (presumably mostly Cav1.2) are inhibited at therapeutic doses of DHP CCBs. It is important to note that in the vast majority of animal studies much higher doses of CCBs have been acutely applied s.c or i.p. This results in up to >100-fold higher peak plasma concentrations ($\mu\text{g}/\text{ml}$ range for nifedipine; [90]) than typically obtained in humans (up to 115 ng/ml in the case of nifedipine; Nifediac^R extended release tablets, prescribing information). Such dosing induces a strong aversive and fearful state [90, 91] that results from inhibition of peripheral Cav1.2 channels [44], presumably excessive cardiovascular depression. Therefore pharmacological effects on *in vivo* brain function reported in these studies cannot be unequivocally attributed to inhibition of brain LTCCs and should be interpreted with caution.

4.1.1. Neuroprotection in PD

The strong preclinical findings regarding a key role of LTCC-mediated Ca^{2+} load in SNc neurons (chapter 3.2.3) has already led to the initiation of a phase 3 clinical trial (NCT02168842) to study the neuroprotective potential of the DHP isradipine in early PD. It is based on a phase 2 trial aimed at finding the highest tolerable dose (10 mg daily; [92]). These clinical studies are also motivated by case-control and cohort studies from Denmark and the United Kingdom that found a significant association between long-term use of brain permeable CCBs as antihypertensives and reduced risk for a first-time diagnosis of PD (odds ratios of 0.71 – 0.78; [93-95]). At present the preclinical *in vivo* findings from neurotoxin PD models do not allow to predict if

Cav1.2, Cav1.3 or both isoforms contribute to the proposed Ca^{2+} toxicity. In clinical trials Cav1.2-mediated side effects, hypotension and/or peripheral edema, limit long-term treatment of PD with higher doses of DHPs [92] providing a strong argument for efforts to discover Cav1.3 selective inhibitors. However, it is at present unknown if Cav1.3-selective inhibitors would miss a neuroprotective component mediated by Cav1.2 channels. On the other hand, Cav1.3 block may have other beneficial CNS effects, such as antidepressant actions (see chapter 3.2.2) that could also benefit PD patients.

4.1.2. Autism Spectrum Disorders

In addition to PD, inhibition of pathologically increased Cav1.3 activity may also be of therapeutic benefit in other brain disorders. We have recently reported that two *de novo* mutations in the CACNA1D gene (Cav1.3 α 1-subunit) identified in humans with ASD cause a pronounced gain-of-channel-function as shown by whole-cell patch-clamp recordings after transient expression in tsA-201 cells [96]. Mutation G407R, localized at the cytoplasmic end of segment IS6 (Fig. 2) results in a dramatic slowing of channel inactivation during prolonged depolarization, similar to mutations in CACNA1C (Cav1.2 α 1) causing Timothy syndrome, a severe multi-organ disorder with high penetrance for autism [68].

Mutation A749G positioned at the intracellular end of segment IIS6 instead results in a shift of the voltage dependence of activation and inactivation to more negative voltages and causes a 3-fold increase in the current amplitude and the estimated open probability [96]. Biophysical changes in both mutants strongly predict enhanced Ca^{2+} entry through Cav1.3 LTCCs, although its extent may vary depending on neuronal firing properties [96].

The disease relevance of such mutations is further supported by two other *de novo* mutations in CACNA1D identified in probands with PASNA, causing even stronger functional changes than the autism associated mutations. Like A749G, PASNA mutations G403R (IS6) and I750M (IIS6) (Fig. 2) lead not only to a hyperpolarized shift of voltage dependence of activation and inactivation, but also cause a dramatic reduction of the channel inactivation as observed for G407R [74].

Together these findings and the important role of normal Cav1.3 function for neuronal development and synaptic morphology (chapter 3.2) strongly indicate that Cav1.3 gain-of-function mutations are associated with an increased risk for neurodevelopmental disorders. However, if the mutations even play a causal role for the development of ASD or PASNA still needs to be investigated. Cav1.2 and Cav1.3 are expressed on the soma and dendrites of neurons [39, 40]. Enhanced activity may therefore interfere with their known role for neuronal firing and gene transcription [98].

The fact that ASD and PASNA patients have been identified with these activating *de novo* mutations also raises the clinically relevant question if these individuals would benefit from treatment with Cav1.3-selective blockers. Primary candidate drugs would be nimodipine, for which effects on human brain metaplasticity at therapeutic doses have been

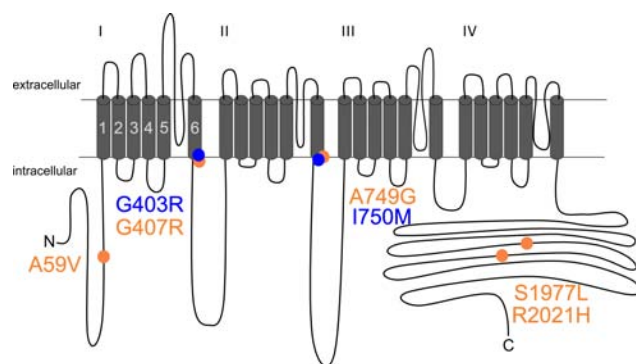


Fig. (2). Human Cav1.3 mutations associated with ASD and PASNA. The transmembrane domain structure of Cav1.3 α 1-subunit is illustrated. α 1-subunits of voltage gated Ca^{2+} channels consist of four homologous repeats (I-IV), each comprising six transmembrane segments. Segments 1-4 of each domain form the voltage-sensor whereas segments 5 and 6 build the pore and activation gates. ASD-associated Cav1.3 mutations are highlighted in orange [72, 73, 97], PASNA-linked mutations shown in blue [74]. A59V, S1977L, and R2021H have not yet been analyzed functionally.

verified, or isradipine, which is in clinical phase 3 trials for PD (see chapter 4.1.1). Although neurodevelopmental aberrations may be largely irreversible, symptomatic improvement (*e.g.* of communicative behaviors or of intellectual functions) cannot be excluded and justifies treatment attempts in these individuals.

4.1.3. Other Neuropsychiatric Disorders

As outlined above normal Cav1.2 expression is required for normal brain function in rodents and gain-of-function mutations in humans cause Timothy syndrome. This is supported by several large-scale genome-wide association studies that revealed a strong association between susceptibility for various psychiatric disorders, including bipolar disease, schizophrenia and major depression, and SNPs in the CACNA1C gene. These are located within intronic regions [71]. SNP rs1006737, a common intronic risk haplotype, is one of the most consistent associations in psychiatric genetics [71, 99]. It also has an impact on task-based human behaviors, and human brain morphology, such as grey matter volume of specific regions (for references see [99]). A recent study investigated the functional consequences of this SNP by generation of proband-derived induced neurons (iN). This allowed demonstration of increased CACNA1C mRNA levels and enhanced current densities in iN from probands which were homozygous for the risk allele [99]. The demonstration of a Cav1.2 gain-of-function by this psychiatric risk haplotype further confirms the hypothesis that dysregulation of Cav1.2 and Cav1.3 LTCC function confers neuropsychiatric disease risk and underscores the requirement for tight regulation of these channels in neurons. Furthermore, these findings also motivate the re-evaluation of CCBs for the treatment of bipolar disease, schizophrenia and major depression [100, 101] in selected patient cohorts homozygous for the risk allele, who may benefit most from the addition of CCBs to standard therapy. The IC_{50} of 33 nM determined for half maximal inhibition of L-type current by isra-

dipine in iN [99] suggests that some Cav1.2 inhibition should be possible by peak plasma (and brain) concentrations likely achieved by high therapeutic doses in humans (up to 10 ng/ml = about 27 nM; www.drugs.com, Dynacirc^R professional drug information), at least in neurons with depolarized resting membrane potentials (recordings in iN were performed at holding potentials of -50 mV, [99]).

Altogether these findings indicate that alterations in L-type Ca²⁺ channel function can lead to various neurological disorders, thereby underlining the necessity for devising strategies to efficiently engage LTCCs in the brain by existing or new drugs.

4.1.4. Febrile Seizures

A recent study [102] discovered a critical role of Cav1.2 channels in the generation of febrile seizures using patch-clamp recordings from hippocampal pyramidal cells in acute rat pup brain slices. L-type currents (likely mediated by Cav1.2 but not by Cav1.3) activated at more negative voltages at supraphysiological temperatures and contributed to abnormal spontaneous temperature-dependent intrinsic firing elicited at > 38 °C which was blocked by nimodipine. In an *in vivo* model nimodipine also dramatically reduced the incidence and duration of febrile seizures in rat pups. Nimodipine was applied i.p. acutely at a dose of 2.5 mg/kg, which was carefully selected to prevent side effects, but must still be considered as high. Based on pharmacokinetic data with nifedipine [90] this acute dose is expected to reach peak plasma concentrations above 1 µg/ml, compared to around 30 ng/ml in humans (60 mg dose; Nimotop^R, Summary of Product Characteristics). High oral doses approved for treatment of subarachnoidal hemorrhage in humans are about 1 mg/kg, which would correspond to a 10-fold higher dose when given parenterally considering the low (about 10 %) oral bioavailability of the drug. Moreover, nimodipine unlike other CCBs is also a potent inhibitor of adenosine uptake [103] and therefore a contribution of this mechanism to the observed *in vivo* protection of febrile seizure cannot be excluded, especially when applied at high doses. Irrespective of these considerations, this study provided compelling evidence for a role of Cav1.2 in febrile seizures and for clinical trials to stop or prevent seizures triggered by high fever and to reduce their risk for long-term neurological consequences. This appears especially justified in patients with seizures in which Na⁺-channel blockers are contraindicated [102].

4.2. Targeting LTCCs in Heart Failure and Primary Aldosteronism

The recent discovery that somatic mutations favoring Ca²⁺ entry through voltage-gated Cav1.3 LTCCs induce excessive aldosterone secretion in aldosterone-producing adrenal adenomas led to the discovery of an unexpected new role of this channel for aldosterone secretion. Activation of LTCCs can be both indirect, by mutations in other proteins favoring cellular depolarization, or direct by gain-of-function mutations in the channel's α 1-subunit [74, 79]. Although a role of LTCCs for aldosterone secretion has been described in earlier *in vitro* studies, no robust inhibitory effects of therapeutic plasma concentrations of CCBs on aldosterone secretion are documented in humans. In addition, enhanced

aldosterone plasma levels are reduced only in a minority of patients with aldosterone-producing adrenal adenomas [79]. One possible explanation is the lower sensitivity of Cav1.3 LTCCs to CCBs, especially to widely used DHPs (see Fig. 1). It is therefore possible that, once available, potent Cav1.3-selective inhibitors would also efficiently inhibit aldosterone secretion. As outlined in chapter 3.4, such drugs are unlikely to affect cardiac inotropy due to their absence in ventricular myocardium. However, they are expected to cause a bradycardic effect, as predicted from Cav1.3^{-/-} mice [34] and human CACNA1D mutations [31]. This combined mechanism of action could be therapeutically meaningful in patients with heart failure in which heart rate (due to enhanced sympathetic drive) and aldosterone (due to secondary aldosteronism) are elevated. High heart rate is a risk factor in heart failure and selective lowering of heart rates with the HCN (If) - channel blocking bradycardic agent ivabradine improves cardiovascular outcomes [104]. However, a troublesome side effect in this patient cohort may be atrial fibrillation risk which has been shown to be increased in Cav1.3-deficient mice [105]. Notably, this is also a therapy-limiting side effect of ivabradine [106].

Another potential (orphan) indication of Cav1.3-selective blockers is Inappropriate Sinus Tachycardia (IST). This rare condition is characterized by inappropriate tachycardia and increased heart rate (usually \geq 100 bpm) at rest or during minimal exercise with normal P waves [107]. Ivabradine (in combination with β -adrenergic blockers) improves IST symptoms [107]. Such an effect is also expected for Cav1.3-selective blockers which would also reduce heart rate by a mechanism downstream of β -adrenergic receptors and thus act in an additive manner. Notably, hypertensive patients and/or patients with stable angina with high heart rates not controlled by the highest tolerable doses of β -adrenergic blockers may also benefit from these drugs.

5. LTCC DRUGS

Given the fact that currently available CCBs appear useful for novel indications as described above, we will briefly summarize their clinical pharmacology. We will then discuss issues related to the development of Cav1.3-selective blockers.

5.1. Pharmacological Properties of Already Approved CCBs

5.1.1. Established Clinical Use

LTCC blockers are licensed since decades for the treatment of hypertension and myocardial ischemias and belong to the most widely prescribed drugs world-wide. Due to the state-dependent action discussed above (chapter 2.2), DHPs are potent vasodilators. They dilate arterial smooth muscle in resistance vessels, reduce peripheral vascular resistance, lower arterial blood pressure and antagonize vasospasms in coronary or peripheral arteries. By reducing the afterload DHPs also reduce cardiac oxygen demand. Together with their anti-vasospastic effect this explains most of the beneficial actions of DHPs in angina pectoris. Most DHPs are only licensed for the therapy of hypertension, some of them also for the treatment of angina pectoris and vasospastic (Prinzmetal) angina. At therapeutic doses, DHPs lack nega-

tive inotropic actions and do not directly affect SAN and AV-node function.

In addition to the antihypertensive, vasodilating and anti-vasospastic properties, therapeutic doses of verapamil and diltiazem also exert negative inotropic, dromotropic and chronotropic actions. Similar to β -adrenergic antagonists, verapamil and diltiazem also inhibit exercise-induced increases in heart rate and myocardial oxygen consumption. Due to their direct cardiodepressant effects they are suitable for the treatment of angina pectoris in hypertensive patients [108].

5.1.2. Adverse Drug Effects

As discussed above, inhibition of LTCCs in the brain may be of therapeutic value for treating CNS disorders. Use of already approved blockers may cause Cav1.2-mediated side effects preventing their long-term use, especially when considering higher doses required for efficient inhibition of LTCCs in the brain. At therapeutic doses unwanted effects are mostly related to the vasodilating effects of Ca^{2+} channel blockers, such as flushing, headache, dizziness, and hypotension. DHPs frequently cause peripheral edema and ankle swelling upon long-term use which may require discontinuation of therapy in some patients [92]. Constipation is a frequent side effect due to inhibitory action on intestinal smooth muscle [109], especially of verapamil. Bradycardia, atrioventricular block or a decrease in left ventricular function are observed with verapamil (and to a lesser degree diltiazem) especially in patients taking β -adrenergic blockers or who have preexisting cardiac disease (impaired left ventricular function, atrioventricular block). Worsening of angina has also been observed with DHPs, most likely due to a redistribution of coronary blood flow to the non-ischemic myocardium.

5.1.3. Pharmacological Effects Outside the Cardiovascular System

At therapeutic doses CCBs cause no relevant side effects in other tissues where LTCCs serve important functions. There is no evidence for muscle weakness from block of Cav1.1 channels in skeletal muscle, increased hearing thresholds from inhibition of Cav1.3 in cochlear inner hair cells, visual impairment from block of Cav1.4 in retinal photoreceptors or CNS disturbances from block of Cav1.2 and or Cav1.3 in the brain. Suppression of insulin secretion and hyperglycemia occur only during intoxications with CCBs [77]. This is due to the dependence of insulin secretion on LTCCs in pancreatic islet cells [50]. However, this side effect plays no role in clinical practice.

5.2. Pharmacological Considerations for the Specific Targeting of Selected LTCC Functions

To minimize cardiovascular side effects (from blocking cardiovascular Cav1.2 channels) and maximize therapeutic actions in the brain, two strategies can be pursued. One consists in the development of Cav1.3-selective drugs. However, if inhibition of neuronal Cav1.2 channels is also desired, then CNS-selectivity of existing drugs may be enhanced by preferential pharmacokinetic targeting of CCBs to the brain using drug delivery approaches.

5.2.1. Isoform-Selectivity

Despite the high sequence homology between Cav1.2 and Cav1.3 α 1-subunits, isoform-selective channel block should be possible. This is supported by differences in the molecular architecture of the DHP binding pocket which have been reported in radioligand binding studies with recombinant channels. Although the dissociation constant for (+)-[^3H]isradipine is identical for Cav1.2 and Cav1.3, binding kinetics are much faster for Cav1.3 [10]. In addition, some DHPs, such as nifedipine and nitrendipine, bind with 3-4-fold higher affinity to Cav1.2 [35]. In functional studies, isradipine and other DHPs block Cav1.2 channels with about 5-10 fold lower IC_{50} values indicating preferential state-dependent inhibition of Cav1.2 [10, 28]. However, since alternative splicing is also an important determinant of state-dependent inhibition of both Cav1.2 and Cav1.3 channels [85, 110], this isoform-selectivity may vary depending on the splice variants employed in these experiments. Evidence for more potent inhibition of Cav1.2 by isradipine also comes from experiments in isolated SAN cells in which 70 % of the L-type current is Cav1.3-mediated. 50 nM isradipine inhibited only 26 % of the wild-type current (mostly Cav1.3) but 72 % of the Cav1.2 component remaining in Cav1.3^{-/-} SAN cells. This implies an IC_{50} for Cav1.3 well above 50 nM [26].

So far only one study has described Cav1.3-selective blockers. A detailed structure-activity relationship has been reported for novel pyrimidine-2,4,6-triones [111, 112]. The original study [111] described selective inhibition of Cav1.3-mediated Ba^{2+} currents using a fluorometric imaging plate reader (FLIPR) assay as well as patch-clamp studies. The most selective candidate, originally termed Cp8 (also BPN-4689, [112]), showed a more than 600-fold selectivity towards Cav1.3 compared to Cav1.2 in the FLIPR assay. Furthermore, whole-cell patch-clamp recordings on HEK293 cells stably expressing LTCC complexes revealed an IC_{50} of 24.3 μM for Cav1.3 inhibition while Cav1.2 Ba^{2+} currents were nearly unaffected.

A follow-up study [113] could confirm the inhibitory activity of Cp8 on transiently expressed LTCC Ca^{2+} currents in whole-cell patch-clamp recordings. However, it did neither report high potency nor strong Cav1.3-selectivity. In contrast to the originally reported IC_{50} of 24.3 μM , only 30-45 % of Cav1.3 Ca^{2+} currents were inhibited by 50 μM Cp8. Additionally their experiments revealed a dependence of the Cp8-mediated effect on the used auxiliary β -subunit. When co-expressed with palmitoylated β 2a, Cav1.2 Ca^{2+} currents were more sensitive to 5 and 50 μM Cp8 than long and short Cav1.3 splice variants. On the contrary, when β 1, β 3 or β 4 were used, the long Cav1.3 isoform was inhibited to a slightly greater extent than Cav1.2 and C-terminally short Cav1.3 channel constructs.

We found an even more complex modulation of LTCC Ba^{2+} and Ca^{2+} currents by Cp8 [114]. Again, neither high potency nor Cav1.3-selectivity could be reproduced using whole-cell patch-clamp recordings on transiently expressed LTCCs in tsA201 cells. In all Ca^{2+} and the majority of Ba^{2+} current recordings, a pronounced and time-dependent change in gating kinetics was observed (Fig. 3). This partially reversible modulation was characterized by a slowing of the

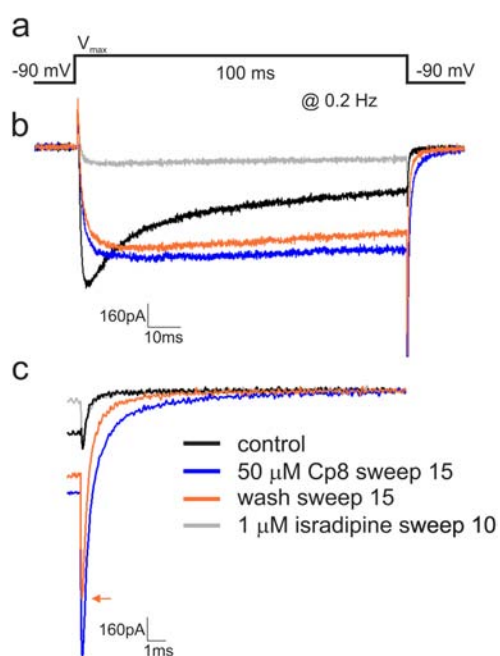


Fig. (3). Cp8-induced change in gating kinetics of full-length rat Cav1.3 (rCav1.3_L) Ca²⁺ currents. rCav1.3_L α1-subunits were transiently expressed in tsA201 cells together with auxiliary β3- and α2δ1-subunits. **a**, Ca²⁺ currents were evoked by a 100-ms long depolarizing step from a holding potential of -90 mV to the voltage of maximal activation (V_{max}) with a frequency of 0.2 Hz using 15 mM Ca²⁺ as charge carrier. **b**, Representative trace showing the modulation by 50 μM Cp8, wash-out and subsequent block by 1 μM isradipine. A pronounced slowing of the activation time course after Cp8 application was observed. **c**, Amplitude and width of tail currents were dramatically increased by Cp8. Subsequent wash-out could partially reverse this effect. The arrow indicates the peak of the tail current after wash-out. For details see [114].

activation, inactivation and deactivation time course and thus closely resembled the activity of known LTCC activators, such as BayK8644 or FPL 64176 [114]. This effect on current kinetics could be further confirmed on native Cav1.2 and Cav1.3 channel complexes in mouse chromaffin cells, in which Cp8 also increased their spontaneous firing frequency. However, in a small subset of recordings using Ba²⁺ as charge carrier, a weak and non-selective inhibition of both, Cav1.2 and Cav1.3, could be observed [114].

In conclusion, these studies suggest that the Cav1.3-selectivity of Cp8 and related pyrimidine-2,4,6-triones is highly dependent on experimental conditions and they may even stabilize gating properties favoring enhanced Ca²⁺ entry through Cav1.2 or Cav1.3 Ca²⁺-channels. Understanding the pharmacodynamic details explaining these differences may provide an important clue to reveal potential Cav1.3-selective actions of this class of drugs. Despite these encouraging results, we conclude that Cav1.3-selective drugs suitable as selective pharmacological tools and with therapeutic potential still have to be discovered.

5.2.2. CCB Brain Drug Delivery

Brain permeation has been demonstrated for several approved DHP LTCC blockers in several species, including

humans [58, 86, 87]. An exception is amlodipine which does not efficiently cross the blood-brain barrier [86]. Isradipine and nimodipine reach total brain concentrations that are similar to those in plasma but increasing the drug concentration in brain relative to plasma would be another strategy to reduce peripheral side effects and allow higher dosing to efficiently engage brain channels. Brain drug delivery approaches are available [115, 116]. One such approach, using a chemical delivery system (CDS), has already been applied to increase brain concentrations of the widely used DHP felodipine [117]. Bodor's lipophilic [(1-methyl-1,4-dihydropyrid-3-yl)-carbonyl]oxy (amino) acts as a chemical delivery system that, after entry into brain is oxidized to a polar pyridinium species. This is trapped in the brain and undergoes ester or amide cleavage to release the active drug. When coupled to felodipine (felodipine-CDS) *in vivo* biodistribution studies after i.v. injections could demonstrate the expected rapid decline of peak felodipine-CDS concentrations in the brain and in plasma. In plasma the oxidized pyridinium salt species also decreased to undetectable concentrations 2 h after reaching peak plasma concentrations. In contrast, in the brain the oxidized pyridinium species persisted at high concentrations providing detectable concentrations up to 4 days. The concentration of the hydrolysis product which corresponds to the coupled CCB released from the CDS in the brain remained very low throughout the study (due to slow hydrolysis and/or rapid egression from the brain). Therefore in this approach a high LTCC blocking activity in the brain would also benefit from high affinity of the oxidized felodipine-CDS for the channel which has not been determined in this study.

CONCLUSION

As antianginal and antihypertensive drugs LTCC blockers (especially DHPs) belong to the most widely prescribed drugs world-wide. Research on different LTCC isoforms has provided us with extensive information about the role of these channels in physiology and human disease suggesting that also LTCCs outside the cardiovascular system could serve as novel targets for these old drugs.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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