Voltage-Dependent Calcium Channels

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Abstract. Voltage-activated calcium channels can be divided into two subgroups based on their activation threshold, low-voltage-activated (LVA) and high-voltage-activated (HVA). Auxiliary subunits of the HVA calcium channels contribute significantly to biophysical properties of the channels. We have cloned and characterized members of two families of auxiliary subunits: $\alpha_2\delta$ and $\gamma$. Two new $\alpha_2\delta$ subunits, $\alpha_2\delta$-2 and $\alpha_2\delta$-3, regulate all classes of HVA calcium channels. While the ubiquitous $\alpha_2\delta$-2 modulates both neuronal and non-neuronal channels with similar efficiency, the $\alpha_2\delta$-3 subunit regulates $\text{Ca}_{\text{v}}$2.3 channels more effectively. Furthermore, $\alpha_2\delta$-2 may modulate the LVA $\text{Ca}_{\text{v}}$3.1 channel. Four new $\gamma$ subunits, $\gamma$-2, $\gamma$-3, $\gamma$-4 and $\gamma$-5, were characterized. The $\gamma$-2 subunit modulated both the non-neuronal $\text{Ca}_{\text{v}}$1.2 channel and the neuronal $\text{Ca}_{\text{v}}$2.1 channel. The $\gamma$-4 subunit affected only the $\text{Ca}_{\text{v}}$2.1 channel. The $\gamma$-5 subunit may be a regulatory subunit of the LVA $\text{Ca}_{\text{v}}$3.1 channel.

The $\text{Ca}_{\text{v}}$1.2 channel is a major target for treatment of cardiovascular diseases. We have mapped the interaction site for clinically important channel blockers – dihydropyridines (DHPs) – and analysed the underlying inhibition mechanism. High-affinity inhibition is characterized by interaction with inactivated state of the channel. Its structural determinants are amino acids of the IVS6 segment, with smaller contribution of the IS6 segment, which contributes to voltage-dependence of DHP inhibition. Removal of amino acids responsible for the high-affinity inhibition revealed a low-affinity open channel block, in which amino acids of the IIIIS5 and IIIIS6 segments take part. Experiments with a permanently charged DHP suggested that there is another low-affinity interaction site on the $\alpha_1$ subunit.

We have cloned and characterized murine neuronal LVA $\text{Ca}_{\text{v}}$3.1 channel. The channel has high sensitivity to the organic blocker mibebradil, moderate sensitivity to phenytoin, and low sensitivity to ethosuximide, amiloride and valproat. The channel is insensitive to tetrodotoxin and DHPs. The inorganic blockers $\text{Ni}^{2+}$ and $\text{Cd}^{2+}$ are moderately effective compared to $\text{La}^{3+}$. The current through the $\text{Ca}_{\text{v}}$3.1 channel inactivates faster with $\text{Ba}^{2+}$ compared to $\text{Ca}^{2+}$. Molecular determinants of fast inactivation are located in amino side of the intracellular carboxy terminus. The voltage dependence of charge movement is very shallow compared to the voltage dependence of current activation. Transfer of 30% of charge correlates with activation of 70% of measurable macroscopic current. Prolonged depolarization does not immobilize charge movement of the $\text{Ca}_{\text{v}}$3.1 channel.

Key words: Calcium channel — $\alpha_1$ subunit — Auxiliary subunits — Low-voltage-activated — High-voltage-activated — Dihydropyridines

Abbreviations: AID, $\alpha$ interaction domain; BID, $\beta$ interaction domain; BTZ, benzothiazepine; CaM, calmodulin; DHP, dihydropyridine; GBP, gabapentin; HEK, human embryonic kidney; HP, holding potential; HVA, high-voltage-activated; IC$_{50}$, concentration for 50% inhibition; LVA, low-voltage-activated; MPS, $\alpha$-methyl-$\alpha$-phenylsuccinimide; PAA, phenylalkylamine; TTX, tetrodotoxin; VDCC, voltage-dependent calcium channels; VACC, voltage-activated calcium channel.
Introduction

Living cells are surrounded by impermeable membranes containing specialized proteins providing for exchange of various atoms and molecules between the extracellular and intracellular spaces. Traditionally, two basic mechanisms of transmembrane transport have been recognized: carriers and channels. Carriers, such as the Ca$^{2+}$ pump, Na$^+$.Ca$^{2+}$ exchanger, or Na$^+$.K$^+$ pump, transport ions against concentration and/or electrical gradients and are coupled to metabolic energy consumption. Membrane channels are viewed as pores, which, when opened, allow passive transport downhill the electric and/or concentration gradients. Opening of a channel can be accomplished in two ways: i) by binding of a specific ligand either directly to the channel or to another membrane protein coupled to the channel, and ii) by a change in transmembrane voltage. The first pathway is characteristic for ligand-gated channels, such as the glutamate or acetylcholine receptors. The second pathway activates the so-called voltage-gated channels. The foundation of biophysical analyses of voltage-gated ion channels was laid in the pioneering works of Hodgkin and Huxley in the 1930’s and culminated in the 1950’s by formulating the Hodgkin-Huxley model of action potential (Hodgkin and Huxley 1952a,b,c,d). Initially, the Na$^+$ and K$^+$ selective voltage-gated channels and a leakage channel were recognized. Later, the family of voltage-gated ion channels was extended to include Ca$^{2+}$ and Cl$^-$ channels.

Voltage-gated calcium channels were first identified by Fatt and Katz (1953). Later it was discovered that there are different channel subtypes in excitable cells and, consequently, voltage-gated calcium channels were classified and named according to various schemes.

In the 1980’s, the calcium channel protein was purified and shown to consist of several subunits (Borsotto et al. 1985; Flockerzi et al. 1986; Sieber et al. 1987; Takahashi et al. 1987; Vaghy et al. 1987; Leung et al. 1988). The principal subunit of the voltage-dependent calcium channel (VDCC) was named $\alpha_1$, and auxiliary subunits were named $\beta$, $\alpha_2$, $\delta$, and $\gamma$. Cloning of the genes encoding individual subunits followed soon. So far, ten genes for $\alpha_1$ subunits, four for $\beta$ subunits, four for the $\alpha_2\delta$ complex and eight for $\gamma$ subunits were identified (for review, see Hofmann et al. 1999).

Mutation analyses during the 1990’s allowed a precise identification of interaction sites for major calcium channel ligands like dihydropyridines (DHPs), phenylalkylamines (PAAs) and benzothiazepines (BTzs), of phosphorylation sites, and of the molecular structures responsible for voltage- and calcium-dependent regulation of channel activation and inactivation. Most recently, investigation of transgenic mouse models has allowed analysis of the physiological role of calcium channels in an organism at large.

Classification of voltage-gated calcium channels

The very first channel classification was based on basic electrophysiological and pharmacological properties. An observation was made that some calcium channels
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need only a small depolarization to be activated, while other require a relatively high step in membrane voltage to open (Hagiwara et al. 1975; Llinás and Yarom 1981). According to this criterion, calcium channels were divided into low-voltage-activated (LVA) and high-voltage-activated (HVA). LVA calcium channels activate at a membrane voltage positive to −70 mV. Because of the small amplitude of single channel conductance and its fast decay, these channels were also called T-type calcium channels (T for tiny and transient). HVA channels have an activation threshold at membrane voltages positive to −20 mV. The first generally known representative of the HVA channel family was found abundantly in skeletal, smooth and cardiac muscles as well as in neurons. Because of its large-single channel conductance amplitude and slow kinetics of current decay, it was named L-type calcium channel (L for large and long-lasting) in contrast to the T-type. A pharmacological hallmark of all L-type channels is their sensitivity to 1,4-DHPs – a wide class of drugs with either inhibitory (nifedipine, nisoldipine, isradipine) or activatory (Bay K 8644) action on the channel.

In the 1980’s, experiments with neuronal cells revealed novel calcium channels, insensitive to DHPs and with single-channel conductances between those of T-type and L-type channels (Nowycky et al. 1985; Fox et al. 1987). These channels were named N-type calcium channels (N for neuronal). Later, it was shown that neuronal non-L-type channels could be further classified into subtypes according to their sensitivity to peptide toxins isolated from cone snails and spiders. The channel sensitive to ω-conotoxin GVIA, kept the name N-type channel, while the channel sensitive to ω-Aga IVA toxin was named P/Q-type calcium channel (P for Purkinje cells, where this channel was characterized by Llinás and co-authors in 1989). The channels resistant to these toxins were named R-type calcium channel (R for resistant).

The second classification of voltage-gated calcium channels was developed in the 1980’s and was based on cloning of cDNAs encoding individual channel types. The cloning experiments revealed that calcium channels consist of the principal α₁ subunit and several auxiliary subunits β, α₂ δ, and γ, which have regulatory functions and each of them has several subtypes. The α₁ subunit is responsible for basic electrophysiological and pharmacological properties that formed the basis of early channel classifications. Therefore, investigators tried to establish links between the newly cloned subunits and channel complexes identified earlier by traditional electrophysiological experiments in native tissues. Several α₁ subunits representing class L calcium channels were identified. As the first one, the α₁ subunit from skeletal muscle was purified (Curtis and Catterall 1984), cloned and named α₁S (Tanabe et al. 1987). Later, α₁ subunit was cloned from cardiac (α₁C-a, Mikami et al. 1989) and smooth (α₁C-b, Biel et al. 1990) muscle. Both splice variants of the α₁C subunit are structurally closely related and share 95% of identical amino acids. Two further representatives of the L-type subfamily were identified later: α₁D (Seino et al. 1992; Williams et al. 1992a) and α₁F (Bech-Hansen et al. 1998; Strom et al. 1998).

Three α₁ subunits, representing the three neuronal types of calcium channels,
were cloned. $\alpha_{1A}$ subunit (Mori et al. 1991; Starr et al. 1991) corresponds to the P/Q-type channel. The properties of $\alpha_{1B}$ subunit (Williams et al. 1992b; Dubel et al. 1992) match those of the N-type channel. $\alpha_{1E}$ subunit (Niïdome et al. 1992; Soong et al. 1993) was initially characterized as an LVA T-type channel; however, later studies demonstrated that it has the properties of R-type channel.

Three members of the LVA T-type subfamily were identified so far: $\alpha_{1G}$ (Perez-Reyes et al. 1998), $\alpha_{1H}$ (Cribbs et al. 1998), and $\alpha_{1I}$ (Lee et al. 1999a).

As the number of cloned calcium channel $\alpha_1$ subunits has been increasing, a need has arisen for a systemic nomenclature. It was agreed that individual $\alpha_1$ subunits will be named according to the $Ca_v.x.y$ scheme (Ertele et al. 2000), where $Ca_v$ stands for voltage-activated calcium channel (VACC), $x$ is a number designating the channel subfamily (i.e., L-type, neuronal, and T-type in the initial classification), and $y$ is a number designating individual members of subfamilies. An overview of VACC known to-date is given in Table 1.

<table>
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<td>Nomenclature according to $\alpha_1$</td>
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<tr>
<td>L-type</td>
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<tr>
<td>HVA channels</td>
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<tr>
<td>P/Q-type</td>
</tr>
<tr>
<td>$\alpha_{1A}$</td>
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<tr>
<td>Neuronal</td>
</tr>
<tr>
<td>N-type</td>
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<tr>
<td>$\alpha_{1B}$</td>
</tr>
<tr>
<td>R-type</td>
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<tr>
<td>$\alpha_{1E}$</td>
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<tr>
<td>LVA channels</td>
</tr>
<tr>
<td>T-type</td>
</tr>
<tr>
<td>$\alpha_{1G}$</td>
</tr>
<tr>
<td>$\alpha_{1H}$</td>
</tr>
<tr>
<td>$\alpha_{1I}$</td>
</tr>
</tbody>
</table>

$^1$ Tsien et al. (1988); $^2$ Snutch et al. (1990); $^3$ Birnbaumer et al. (1994); $^4$ Ertele et al. (2000).

Comparison of amino acid sequences of individual calcium channels revealed an evolutionary relationship between channel classes. An early evolutionary event separated the $\alpha_1$ subunits into LVA and HVA channels, with less than 30% of sequence homology. A later evolutionary event divided HVA channels into two subfamilies: L-type and neuronal types, with about 50% sequence homology. Individual members of both subfamilies share more than 80% of sequence homology (Figure 1). Virtually all known $\alpha_1$ subunits have more splice variants. These share usually more than 95% sequence homology and will be described in chapters devoted to individual channels.
Subunit composition of the calcium channel complex

HVA channels are heterooligomeric complexes, consisting of up to five proteins from four genes (Figure 2). α1 subunit is a principal subunit, localized in the cell membrane and forming a conductive pore. β, α2, δ, and γ are auxiliary subunits. Not all four modulatory proteins are necessarily present in each channel complex. Regulation by γ subunit is undoubtedly proven only for the Ca_{v}1.1 channel. While all α1 subunits of HVA channels are most probably associated with β and α2δ subunits (Hofmann et al. 1999), the subunit composition of LVA channels is not known yet (Lacinová et al. 2000a). β, α2δ, and γ subunits create gene families with 4, 4 and 8 members, respectively. For many subunits, splice variants are known which may differ dramatically in functional characteristics.

α1 subunit

α1 subunits VACCs of are large proteins with molecular weight between 212 and 273 kDa. They belong to the same multigene family as voltage-activated sodium and potassium channels. Hydrophobicity analysis predicted transmembrane topology with four homologous transmembrane domains, each containing six membrane-spanning putative α-helices, creating six transmembrane segments and a pore region between segments S5 and S6 (Figure 3). The length of α1 subunit ranges approximately between 1870 and 2420 amino acids. Sizes of several cloned α1 subunits are given in Table 2. Individual transmembrane segments contain approximately
Figure 2. A proposed schematic structure of VACC. The principal $\alpha_1$ subunit is a transmembrane protein containing a conducting pore, through which calcium ions can pass upon opening. $\alpha_1$ subunit is further regulated by auxiliary subunits: intracellular $\beta$ subunit, transmembrane $\gamma$ subunit and a complex of extracellular $\alpha_2$ subunit and transmembrane $\delta$ subunit, connected by a disulphide bridge.

Figure 3. Suggested membrane topology of $\alpha_1$ subunit of the VACC. The subunit consists of four homologous domains I–IV, each containing six transmembrane segments S1–S6 and a pore region between segments S5 and S6. Putative $\alpha$-helices are shown as cylinders. The fourth transmembrane segment S4 in each domain bears a net positive charge.

20 amino acids. S4 segments contain five to six positively charged arginins and lysines (Figure 4), so that these segments bear a net positive charge and can act as voltage sensors controlling VDCC gating. Movement of the S4 segment during
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<table>
<thead>
<tr>
<th>S4</th>
<th>IIS4</th>
<th>IIIS4</th>
<th>IVS4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca1.1 / rabbit skeletal muscle</td>
<td>ISWLRTELLFPEITTYW</td>
<td>VELLSVYSLPSAILWA</td>
<td>SAFTEYFVWLELQDBE</td>
</tr>
<tr>
<td>Ca1.2 / rabbit heart</td>
<td>ISWLRTELLFPEITTYW</td>
<td>VELLSVYSLPSAILWA</td>
<td>SAFTEYFVWLELQDBE</td>
</tr>
<tr>
<td>Ca1.3 / rabbit lung</td>
<td>ISRCAVLRLEPVMTNW</td>
<td>VELLSVYSLPSAILWA</td>
<td>IEFTEYFVWLELQDBE</td>
</tr>
<tr>
<td>Ca1.4 / rat aorta</td>
<td>ISRCAVLRLEPVMTNW</td>
<td>VELLSVYSLPSAILWA</td>
<td>IEFTEYFVWLELQDBE</td>
</tr>
<tr>
<td>Ca1.3 / human pancreas</td>
<td>ISRCAVLRLEPVMTNW</td>
<td>VELLSVYSLPSAILWA</td>
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</tr>
<tr>
<td>Ca1.4 / human retina</td>
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<td>VELLSVYSLPSAILWA</td>
<td>IEFTEYFVWLELQDBE</td>
</tr>
<tr>
<td>Ca1.5 / rabbit brain</td>
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<td>VELLSVYSLPSAILWA</td>
<td>IEFTEYFVWLELQDBE</td>
</tr>
<tr>
<td>Ca1.6 / human brain</td>
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<td>VELLSVYSLPSAILWA</td>
<td>IEFTEYFVWLELQDBE</td>
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<tr>
<td>Ca1.7 / human retina</td>
<td>ISRCAVLRLEPVMTNW</td>
<td>VELLSVYSLPSAILWA</td>
<td>IEFTEYFVWLELQDBE</td>
</tr>
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<td>Ca1.8 / mouse brain</td>
<td>ISRCAVLRLEPVMTNW</td>
<td>VELLSVYSLPSAILWA</td>
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<td>Ca1.9 / human heart</td>
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<td>VELLSVYSLPSAILWA</td>
<td>IEFTEYFVWLELQDBE</td>
</tr>
<tr>
<td>Ca1.10 / rat brain</td>
<td>ISRCAVLRLEPVMTNW</td>
<td>VELLSVYSLPSAILWA</td>
<td>IEFTEYFVWLELQDBE</td>
</tr>
</tbody>
</table>

Figure 4. Alignment of S4 segments of VACC. Charged amino acids are highlighted by grey boxes.

Table 2. Sizes of individual cloned α1 subunits of the VACC

<table>
<thead>
<tr>
<th>Subunit / origin</th>
<th>Molecular weight</th>
<th>Number of amino acids</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Ca1.1 / rabbit skeletal muscle</td>
<td>212</td>
<td>1873</td>
<td>Tanabe et al. 1987</td>
</tr>
<tr>
<td>Ca1.2 / rabbit heart</td>
<td>242.8</td>
<td>2171</td>
<td>Mikami et al. 1989</td>
</tr>
<tr>
<td>Ca1.3 / rabbit lung</td>
<td>242.5</td>
<td>2166</td>
<td>Biel et al. 1990</td>
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<tr>
<td>Ca1.4 / rat aorta</td>
<td>243.6</td>
<td>2169</td>
<td>Koch et al. 1990</td>
</tr>
<tr>
<td>Ca1.5 / human pancreas</td>
<td>247.6</td>
<td>2181</td>
<td>Seino et al. 1992</td>
</tr>
<tr>
<td>Ca1.6 / human brain</td>
<td>245.2</td>
<td>2161</td>
<td>Williams et al. 1992</td>
</tr>
<tr>
<td>Ca1.7 / human retina</td>
<td>245.2</td>
<td>2161</td>
<td>Williams et al. 1992</td>
</tr>
<tr>
<td>Ca1.8 / rabbit brain</td>
<td>257.3</td>
<td>2273</td>
<td>Mori et al. 1991</td>
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<tr>
<td>Ca1.9 / rabbit brain</td>
<td>273.2</td>
<td>2424</td>
<td>Horne et al. 1993</td>
</tr>
<tr>
<td>Ca2.2 / human brain</td>
<td>264.5</td>
<td>2326</td>
<td>Williams et al. 1992</td>
</tr>
<tr>
<td>Ca2.3 / rabbit brain</td>
<td>262.5</td>
<td>2339</td>
<td>Williams et al. 1992</td>
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<td>Ca2.4 / human brain</td>
<td>251.8</td>
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<tr>
<td>Ca2.5 / rat brain</td>
<td>252</td>
<td>2222</td>
<td>Soong et al. 1993</td>
</tr>
<tr>
<td>Ca3.1 / mouse brain</td>
<td>205.2</td>
<td>1835</td>
<td>Lee et al. 1999</td>
</tr>
</tbody>
</table>

channel activation was directly proved for the K+ channel (Logothetis et al. 1992; Glauner et al. 1999). Based on structural homology of all voltage-gated channels, we can hypothesize that the S4 segments of the VACC play a similar role. The pore region contains a putative selectivity filter, which ensures high selectivity of the channel pore for calcium ions. Furthermore, the α1 subunit sequence contains sites of interaction with auxiliary subunits, binding sites for various activators and
blockers, including G-proteins, as well as several putative phosphorylation sites. Some of these interaction sites are common, e.g., the site of the interaction with β subunit, while others are channel type specific, e.g., the binding sites for specific channel ligands.

When expressed in heterologous expression system, either in *Xenopus* oocytes or in a mammalian cell line (Chinese hamster ovary, tsa201, and HEK 293 are the most commonly used mammalian expression systems), α1 subunit undergoes voltage-dependent activation and inactivation and is subject to regulation by various blockers and activators. However, quantitative characteristics of voltage- and drug-dependent regulation of expressed α1 subunits of HVA channels differ from those described for the corresponding calcium channel types in native tissues. To mimic the behaviour of native channels, coexpression of auxiliary subunits and other regulatory proteins was necessary. LVA channels are an exception. No remarkable difference between current regulations observed in native tissue and from CaV3.x channels expressed in a mammalian cell line has been reported so far. The subunit composition of the LVA channel remains an open and controversial issue (Lacinová et al. 2000a).

**β subunit**

β subunit is an intracellular auxiliary subunit coexpressed with α1 subunit of all HVA channels (Figure 5). Coexpression of β subunit with α1 subunit of the LVA channel has not been demonstrated (for review, see Lacinová et al. 2000a). So far, four β subunit isoforms (β1–β4), coded by distinct genes, have been identified (for review, see Hofmann et al. 1999). Each isoform has multiple splice variants. Primary structure alignment of β subunits revealed that all share a common central core, whereas their amino (N)- and carboxy (C)-termini and a part of the central region differ significantly.

_Cloning of β subunits family_

The first cloned β1 subunit was that from rabbit skeletal muscle containing 524 amino acids, with calculated molecular weight of 57.9 kD (Ruth et al. 1989). Hydropathicity analysis revealed absence of a typical membrane-spanning region. Its splice variants β1a, β1b, and β1c were identified in skeletal, cardiac and neuronal tissues (for review, see Birnbaumer et al. 1998). Two splice variants of β2 subunits, β2a and β2b, containing 606 and 632 amino acids, respectively, as well as a 477 amino acids long β3 subunit were cloned from rabbit heart by Hullin and co-workers (1992). Perez-Reyes and co-workers (1992) isolated β2 subunit from rat brain (604 amino acids, 68.2 kD). Castellano and co-workers (1993a) cloned a homologue of β3 subunit from rat brain (484 amino acids, 54.6 kD). β4 subunit was first cloned from rat brain (Castellano et al. 1993b). It encodes a protein with 519 amino acids and molecular weight of 58.0 kD.
Interaction of β subunits with α₁ subunits

Purification of the calcium channel protein demonstrated the association of the transmembrane α₁ subunit with the intracellular β subunit (Borsotto et al. 1985; Flockerzi et al. 1986; Sieber et al. 1987; Takahashi et al. 1987; Vaghy et al. 1987; Leung et al. 1988). Individual α₁ subunits associate with various β subunits or their splice variants in a tissue-dependent manner. Interaction between the subunits was tested by analyzing changes in current properties caused by coexpression of a β subunit with a particular α₁ subunits in Xenopus oocytes or in a mammalian cell line. Table 3 summarizes combinations of subunits tested positively for modulatory effects of β subunit on calcium current through a given α₁ subunit. Modulation of the current through expressed Ca₃.x subunits was not detected. Several facts seems to exclude this interaction: i) the amino acid sequence of all three Ca₃.x subunits lacks the α interaction domain (AID) identified in α₁ subunits of HVA channels (for review, see Hofmann et al. 1999); ii) elimination of the four known β subunits by transfection of nodus ganglion neurons (Lambert et al. 1997) or mammalian neuronal NG108-15 cells (Leuranguer et al. 1998) with antisense oligonucleotides did not affect the size or voltage-dependence of native T-type current; iii) overexpression of the neuronal β₂a subunit (Wyatt et al. 1998) did not alter the characteristics of T-type current in undifferentiated mammalian NG108-15 cells.


Table 3. Original papers describing interactions between particular $\alpha_1$ and $\beta$ subunits in a recombinant expression system. Only initial studies are included. For more recent citations, see Birnbaumer et al. (1998) and Hofmann et al. (1999).

<table>
<thead>
<tr>
<th>$\alpha_1$</th>
<th>$\beta_1$</th>
<th>$\beta_2$</th>
<th>$\beta_3$</th>
<th>$\beta_4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca\textsubscript{v}1.1</td>
<td>Varadi et al. 1991</td>
<td>Lacerda et al. 1991</td>
<td>Hullin et al. 1992</td>
<td>Hullin et al. 1992</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.2</td>
<td>Wei et al. 1991</td>
<td>Singer et al. 1991</td>
<td>Perez-Reyes et al. 1992</td>
<td>Castellano et al. 1993a</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.3</td>
<td>Williams et al. 1992a</td>
<td>Koschak et al. 2001</td>
<td>Ihara et al. 1995</td>
<td>Scholze et al. 2001</td>
</tr>
<tr>
<td>Ca\textsubscript{v}1.4</td>
<td>not investigated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca\textsubscript{v}2.1</td>
<td>Stea et al. 1994</td>
<td>De Waard et al. 1994</td>
<td>Stea et al. 1994</td>
<td>Stea et al. 1994</td>
</tr>
<tr>
<td></td>
<td>Parent et al. 1997</td>
<td>Nakashima et al. 1998</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The skeletal muscle $\alpha_1$ subunit is most probably regulated exclusively by the skeletal $\beta_1$ subunit. Coexpression of the $\alpha_1$ subunit of the Ca\textsubscript{v}1.4 channel with auxiliary subunits has not been studied yet. All other HVA $\alpha_1$ subunits can interact in heterologous expression systems with all four $\beta_1$ subunits. However, this does not prove that such regulation takes place also in situ. Other authors used antisense depletion of $\beta$ subunits in cell culture as a functional test for the interaction between $\alpha_1$ and $\beta$ subunits (Berrow et al. 1995; Lambert et al. 1997; Leuranguer et al. 1998). This analysis showed lack of modulation of T-type channels by $\beta$ subunits and participation of all $\beta$ subunits in regulation of neuronal HVA channels. Immunohistological studies (Biel et al. 1991; Hullin et al. 1992; Ludwig et al. 1997;
Volsen et al. (1997) revealed complex tissue distributions of β subunits. The β₁a subunit is expressed in skeletal muscles. The β₂ gene is expressed abundantly in the heart and, to a lower degree, in aorta, trachea, lungs and brain, whereas the β₃ specific mRNA is detectable in the brain and a variety of smooth muscle tissues.

The interaction site of α₁ and β subunits was identified on the connector between the first and second homologous domains of α₁ subunit (Pragnell et al. 1994). The consensus sequence of this AID is 428QQ–E–L–GY–WI—E445, in terms of amino acid numbering according to the α₁Cₐ sequence (Biel et al. 1990). The corresponding β interaction domain (BID) is located between the second and third predicted α-helices of β subunit (Figure 5). The amino acid sequence of the BID is —E—PYDVVPSMRP-LVGPSLKGYEVTDMMQKALFDF (De Waard et al. 1994, 1996). However, the most recent studies describing crystal structure of β₂a subunit (Van Petegem et al. 2004) and β₃ and β₄ subunits (Chen et al. 2004) suggested that BID is buried in the core of crystallized protein and is unavailable for protein–protein interaction. Instead, AID interacts with a hydrophobic groove in a guanylate kinase domain.

The stoichiometry of α₁/β interaction is supposedly 1 : 1. However, identification of low-affinity α₁/β interaction site at the carboxy terminus of α₁ subunit of the Caᵥ₂.3 (Qin et al. 1996; Tareilus et al. 1997) or Caᵥ₂.1 channel (Walker et al. 1998), and at the amino terminus of α₁ subunit of the Caᵥ₂.1 channel (Walker et al. 1999) and Caᵥ₂.2 channel (Stephens et al. 2000) raised the possibility that more than one β subunit may interact with a single α₁ subunit. Hümmer and collaborators (2003) demonstrated that β₁b subunit interacts with the C terminus of Caᵥ₂.1 channel α₁ subunit. Nevertheless, deletion of the C terminus of Caᵥ₂.2 channel α₁ subunit did not affect its interaction with the β₃ subunit (Stotz et al. 2004). Garcia and co-authors (2002) injected purified β₁ a subunits into vesicles derived from frog and mouse adult skeletal myocytes and observed enhanced current amplitude and altered tail current. Time courses of current activation and inactivation and parameters characterizing charge movement were not altered. Authors concluded that these effects were true modulatory effects of the β subunit mediated by interaction with the second interaction site distinct from the site that mediates chaperoning effect of β subunits.

There may be complex modulatory effects of β subunits on α₁ subunits. Co-expression of a β subunit with various α₁ subunits increases peak current (Singer et al. 1991), most likely by increasing the number of functional surface membrane channels and by facilitating channel pore opening (Neely et al. 1993; Josephson and Varadi 1996; Kamp et al. 1996). With the exception of the rat brain β₂a, all other β subunits accelerate channel activation and inactivation and shift the steady state inactivation curve to hyperpolarized membrane voltages (Singer et al. 1991; Wei et al. 1991; Hullin et al. 1992; Castellano et al. 1993a). The rat brain β₁a, when coexpressed with the Caᵥ₂.3 channel, slowed down inactivation rate and caused a right shift in the steady-state inactivation curve (Olcese et al. 1994; Qin et al. 1998). The role of β subunits is not restricted to modulatory effects on α₁ subunits. The β₄c subunit is a multifunctional protein that not only regulates α₁ subunit of
Table 4. \( I_{\text{max}} \) for each cell was calculated from the peak current of individual current-voltage relations and was normalized to the cell capacity. The inactivation time constants \( \tau_1 \) and \( \tau_2 \) were calculated from double exponential fits to the current traces measured during 800 ms depolarizing pulses from HP \(-80 \text{ mV} \) to \(+30 \text{ mV} \). \( V_{0.5} \) represents the half-maximal inactivation voltage calculated from a Boltzmann fit of the current-voltage relation.

<table>
<thead>
<tr>
<th>Channel</th>
<th>( I_{\text{max}} ) (pA/pF)</th>
<th>( \tau_1 ) (ms)</th>
<th>( \tau_2 ) (s)</th>
<th>( V_{0.5} ) (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Ca}_V^{1.2-a} )</td>
<td>(-11.3 \pm 1.3 ) (11)</td>
<td>254 ± 20&quot; (11)</td>
<td>2.8 ± 0.4&quot;***</td>
<td>(-3.2 \pm 1.3 ) (9)</td>
</tr>
<tr>
<td>( \text{Ca}_V^{1.2-a}\beta_3 )</td>
<td>(-13.8 \pm 1.7 ) (16)</td>
<td>166 ± 14&quot; (16)</td>
<td>0.90 ± 0.07&quot;***</td>
<td>(-8.4 \pm 0.8 ) (13)</td>
</tr>
</tbody>
</table>

* and *** indicate a significant difference between the two cell lines at \( p < 0.05 \) and \( p < 0.001 \), respectively. Numbers of cells measured are given in brackets.

**Modulation of interactions of \( \alpha_1 \) subunit with calcium channel inhibitors by \( \beta \) subunits**

Coexpression of a \( \beta \) subunit may also affect the interaction of a calcium channel with its blockers (Lacinová et al. 1995; Welling et al. 1995). We have shown that coexpression of the \( \beta_3 \) subunit with a heart-specific splice variant of the \( \text{Ca}_V^{1.2-a} \) channel shifted the average current-voltage relation by \(-3.2 \text{ mV} \) in the hyperpolarizing direction, significantly accelerated the kinetics of current inactivation and shifted the half-maximal inactivation voltage by \(-5.2 \text{ mV} \) toward hyperpolarizing potentials (Lacinová et al. 1995 and Table 4).

In addition to its effects on the basic electrophysiological properties of the \( \text{Ca}_V^{1.2} \) channel, the \( \beta_3 \) subunit significantly increased the channel affinity for the PAAs verapamil and gallopamil. The inhibitory potency of the DHP isradipine and of mibebradil, an organic calcium channel blocker, which does not belong to the above two classes of channel blockers, was not affected by the presence of the \( \beta_3 \) subunit (Figure 6). Because the action of all tested channel blockers is known to be voltage-dependent, we have tested all of them at a hyperpolarized membrane potential of \(-80 \text{ mV} \) and at a relatively depolarized membrane potential of \(-40 \text{ mV} \). Current inhibition was measured at five different concentrations of each drug at each holding potential (HP). The data measured on 5 to 10 cells for each compound and channel type were averaged and fitted by the Hill equation. The resulting values of concentration for 50% inhibition (IC\(_{50}\)) are summarized in Table 5.

Furthermore, we have investigated the effect of mibebradil on the \( \text{Ca}_V^{1.2-b} \), smooth muscle splice variant of the \( \text{Ca}_V^{1.2} \) channel alone or coexpressed either with the skeletal muscle \( \beta_1 \) or the neuronal \( \beta_3 \) subunits (Welling et al. 1995). Consistent with our observation on the \( \text{Ca}_V^{1.2-a} \), the cardiac splice variant of the

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**Note:** The table and text are extracted from a scientific research document, focusing on the electrophysiological properties and interactions of calcium channel blockers with \( \alpha_1 \) and \( \beta \) subunits.
Figure 6. In each pair of traces, the control trace was recorded just before the indicated drug concentration was added to the bath solution. The second trace shows $I_{Ba}$ three min after drug application, when a new stable current amplitude was reached. The $I_{Ba}$ was activated by a 40 ms long pulse from the HP $-80$ mV to $+30$ mV, or from the HP $-40$ mV to $+30$ mV with frequency of 0.2 Hz. Calibration bars represent 10 ms (horizontal) and 100 pA (vertical). Modified from Lacinová et al. (1995).

$\text{Ca}_{\nu}.1.2$ channel $\beta_3$ subunit did not significantly affect the current inhibition by mibebradil. When $\beta_1$ subunit was coexpressed together with the $\text{Ca}_{\nu}.1.2$-b channel, the interaction between the channel and the drug was altered in a complex man-
Table 5. IC₅₀ values were calculated according to the Hill equation. Significance of differences between the two channels was evaluated using non-paired Student’s t-test

<table>
<thead>
<tr>
<th>Compound</th>
<th>HP (mV)</th>
<th>IC₅₀ [µmol/l]</th>
<th>p ≤</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Caᵥ₁.₂</td>
<td>Caᵥ₁.₂β₃</td>
<td></td>
</tr>
<tr>
<td>Isradipine</td>
<td>−80</td>
<td>0.025 ± 0.002</td>
<td>0.020 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>−40</td>
<td>0.0027 ± 0.0010</td>
<td>0.001 ± 0.001</td>
</tr>
<tr>
<td>Gallopamil</td>
<td>−80</td>
<td>51 ± 4</td>
<td>23 ± 4</td>
</tr>
<tr>
<td></td>
<td>−40</td>
<td>3.7 ± 0.3</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Verapamil</td>
<td>−80</td>
<td>225 ± 60</td>
<td>84 ± 15</td>
</tr>
<tr>
<td></td>
<td>−40</td>
<td>15 ± 3</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Mibefradil</td>
<td>−80</td>
<td>4.9 ± 0.7</td>
<td>4.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>−40</td>
<td>1.4 ± 0.3</td>
<td>0.9 ± 0.1</td>
</tr>
</tbody>
</table>

n.s., not significant.

...ner. β₁ subunit partly prevents the drug-induced negative shift in the steady-state inactivation and acceleration of the current inactivation observed for the Caᵥ₁.₂-a or Caᵥ₁.₂-a/β₃ channel. Furthermore, in a part of all investigated cells, low concentrations of mibefradil caused current activation rather than current inhibition.

The shifts in steady-state inactivation and acceleration of current decay in the presence of the drug are consistent with a state-dependent channel block. Our experiments demonstrated that coexpression of β-subunit significantly influences not only the basic properties of calcium current, but also the state-dependent inhibition of the channel by certain classes of pharmacological agents. β-subunit-dependent modulation of neuronal calcium channel blocks by piperidine was reported by Zamponi and co-authors (1996). The block of expressed Caᵥ₁.₂, Caᵥ₂.1 and Caᵥ₂.3 channels by mibefradil was strongly affected by coexpression of the β₁b subunit (Jimenez et al. 2000). Sokolov et al. (2001) investigated the mechanism of regulation of the PAA gallopamil block of the expressed Caᵥ₁.₂ channel by various β subunits and found a correlation between the β-subunit-dependent amplification of channel inactivation and amplification of drug-induced inhibition. Altogether, we can conclude that auxiliary β subunits significantly affect both the gating-related properties of HVA channels and their interaction with pharmacologically relevant inhibitors.

**α₂δ subunit**

*Cloning of the α₂δ subunit family*

The α₂δ subunit with a molecular size of 125.0 kD was cloned by Ellis and co-authors (1988). Structurally, the α₂δ subunit is a heavily glycosylated 175 kDa...
protein that is post-translationally cleaved to yield a disulfide-linked α2 and δ proteins (De Jongh et al. 1990; Jay et al. 1991). The δ part anchors the α2 protein to membrane via a single transmembrane segment (Gurnett et al. 1996). Originally, two transmembrane segments were suggested for the α2 protein based on hydropathy analysis (Ellis et al. 1988) and biochemical experiment (Jay et al. 1991). Later experiments with site-directed antibodies (Brickley et al. 1995; Wiser et al. 1996) and in vitro translation (Gurnett et al. 1996) revealed a purely extracellular location of the α2 protein, which is anchored to the calcium channel complex by the membrane-located δ protein (Figure 7). Structural studies have shown that the extracellular α2 domain provides the structural elements required for channel stimulation (Gurnett et al. 1996). The δ domain, which contains the only transmembrane segment of the α2δ complex, harbours the regions important for the shift in the voltage-dependent activation and steady-state inactivation, and for the modulation of the inactivation kinetics (Felix et al. 1997).

Much later, three new members of the α2δ subunit family were cloned (Klugbauer et al. 1999b; Gao et al. 2000; Hobom et al. 2000; Hanke et al. 2001; Qin et al. 2002). All four proteins are summarized in Table 6.

Table 6. Sizes of individual cloned α2δ subunits of the VACC

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Molecular weight (kDa)</th>
<th>Number of amino acids</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α2δ-1</td>
<td>125.0</td>
<td>1106</td>
<td>Ellis et al. 1988</td>
</tr>
<tr>
<td>α2δ-2</td>
<td>129.3</td>
<td>1145</td>
<td>Hobom et al. 2000; Gao et al. 2000</td>
</tr>
<tr>
<td>α2δ-3</td>
<td>1091</td>
<td>1091</td>
<td>Klugbauer et al. 1999b</td>
</tr>
<tr>
<td>α2δ-4</td>
<td>126.0</td>
<td>1120</td>
<td>Qin et al. 2002</td>
</tr>
</tbody>
</table>

Modulatory role of α2δ subunits

The role of the α2δ-1 subunit in regulation of channel function was less extensively investigated than the role of diverse β subunits, and the reported results are
partially inconsistent. Functional coexpression of the \( \alpha_2 \delta-1 \) subunit with various combinations of \( \alpha_1 \) and \( \beta \) subunits resulted in an increase in the current amplitude in most (Singer et al. 1991; De Waard et al. 1995; Shistik et al. 1995; Bangalore et al. 1996; Garnet et al. 1996; Felix et al. 1997; Parent et al. 1997), but not in all cases (Welling et al. 1993a; Qin et al. 1998). Welling and co-authors (1993a) and De Waard and collaborators (1995) also failed to observe any effects of \( \alpha_2 \delta-1 \) coexpression on the time course and voltage dependence of current activation. In contrast, a remarkable acceleration of current activation by \( \alpha_2 \delta-1 \) subunit was reported by Singer et al. (1991), Bangalore et al. (1996) and Qin et al. (1998). A shift of the current-voltage curve in hyperpolarizing direction was observed by Singer et al. (1991) and Felix et al. (1997); however, a shift in the opposite – depolarizing direction was reported by Qin et al. (1998). Acceleration of the time course of current inactivation was consistently observed by all authors who investigated it (Singer et al. 1991; Welling et al. 1993a; De Waard et al. 1995; Felix et al. 1997; Qin et al. 1998; Shirokov et al. 1998). Nevertheless, a hyperpolarizing shift in the steady-state inactivation curve evoked by coexpression of the \( \alpha_2 \delta-1 \) subunit was reported by Singer et al. 1991; Welling et al. 1993a; Felix et al. 1997), or was not (De Waard et al. 1995; Parent et al. 1997; Qin et al. 1998) observed. The cited authors used different expression systems (Xenopus oocytes or mammalian cell lines), different charge carriers (Ba\(^{2+}\) or Ca\(^{2+}\)), and different combinations of \( \alpha_1 \) (L-type \( \text{Ca}_v1.2 \) or \( \text{Ca}_v2.1 \), neuronal \( \text{Ca}_v2.3 \) channels) and \( \beta \) (\( \beta_1 \), \( \beta_2 \), \( \beta_3 \) or \( \beta_4 \)) subunits. It is not possible to trace a common pattern in the relation between experimental conditions and observed effects of the \( \alpha_2 \delta-1 \) subunit. However, one should note that though the reported results are inconsistent, they are not contradictory. The authors do not report opposite effects, but rather report presence or absence of individual effects. The only exception is the work of Qin et al. (1998) who observed a depolarizing shift in the current-voltage relation, in contrast to all other authors who observed a hyperpolarizing shift or no shift at all.

The increase in current density could be attributed to improved targeting of expressed \( \alpha_1 \) subunit to the cell membrane. Indeed, this effect was directly demonstrated by Shistik et al. (1995). The effects of coexpression of the \( \alpha_2 \delta-1 \) subunit on the time course and/or voltage dependence on current activation and inactivation suggest a more specific modulation of the behaviour of the channel’s voltage gate. Shistik et al. (1995) demonstrated that in presence of the \( \alpha_2 \delta-1 \) subunit, the open probability of the channel increases without a change in the mean open time. Both Bangalore et al. (1996) and Qin et al. (1998) showed that coexpression of the \( \alpha_2 \delta-1 \) subunit increases the amount of charge moved during channel activation. This increase was coupled with increased maximal conductance in the L-type \( \text{Ca}_v1.2 \) channel (Bangalore et al. 1996), while no change at maximal conductance was observed in the neuronal \( \text{Ca}_v2.3 \) channel (Qin et al. 1998). Shirokov et al. (1998) reported that \( \alpha_2 \delta-1 \) speeds up the transition of the \( \text{Ca}_v1.2 \) channel into the slow inactivated state and slows down the recovery from this state. These changes in channel gating probably underlie the observed effects on inactivation of whole cell current. The importance of the role of \( \alpha_2 \delta-1 \) in regulation of channel gating is
supported also by the work of Wyatt et al. (1998), who over-expressed the α2δ-1 subunit in undifferentiated mammalian neuronal NG108-15 cell line and were able to demonstrate the modulation of activation and inactivation of both the L- and T-type channels (however, cf. Lacinová et al. 1999c).

Interactions of various α1 subunits of the VDCC with α2δ-2, -3 and -4 subunits have been described only recently. It is possible that the α2δ-2 subunit interacts with LVA T-type channels by increasing the number of membrane-targeted channels (Gao et al. 2000; Hobom et al. 2000) and accelerating channel inactivation (Hobom et al. 2000). Both the α2δ-2 and -3 modulate the neuronal HVA channels (Klugbauer et al. 1999b; Hobom et al. 2000). The α2δ-4 subunit was shown to increase current density of the CaV1.2/β3 channel (Qin et al. 2002).

It was suggested that the mechanism of current enhancement by α2δ subunits mimics the mechanism of channel facilitation. When α2δ-1 or α2δ-3 were coexpressed together with the CaV1.2 channel, pre-pulse facilitation of Ca2+ current was abolished (Dai et al. 1999; Platano et al. 2000).

Similar to β subunits, α2δ subunits may modulate interaction of calcium channels with blockers. The α2δ-1 subunit was shown to reduce both on-rates and equilibrium inhibition of CaV2.2 channel by ω-conotoxins GVIA, MVIIA, CVID and analogues (Mould et al. 2004.). Because the expression of the α2δ subunit is up-regulated in certain pain states, this finding may have direct clinical implications.

**Tissue distribution of α2δ subunits**

α2δ subunits are widely distributed in excitable tissues. The α2δ-1 subunit is predominantly expressed in skeletal muscles, heart and brain. α2δ-2 is prominently expressed in the heart, skeletal muscles, lungs, pancreas, testes and brain (Klugbauer et al. 1999b; Gao et al. 2000; Hobom et al. 2000). The α2δ-3 subunit is mostly expressed in the brain (Klugbauer et al. 1999b). Northern blot analyses revealed expression of the α2δ-4 gene in the heart and skeletal muscles. Immunohistochemical labelling showed presence of the α2δ-4 protein in the pituitary gland, adrenal gland, small intestines and foetal liver tissue (Qin et al. 2002).

**Regulation of high-voltage-activated calcium channels by the α2δ-1, -2 and -3 subunits**

To elucidate the possible role of the new α2δ subunits on the L-type and neuronal HVA channel subfamilies, CaV1.2 and CaV2.3 subunits were coexpressed together with β2a or β3 subunits, respectively, and effects of α2δ-1 and α2δ-3 on channel gating were compared. The effect of both α2δ subunits on current density was equivalent (Figure 8).

The changes in gating of the CaV1.2 channel elicited by both subunits were qualitatively similar. The effects of α2δ-1 and α2δ-3 on current density, voltage dependence of current activation and inactivation as well as on the time course
α2δ-1 and α2δ-3 subunits affect the amplitude of current through the HVA Ca\textsubscript{v}1.2 (left panel) and Ca\textsubscript{v}2.3 (right panel) channels. The differences between current amplitudes in the absence and in presence of each α2δ subunit were significant. Original current records are illustrated in lower parts of each panel. Squares represent individual measurements of current amplitude.

of current activation were not quantitatively different. Both the α2δ-1 and α2δ-3 subunits accelerated the time course of current inactivation at membrane potential of +20 mV by increasing the proportion of the fast time constant (τ\textsubscript{1}). α2δ-3 changed this time constant from 260 ± 19 ms (Ca\textsubscript{v}1.2β2αyδ-1 channel; \(n = 11\)) to 156 ± 10 ms (Ca\textsubscript{v}1.2β2α2δ-3 channel; \(n = 9\)). Surprisingly, α2δ-1 significantly increased the slow time constant (τ\textsubscript{2}) from 1.16 ± 0.08 s (Ca\textsubscript{v}1.2β2 channel; \(n = 12\)) to 3.54 ± 0.46 s (Ca\textsubscript{v}1.2β2α2δ-1 channel; \(n = 11\)), though this effect is largely screened by the effect of an increased proportion of current inactivating by τ\textsubscript{1} and causing the overall time course of inactivation to be accelerated (Klugbauer et al. 1999b). The effects of both the α2δ-3 and α2δ-1 subunits on the whole cell current parameters, which reflect gating of the Ca\textsubscript{v}1.2 channel, are virtually identical and require the presence of β, in this case, the β\textsubscript{2a} subunit, to become prominent. In contrast to some previous works (Welling et al. 1993a; Shistik et al. 1995), we found statistically significant effects of α2δ coexpression on all measured parameters. However, a direct comparison of our results is not possible because of different experimental conditions – Welling et al. (1993a) used Ca\textsuperscript{2+} as a charge carrier and stably expressed cell lines, and Shistik et al. (1995) used Xenopus oocytes as expression system. Moreover, the reported enhancement of charge movement related to both current activation (Bangalore et
Voltage-Dependent Calcium Channels

al. 1996; Qin et al. 1998) and inactivation (Shirokov et al. 1998) suggests that, in the whole cell current measurements, alteration of all related activation and inactivation parameters could be expected, as we indeed observed in our experiments.

While both the Ca\(_{v}\)1.2 and \(\alpha_2\delta\)-1 subunits are fairly abundant in mammalian tissues, Ca\(_{v}\)2.3 and \(\alpha_2\delta\)-3 are predominantly expressed in neuronal tissue. Therefore, we have selected the Ca\(_{v}\)2.3 channel for studies of the effects of \(\alpha_2\delta\) subunits. In all experiments, \(\beta_3\) subunit was coexpressed. This subunit had been suggested to modulate the current through the Ca\(_{v}\)2.3 channel (Ludwig et al. 1997). As with the Ca\(_{v}\)1.2 channel, both \(\alpha_2\delta\)-1 and \(\alpha_2\delta\)-3 affected most of the gating-related parameters except for the time course of current activation during membrane depolarization. Nevertheless, in this case the time constant of activation in absence of \(\alpha_2\delta\) already reached the values, which in the case of the Ca\(_{v}\)1.2/\(\beta_{2a}\) channel required coexpression of \(\alpha_2\delta\). Furthermore, the effects of \(\alpha_2\delta\)-3 and \(\alpha_2\delta\)-1 on voltage dependence of current activation and inactivation of the Ca\(_{v}\)2.3/\(\beta_3\) channel were significantly different. The \(\alpha_2\delta\)-1 subunit shifted both the activation and steady-state inactivation curves in a hyperpolarizing direction, but the change in current activation was not statistically significant. In both curves, the shift invoked by \(\alpha_2\delta\)-3 was significantly larger. In contrast, the effects of both \(\alpha_2\delta\)'s on the time course of current inactivation at a membrane potential of +20 mV were identical. Altogether, \(\alpha_2\delta\)-1 had smaller effect on the Ca\(_{v}\)2.3 channel than on the Ca\(_{v}\)1.2 channel. \(\alpha_2\delta\)-3 was found to be equally efficient in modulation of both calcium channel \(\alpha_1\) subunits in the presence of a \(\beta\) subunit. The regulation of the neuronal Ca\(_{v}\)2.3 channel by a \(\alpha_2\delta\) subunit was not extensively studied before. Parent et al. (1997) reported, consistent with our results, an increase in current density upon coexpression of \(\alpha_2\delta\) and, in contrast to our observations, no effect on voltage dependence of the steady-state inactivation curve. However, because of the voltage protocol used in the said study, one may expect a certain level of contamination of inactivated current by tail current. Qin et al. (1998) observed an acceleration of current inactivation, which is apparent in our experiments, too. Nevertheless, in contrast to our results, these authors observed also an acceleration of current activation and no effect on current density or voltage dependence of current inactivation. This discrepancy may again be a result of different expression systems (Xenopus oocytes in Qin et al. 1998) and/or different coexpressed \(\beta\) subunit (\(\beta_1\) in Qin et al. 1998). In our experiments with the Ca\(_{v}\)2.3 channel, \(\alpha_2\delta\)-3 but not \(\alpha_2\delta\)-1 subunit affected all whole cell parameters related to channel gating except for the time course of channel activation, which was fast already in its absence (see discussion above). Therefore, one could speculate that, as follows also from the works of Bangalore et al. (1996) and Shirokov et al. (1998), \(\alpha_2\delta\) subunits in the presence of a \(\beta\) subunit affect both activation and inactivation gating of the \(\alpha_1\) subunit, and that in the neuronal Ca\(_{v}\)2.3 channel this is predominantly the role of the new neuronal \(\alpha_2\delta\)-3 subunit, for which the ubiquitous \(\alpha_2\delta\)-1 subunit is not an adequate replacement.
Regulation of low-voltage-activated calcium channels by α2δ-1, -2 and -3 subunits

Functional modulation of a LVA channel by α2δ subunits was tested upon coexpression of α2δ-1, -2 and -3 subunits together with the CaV3.1 channel in human embryonic kidney (HEK) 293 cells. The current density-voltage relationship for peak and sustained current, kinetics of current activation and inactivation, voltage dependence of current inactivation and time course of recovery from inactivation were analyzed for each type of expressed channel. No significant difference was found for any of the examined parameters.

The lack of modulatory effects of α2δ-1 and α2δ-3 subunits contrasts with the finding of Wyatt and co-authors (1998) who overexpressed α2δ-1 subunit in undifferentiated mammalian neuronal NG108-15 cell line and reported modulation of activation and inactivation of LVA channels. It is possible that the effects observed by Wyatt et al. (1998) upon coexpression of α2δ subunit during depolarizing pulses positive to −30 mV reflect the up-regulation of a previously immeasurable L-type channel.

In contrast to α2δ-1 and α2δ-3, the α2δ-2a subunit caused a significant alteration of several CaV3.1 channel characteristics. Current density was significantly increased (Table 7). A similar finding was reported by Dolphin et al. (1999). Steady-state inactivation was significantly shifted by +4.5 mV in the depolarizing direction (Table 7 and Figure 9). Kinetics of current activation was not significantly altered, but the time course of inactivation was significantly accelerated (Figure 9). The time constant of inactivation saturated at approximately 14.5 ms and 12.5 ms in the absence and in presence of the α2δ-2 subunit, respectively. Voltage dependence of current activation was slightly shifted in hyperpolarizing direction, but this shift was not significant (Figure 9). From such minute effects, it is hard to judge whether

Table 7. Effect of coexpression of α2δ-1, α2δ-2a, and α2δ-3 subunits on the parameters of current density-voltage relationship of the CaV3.1 channel. V0.5 is the potential of half-maximal current activation or inactivation and k is the corresponding slope factor. Numbers of experiments are given in brackets. Significance of differences between individual parameters for cells expressing the CaV3.1 subunit only and cells coexpressing an α2δ subunit was tested using unpaired Student’s t-test.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Charge carrier</th>
<th>Activation</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pA/pF)</td>
<td>V0.5 (mV)</td>
<td>k (mV)</td>
</tr>
<tr>
<td>CaV3.1</td>
<td>Ba2+ 40 ± 10 (9)</td>
<td>−28.4 ± 0.6 (9)</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>CaV3.1α2δ-1</td>
<td>Ba2+ 54 ± 12 (10)</td>
<td>−28.1 ± 0.5 (10)</td>
<td>4.4 ± 0.4</td>
</tr>
<tr>
<td>CaV3.1α2δ-3</td>
<td>Ba2+ 58 ± 12 (11)</td>
<td>−29.8 ± 0.5 (11)</td>
<td>4.1 ± 0.5</td>
</tr>
<tr>
<td>CaV3.1</td>
<td>Ca2+ 62 ± 7 (25)</td>
<td>−24.9 ± 1.2 (12)</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>CaV3.1α2δ-2a</td>
<td>Ca2+ 93 ± 15* (17)</td>
<td>−22.3 ± 0.8 (12)</td>
<td>4.1 ± 0.2</td>
</tr>
</tbody>
</table>

* p < 0.5; ** p < 0.01; numbers of cells measured are given in brackets.
the $\alpha_2\delta$-2 subunit simply facilitates membrane targeting of the Ca$_{v}$3.1 channel or indeed has a genuine modulatory effect on calcium channel gating.

Modulation of the Ca$_{v}$3.1 channel as a representative of the LVA channels family, is summarized in Table 8.
Table 8. Effects of coexpression of various $\alpha_2\delta$ subunits on the gating properties of the Ca$_{v}$3.1 channel. The positive and negative numbers represent shifts of half-maximal voltage for current activation ($V_{0.5\text{act}}$) or half-maximal voltage for steady-state inactivation of the current ($V_{0.5\text{inact}}$) in the depolarizing and hyperpolarizing direction, respectively. Time constants of current activation ($\tau_{\text{act}}$) and inactivation ($\tau_{\text{inact}}$) were significantly affected by $\alpha_2\delta$ coexpression either at individual voltages or in the whole range of voltages (marked by “…”).

<table>
<thead>
<tr>
<th>Enhancement of $I_{\text{max}}$</th>
<th>Shift of $V_{0.5\text{act}}$</th>
<th>Shift of $V_{0.5\text{inact}}$</th>
<th>$\tau_{\text{act}}$</th>
<th>$\tau_{\text{inact}}$</th>
<th>$\tau_{\text{deact}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha_2\delta$-1</td>
<td>0.95</td>
<td>+1.5</td>
<td>+1.4</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>$\alpha_2\delta$-2</td>
<td>1.5</td>
<td>+2.6</td>
<td>$+4.5$</td>
<td>$-30\ldots+30$ mV</td>
<td>$-20\ldots+20$ mV</td>
</tr>
<tr>
<td>$\alpha_2\delta$-3</td>
<td>1.2</td>
<td>+1.4</td>
<td>+0.1</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

n.s., not significantly altered; gray background, $p < 0.01$; bold italics, $p < 0.001$.

Interaction of $\alpha_2\delta$ subunit with gabapentin

Gabapentin (GBP) is an antiepileptic drug that has found application also in pain relief and as an anxiolytic (Welty et al. 1993; Beydoun et al. 1995). GBP was found to bind specifically to $\alpha_2\delta$-1 of VACCs (Gee et al. 1996). This was the first described interaction between a regulatory subunit of a VACC and a pharmaceutical agent. Because VACCs are involved in the control of electrical excitability of neurons, it has been postulated that this drug reduces calcium current by modulating $\alpha_1$ subunit of HVA channel indirectly through its association with $\alpha_2\delta$-1 (Gee et al. 1996).

The $K_d$ of GBP binding to the porcine brain $\alpha_2\delta$-1 was reported as 9.4 nmol/l (Brown et al. 1998), but as 37.5 nmol/l for porcine $\alpha_2\delta$-1 expressed in COS-7 cells (Brown and Gee 1998) and 16 nmol/l for rabbit $\alpha_2\delta$-1 in COS-7 cells (Gee et al. 1996). Marais and co-authors (2001) found $K_d$ values of 59 nmol/l for $\alpha_2\delta$-1 and 153 nmol/l for $\alpha_2\delta$-2. No specific binding of GBP to $\alpha_2\delta$-3 subunit was found. The reason of variations in $K_d$ values is not clear, but may be attributable to species differences and assay methods.

The effect of GBP on physiological activity of calcium channels is not clearly understood. In patch-clamp studies with hippocampal granule cells, no effect of GBP was reported (Schumacher et al. 1998). However, in other studies, modest to dramatic changes in calcium current were noted. A reduction in the calcium current in isolated neurons (Stefani et al. 1998) and in rat neocortical slices (Fink et al. 2000) upon application of GBP has been described. Calabresi et al. (1999) found GBP to reduce most excitatory properties of striatal spiny neurons, which could account for the anticonvulsant effect of the drug. The calcium channels affected are not known, but candidates are L-type (Stefani et al. 1998) and P/Q-type channel (Fink et al. 2000; Meder and Dooley 2000). We have observed no consistent effect of GBP on Ca$_{v}$1.2, Ca$_{v}$2.1, and Ca$_{v}$3.2 currents in a heterologous HEK 293 expression system (Marais et al. 2001).
Initial studies on GBP binding using rat tissue homogenates showed strong binding in skeletal muscles and the brain, where \( \alpha_2\delta-1 \) is most highly expressed (Gee et al. 1996). A much lower binding of the drug was seen in the liver and kidney, which express considerable levels of the protein, as judged by Western blotting. A possible explanation for these conflicting results is that the binding of GBP to \( \alpha_2\delta-1 \) is modulated by other subunits (e.g., the \( \alpha_1 \) pore protein). It is possible that the effects of GBP depend on the composition and environment of the channel. The lack of clinical side effects of the drug on skeletal muscles and other \( \alpha_2\delta-1 \) expressing tissues supports this view (Beydoun et al. 1995). GBP has also been shown to be an agonist of certain GABA\(_B\) receptors, and this has also been postulated to be involved in the clinical action of the drug (Ng et al. 2001).

\( \gamma \) subunit

The \( \gamma \) subunit is an integral membrane protein. Originally the \( \gamma-1 \) subunit consisting of 222 amino acids with a predicted molecular mass of 25.1 kDa was purified from rabbit skeletal muscle (Bosse et al. 1990; Jay et al. 1990). Hydrophobicity analysis revealed the existence of four putative transmembrane helices with intracellularly located N- and C-terminals (Figure 10A). The presence of two extracellular potential N-glycosylation sites is consistent with the observed strong glycosylation of these subunits (Bosse et al. 1990; Jay et al. 1990).

For a long time, the \( \gamma \) subunit was considered unique to skeletal muscles. Later, a second \( \gamma-2 \) subunit was identified in brain. The \( \gamma-2 \) subunit has 25% identity with the skeletal muscle \( \gamma-1 \) and is expressed mostly in the cerebellum, olfactory bulb, cerebral cortex, thalamus, CA3, and dentate gyrus of the hippocampus (Letts et al. 1998).

Additional putative \( \gamma \) subunits \( \gamma-3, \gamma-4, \) and \( \gamma-5 \) have been identified (Klugbauer et al. 2000). The \( \gamma-3 \) clone is highly homologous to the \( \gamma-2 \) subunit. The \( \gamma-4 \) clone has 25% identity with the \( \gamma-1 \) subunit. The \( \gamma-3 \) subunit is only distantly related to \( \gamma-2, \gamma-3, \) and \( \gamma-4 \) (Figure 10B). Burgess and co-authors (2001) identified a cluster of three novel \( \gamma \) subunit genes. Structurally, \( \gamma-6 \) subunit is related to \( \gamma-1, \gamma-7 \) subunit is related to \( \gamma-5, \) and \( \gamma-8 \) subunit is related to \( \gamma-4. \)
Chu and co-workers (2001) analysed γ subunit family in rat, mouse and human. They identified eight γ subunits expressed in rat tissues including three new genes and identified orthologs of these genes in mouse and human. Based on this analysis, authors suggests that protein identified previously as mouse γ-5 subunit (Klugbauer et al. 2000) is not a member of the γ subunit family and introduces the term “protein distantly related to the γ subunit family” (Chu et al. 2001).

Tissue distribution of the novel putative γ subunits was analyzed by Northern blot. γ-3 and γ-4 mRNAs were only detectable in mouse brain and, even after a long time of autoradiography, no signals were seen in other tissues. The γ-5 subunit is highly expressed in the liver, kidney, heart, lung, skeletal muscles, and, with a lower abundance, in testes (Klugbauer et al. 2000).

Coexpression of each γ subunit together with α1, α2δ and β subunits in oocytes induces a left shift in steady-state inactivation curves (Singer et al. 1991; Letts et al. 1998). We have tested for regulatory effects of γ-2, γ-4, and γ-5 upon their coexpression with the Cav1.2, CaV2.1, or CaV3.1 subunits, and also in combination with other auxiliary subunits (Klugbauer et al. 2000). The sequence of the γ-3 subunit is highly homologous with γ-2, so one can expect that they behave similarly.

In the combination of CaV2.1, β1a, and α2δ-1, only γ-2 showed a small but significant shift of voltage-dependent activation to more positive potentials. A shift of the steady-state inactivation curve towards more hyperpolarized potentials was observed upon coexpression for the γ-2 and γ-4, but not for the γ-5 subunit. When these experiments were repeated with the neuronal β2a subunit, the same effect on the steady-state inactivation as seen with β1a could be observed with Ca2+, but not with Ba2+ as charge carrier (Klugbauer et al. 2000).

Furthermore, we compared the effects of the novel γ subunits with previously published results on the L-type CaV1.2 channel. Upon coexpression of γ-1 with CaV1.2-a, cardiac β2a and α2δ-1, the steady-state inactivation curve was shifted about 30 mV to more negative potentials and current inactivation was accelerated. Using the newly identified subunits, γ-2 and γ-5, we performed coexpression studies with CaV1.2-a, cardiac β2a and α2δ-1 with Ba2+ as charge carrier. Small but significant shifts of the voltage dependence of activation and inactivation were observed with γ-2, but not with the γ-5 subunit. These results suggest that there is also an interaction between the γ-2 subunit and the L-type CaV1.2 channel. Coexpression studies with γ-5 have also been performed in the absence of α2δ-1. The absence of this subunit did not uncover any effects of γ-5 on voltage dependence of activation or on steady-state inactivation of current through the CaV1.2 channels. Although γ-5 is expressed in at least some of the tissues that also express CaV1.2 channel, it does not seem to modulate this L-type channel. For these reasons, we suggest that the γ-5 subunit may not be an auxiliary subunit of the HVA channel.

The possibility that the novel γ subunits are auxiliary subunits of the LVA T-type channels was considered and the effects of the γ-2, γ-4 and γ-5 subunits on the CaV3.1 channel were analyzed. Current density was enhanced upon coexpression of each γ subunit, but this increase was not statistically significant (Table 9). Voltage dependence of current activation was unaffected. Steady-state inactivation
Table 9. Effects of γ subunits on the voltage-dependent activation and inactivation of LVA calcium channels. $I_{\text{max}}$ represents averaged current densities for all tested channels. Activation and steady-state inactivation experimental data were fitted by the Boltzmann equation and the resulting $V_{0.5}$ and k values were averaged. The last column shows time constants of recovery from voltage-dependent inactivation. Individual measurements were fitted to an exponential association curve and the resulting $\tau$'s were averaged. Significance of the differences between parameters measured in the absence and in presence of various auxiliary subunits was tested by unpaired Student’s $t$-test. Data are given as mean ± S.E.M. with number of cells in brackets. 20 mmol/l Ca$^{2+}$ was used as a charge carrier in all experiments.

<table>
<thead>
<tr>
<th>Channel</th>
<th>$I_{\text{max}}$ (pA/pF)</th>
<th>Activation</th>
<th>Inactivation</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$V_{0.5}$ (mV)</td>
<td>k (mV)</td>
<td>$V_{0.5}$ (mV)</td>
</tr>
<tr>
<td>$\text{Ca}_v 3.1$</td>
<td>61 ± 7 (20)</td>
<td>−24.9 ± 1.2 (12)</td>
<td>3.9 ± 0.3</td>
<td>−54.9 ± 1.2 (9)</td>
</tr>
<tr>
<td>$\text{Ca}_v 3.1\gamma-2$</td>
<td>87 ± 13 (13)</td>
<td>−24.4 ± 1.6 (10)</td>
<td>3.8 ± 0.2</td>
<td>−51.4 ± 1.4 (10)</td>
</tr>
<tr>
<td>$\text{Ca}_v 3.1\gamma-4$</td>
<td>79 ± 12 (14)</td>
<td>−23.9 ± 1.3 (10)</td>
<td>4.1 ± 0.2</td>
<td>−50.0 ± 1.4* (11)</td>
</tr>
<tr>
<td>$\text{Ca}_v 3.1\gamma-5$</td>
<td>73 ± 9 (22)</td>
<td>−24.1 ± 1.0 (12)</td>
<td>3.9 ± 0.2</td>
<td>−51.7 ± 1.2 (11)</td>
</tr>
<tr>
<td>$\text{Ca}<em>v 3.1\alpha</em>\delta-2a$</td>
<td>93 ± 15* (17)</td>
<td>−22.3 ± 0.8 (12)</td>
<td>4.1 ± 0.2</td>
<td>−50.4 ± 0.7** (11)</td>
</tr>
<tr>
<td>$\text{Ca}<em>v 3.1\alpha</em>\delta-2a\gamma-5$</td>
<td>85 ± 12 (18)</td>
<td>−21.4 ± 1.1 (19)</td>
<td>4.2 ± 0.2</td>
<td>−49.3 ± 1.1** (11)</td>
</tr>
</tbody>
</table>

* $p < 0.05$; ** $p < 0.01$. 
Figure 11. A. Coexpression of the $\gamma$-2, $\gamma$-4, and $\gamma$-5 subunits together with the Ca$_{v}$3.1 channel enhanced current density (left), but this effect was not significant. Voltage dependence of current activation was not affected (right). B. Kinetics of current activation during depolarization was significantly accelerated by the $\gamma$-5 subunit, partly also by $\gamma$-4, but not by the $\gamma$-2 subunit (left). Current inactivation was significantly accelerated by the $\gamma$-5 subunit (right). Significance of differences between the parameters observed in the absence and in presence of individual $\gamma$ subunits was tested by unpaired Student’s $t$-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. C. Shift of the steady-state inactivation curve caused by coexpression of $\gamma$ subunits was significant for the $\gamma$-4 subunit (left). Recovery from the inactivation was significantly slowed down by the $\gamma$-2 and $\gamma$-4 subunits (right). Modified from Klugbauer et al. (2000).
curves were slightly shifted to more positive voltages (Table 9 and Figure 11); however, this shift was only significant for the γ-4 subunit. The speed of recovery from voltage-dependent inactivation was significantly slowed down by the γ-2 and γ-4 subunits, but not by the γ-5 subunit (Table 9 and Figure 11). The time course of current activation and inactivation during a depolarizing pulse was fitted by the Hodgkin–Huxley equation. The γ-5 subunit significantly accelerated both processes over the whole range of voltages. This effect indicates that γ-5 could be an auxiliary subunit of LVA channels. In contrast, the γ-4 subunit had similar effects only at voltages above the threshold for current activation and γ-2 was without effect.

To test the possibility that an interaction of the γ-5 subunit with Ca\textsubscript{v}3.1 requires the presence of an additional auxiliary subunit, the α\textsubscript{2}δ-2a subunit was coexpressed together with Ca\textsubscript{v}3.1 and γ-5. However, the properties of the Ca\textsubscript{v}3.1/α\textsubscript{2}δ-2a/γ-5 channel were similar to those of the Ca\textsubscript{v}3.1/α\textsubscript{2}δ-2a channel (Table 9). Another member of LVA channel family, Ca\textsubscript{v}3.3, is modulated by coexpression of γ-2, but not γ-3 and γ-4 subunits (Green et al. 2001).

In summary, γ-2, γ-3 and γ-4 could be associated with the Ca\textsubscript{v}2.1, Ca\textsubscript{v}2.2, and Ca\textsubscript{v}2.3 channels. γ-5 subunit may modulate LVA T-type channels. Rousset et al. (2001) demonstrated minor modulatory effects of γ-2, γ-3, and γ-4 subunits on electrophysiological characteristics of the Ca\textsubscript{v}2.1 channel. The role of γ-2 and γ-3 subunits in the neuronal channel complex was underlined by Kang and co-authors (2001), who demonstrated its co-immunoprecipitation with the Ca\textsubscript{v}2.1/2.2 channels. Furthermore, the γ-2 subunit regulated the Ca\textsubscript{v}2.2 channel expressed in Xenopus oocytes (Kang et al. 2001). The γ-2 subunit downregulated activity of both Ca\textsubscript{v}2.1 and Ca\textsubscript{v}2.2 channels. Similarly, the γ-1 subunit downregulates calcium current through the Ca\textsubscript{v}1.1 channel (Freise et al. 2000; Arikkath et al. 2003). This property contrasts with the regulatory role of β and α\textsubscript{2}δ subunits, which generally enhance the activity of VACC.

It is possible that while the modulatory effects of γ-2 through γ-5 on the VACC

Table 10. Effects of coexpression of various γ subunits on gating properties of the Ca\textsubscript{v}3.1 channel. Positive and negative numbers represent shift of half-maximal voltage for current activation (V\textsubscript{0.5act}) or half-maximal voltage for steady-state inactivation of current (V\textsubscript{0.5inact}) in the depolarizing and hyperpolarizing direction, respectively. Time constants of current activation (τ\textsubscript{act}) and inactivation (τ\textsubscript{inact}) were significantly affected by γ coexpression either at individual voltages or in the whole range of voltages (marked by ‘...’).

<table>
<thead>
<tr>
<th>γ</th>
<th>Enhancement of I\textsubscript{max}</th>
<th>Shift of V\textsubscript{0.5act}</th>
<th>Shift of V\textsubscript{0.5inact}</th>
<th>τ\textsubscript{act}</th>
<th>τ\textsubscript{inact}</th>
<th>τ\textsubscript{deact}</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ\textsubscript{2}</td>
<td>1.4</td>
<td>+0.5</td>
<td>+3.5</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>γ\textsubscript{4}</td>
<td>1.3</td>
<td>+1.0</td>
<td>+4.9</td>
<td>-20, -10 mV</td>
<td>-20 mV</td>
<td>n.s.</td>
</tr>
<tr>
<td>γ\textsubscript{5}</td>
<td>1.2</td>
<td>-0.8</td>
<td>+3.2</td>
<td>-30...+30 mV</td>
<td>-10...+30 mV</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

n.s., not significantly altered; bold italics, p < 0.05; grey background, p < 0.01.
are modest, their principal role is in interaction with other membrane proteins. Chen and co-authors (2000) have shown that \( \gamma \)-2 is responsible for proper targeting of AMPA receptor to the synaptic space.

The possible role of \( \gamma \) subunits in the LVA channel complex is summarized in Table 10.

**L-type calcium channel**

**Subunit composition of the calcium channel**

The L-type channel family has currently four known members (see Table 1). The first cloned skeletal muscle \( \text{Ca}_v1.1 \) channel consists of five proteins, the principal \( \alpha_1 \) and auxiliary \( \beta_1, \alpha_2\delta-1 \) and \( \gamma-1 \) subunits. Subunit composition of another three L-type channels, \( \text{Ca}_v1.2, \text{Ca}_v1.3 \) and \( \text{Ca}_v1.4 \), is less certain. \( \text{Ca}_v1.2 \) may be coexpressed with various \( \beta \) and \( \alpha_2\delta \) subunits in a tissue-dependent manner. Coexpression studies in heterologous systems demonstrated modulation of the \( \text{Ca}_v1.2 \) channel by all four known \( \beta \) subunits. Our results (Klugbauer et al. 2000) suggest that the \( \gamma-2 \) subunit may regulate the \( \text{Ca}_v1.2 \) channel. Subunit composition of the \( \text{Ca}_v1.3 \) channel was not investigated systematically, but coexpression studies confirmed regulation of the channel by the \( \beta_2, \beta_3 \) and \( \alpha_2\delta-1 \) subunits. No expression studies have been done so far with the \( \text{Ca}_v1.4 \) channel.

**Regulation by calcium channel blockers and activators**

**Dihydropyridines**

Ligands of the L-type channel represent a clinically and experimentally important set of blockers and agonists. The major classes of these drugs are organic channel blockers, such as dihydropyridines (DHP), phenylalkylamines (PAA) and benzothiazepines (BTZ). Different techniques have been used to localize potential binding sites of these drugs on the calcium channel complex. Earlier experimental observations from photoaffinity labelling and peptide mapping studies on the skeletal muscle channel revealed that all three classes bind to the transmembrane region of repeat IV of the \( \alpha_1 \) subunit (Regulla et al. 1991; Catterall and Striessnig 1992; Kuniyasu et al. 1998) with additional sites on repeat III (Catterall and Striessnig 1992; Kalasz et al. 1993) and repeat I (Kalasz et al. 1993) for the DHPs. Custom-synthesized DHPs, in which the DHP moiety was separated from the permanently charged head by a spacer chain with variable length, were most effective in channel inhibition when the length of the spacer chain was ten methylene residues (Baindur et al. 1993; Bangalore et al. 1994). While the permanently charged hydrophilic head stays outside of cell membrane, the active DHP moiety can reach about 11–14 Å inside the lipid bilayer under these conditions.

The construction of chimeric \( \text{Ca}_v1.2/\text{Ca}_v2.1 \) and \( \text{Ca}_v1.2/\text{Ca}_v2.3 \) channels and site directed mutagenesis of single amino acids in the \( \text{Ca}_v1.1 \) or \( \text{Ca}_v1.2 \) subunit further refined the location of the DHP-interaction site. Tyr1485, Met1486 and Ile1493 of IVS6 (amino acid numbering is according to the \( \text{Ca}_v1.2 \)-b sequence (Biel
**Figure 12.** Important determinants of DHP binding to the Ca\(_{\text{v}}\)1.2 channel are the amino acids of the IVS6 segment. Panel **A.** Amino acid exchange between the Ca\(_{\text{v}}\)1.2 channel and the DHP insensitive Ca\(_{\text{v}}\)2.3 channel, yielding the Ch30 channel. Panel **B.** The effect of mutations of the Ch30 channel on IC\(_{50}\) for inhibition of the expressed channel by (+)isradipine. The dose-response curve measured at HP of –80 mV is in the left part of the panel. Examples of current traces measured in the absence (black) and in presence (grey) of 1 µmol/l (+)isradipine from either the wild type Ca\(_{\text{v}}\)1.2 or the Ch30 channel are on the right part of the panel. Panel **C.** The effects of mutations of the Ch30 channel on current enhancement by the Bay K 8644 agonist. Current-voltage relationships and current traces measured under the control conditions and in the presence of 1 µmol/l Bay K 8644 are shown in black and grey, respectively. Reprinted by permission from EMBO J., Schuster et al. (1996), copyright 1996 Macmillan Publishers Ltd.
et al. 1990)) contribute to the DHP-interaction site in a decisive way. Their replacement by the corresponding amino acids from the DHP-insensitive Ca\textsubscript{v}2.3 channel in chimera 30 (Ch30) channel increased IC\textsubscript{50} for isradipine inhibition more than 100-fold, from 16 ± 2 nmol/l (wild type channel) to 1.7 ± 0.3 µmol/l (Ch30 channel). These mutations reversed the effect of another DHP Bay K 8644 from agonistic to antagonistic (Figure 12 and Schuster et al. 1996). Both DHPs accelerated current decay of the Ch30 channel.

Further investigations identified additional amino acids participating in the high affinity block of the Ca\textsubscript{v}1.2 mediated barium current (I\textsubscript{Ba}) by antagonist DHPs: Thr1061 and Gln1065 in IIIS5 (Ito et al. 1997; He et al. 1997), Ile1175, Ile1178, Met1183 and the conserved Tyr1174 of IIIS6 (Bodi et al. 1997; Peterson...
et al. 1997) and the conserved Asp1494 in IVS6 (Peterson et al. 1997). The largest effects were observed with mutation of Thr1061 to Tyr, which lowered the affinity for isradipine more than 1000-fold (He et al. 1997; Ito et al. 1997). The removal of stimulation of I\textsubscript{Ba} by the DHP agonists Bay K 8644 or (+)S-202-791 required mutation of less amino acids: Thr1061 in IIIS5 (Ito et al. 1997), Tyr1174 in IIIS6 (Bodi et al. 1997) and Tyr1485, Met1486 in IVS6 (Schuster et al. 1996). In contrast to these mutations, the replacement of the L-type channel specific Phe1484 in IVS6 by Ala decreased the IC\textsubscript{50} for the DHP antagonist isradipine from 6.8 nmol/l to 0.014 nmol/l (Peterson et al. 1997). High affinity binding of DHPs requires Ca\textsuperscript{2+} ions (Schneider et al. 1991), which are coordinated by the pore region glutamates (Mitterdorfer et al. 1995). Mutation of the respective Glu to Gln in the Ca\textsubscript{v}1.1 pore region III and IV decreased the affinity for isradipine 10- to 40-fold (Peterson and Catterall 1995). Although not completely excluded, it is unlikely that the high affinity binding of DHPs involves direct binding to the pore region glutamates. Most

**Figure 13. A.** Effect of (+)isradipine on I\textsubscript{Ba} waveform. The currents recorded from the wild type Ca\textsubscript{v}1.2 channel (left) or Ch30 channel (right) during a 1 s long depolarizing pulse from the holding potential of −80 mV to +20 mV under control conditions (solid line) and in the presence of 30 nmol/l (wild type) or 1 µmol/l (Ch30) of (+)isradipine (dashed line) are shown in insets. The main graphs were obtained by dividing individual points of the current trace sampled in the presence of (+)isradipine by the corresponding current samples measured under the control conditions. The points preceding the peak of the current trace were omitted. The first 200 ms of each record were analyzed. The solid line for the wild type channel represents a linear fit with a slope of 0.9 × 10^{-4} ms\textsuperscript{-1}. The solid line for Ch30 channel is a monoexponential fit with a time constant of 30 ms. B. Concentration dependence of I\textsubscript{Ba} decay at the end of a 40 ms long test pulse for Ca\textsubscript{v}1.2 and Ch30 channels. I\textsubscript{Ba} decay was calculated as (1 − I\textsubscript{end}/I\textsubscript{peak}) × 100%, where I\textsubscript{end} is the current amplitude measured at the end of a 40 ms test pulse and I\textsubscript{peak} is the maximal I\textsubscript{Ba} amplitude during the test pulse. n = 5–9 at individual concentrations; ** p < 0.01; *** p < 0.001 (paired t-test) for the difference between I\textsubscript{Ba} decay at a given isradipine concentration and in the control solution for the same calcium channel type. C. Recovery of the two channels from voltage-dependent inactivation. The voltage protocol used in this set of measurements is shown at the top. A 40 ms long test pulse T1 was followed by a 5 s inactivating pulse to +20 mV. After a variable time recovery interval Δ, a second test pulse T2, identical to the T1 pulse, followed. Between the depolarizing pulses, the membrane was held at the HP of −80 mV. The whole sequence was repeated starting 60 s after the end of T2. The recovery interval Δ varied from 5 ms to 79.36 s. The amplitudes of I\textsubscript{Ba} measured during the T2 pulse were normalized to the amplitude measured during the T1 pulse. Averaged results from cells expressing the Ca\textsubscript{v}1.2 channel (upper graph) or Ch30 channel (lower graph) measured under the control conditions (open symbols) or in the presence of isradipine (solid symbols) were fitted to double exponential association curves (solid lines in both panels). The concentration of isradipine was 30 nmol/l (Ca\textsubscript{v}1.2 channel) or 1 µmol/l (Ch30 channel); n = 6 for both calcium channels. Copyright 1998. From: Isradipine interacts with the open state of the L-type calcium channel at high concentrations (Lacinová and Hofmann). Reproduced by permission of Taylor & Francis Group, LLC (http://www.taylorandfrancis.com).
likely, the coordination of Ca$^{2+}$ is required to allow the optimal conformation for high affinity binding.

Removal of high affinity interaction site in the Ch30 channel revealed low affinity interaction with IC$_{50}$ about 1 $\mu$mol/l (Schuster et al. 1996). Mechanism of this interaction has typical features of open channel block (Lacinová and Hofmann 1998). Isradipine accelerated current decay in the Ch30 channel, but not in the wild type Ca$_{v}$2.1 channel, in a concentration-dependent manner (Figure 13A and B). Apparently, prolonged depolarization supported development of channel block in Ch30, but not in the wild type channel. This observation was consistent with an open channel block of the Ch30 channel. Inhibition of current amplitude increased to the same extent with increasing HP in both the wild type and Ch30 channels. Acceleration of current decay by isradipine in the Ch30 channel was independent on HP. Increase in HP increases the proportion of channels in inactivated state and supports the inhibition of current amplitude. We therefore suggested that the inhibition of current amplitude was mainly due to isradipine interaction with an inactivated channel state and that it is this interaction, which is removed by the mutations of the Ch30 channel. The recovery of both channels from voltage-dependent inactivation was biphasic. The presence of isradipine slowed down the recovery of the wild type channel, suggesting that the inactivated channel must unbind the drug before it can recover from inactivation. In contrast, the recovery was faster in the presence of isradipine in the Ch30 channel (Figure 13C), suggesting that the drug-bound channel did not inactivate and could recover fast. Altogether, the mutations in IVS6 yielding the Ch30 channel disturb the high affinity interaction site responsible for DHP interaction with an inactivated channel and reveal an interaction with an open channel state (Lacinová and Hofmann 1998). Possibly, binding to the pore region is involved in this low affinity block.

Identification of DHP interaction site using the loss-of-function chimeric channels was confirmed by construction of gain-of-function chimeras. Transfer of parts of the Ca$_{v}$1.2 sequence to the DHP insensitive neuronal Ca$_{v}$2.1 subunit (Grabner et al. 1996; Hockerman et al. 1997b; Sinnegger et al. 1997) or the Ca$_{v}$2.3 subunit (Ito et al. 1997) showed that the L-type specific and non-conserved amino acids mentioned above had to be present to allow high affinity block and stimulation of these channels by the DHP antagonist isradipine and the Bay K 8644 agonist, respectively. The IC$_{50}$ values for block of the chimeric channels were in the range of 10 to 100 nmol/l. A similar value is obtained with the wild type Ca$_{v}$1.2 channel at a HP of $-80$ mV, suggesting that these amino acids transfer the affinity for a “resting block”. The high affinity block for DHPs requires inactivation of the L-type Ca$^{2+}$ channel. Inhibition of the inactivated state of the channel measured at more positive HPs of $-40$ to $-30$ mV results in IC$_{50}$ values less than 1 nmol/l (Lacinová et al. 2000b). The transfer of the amino acids of the IIIIS5, IIIIS6 and IVS6 segments described above was not sufficient to reconstruct the voltage dependence of the DHP-induced channel block. The involvement of additional amino acids in the high affinity block of the L-type channel by DHPs is supported by the report about a recently cloned L-type channel $\alpha_1$ subunit from jellyfish (Jeziorski et al. 1998).
Figure 14. A. Mutations of Ch30 channel (see Figure 12) affect differently the inhibition of the Caᵥ1.2 channel by the neutral DHP isradipine and charged DHPch. Dose-response curves for wild type (■) and mutated (●) channels are shown in the upper part of the panel. In the lower part, original current records in the absence (solid line) and in presence (dashed line) of 1 μmol/l of each drug are shown. Scale bars, 500 pA. B. Left: IC₅₀ for DHPch measured for the wild type Caᵥ1.2 channel and for the Ch14 and Ch30 channels at two HP, 80 mV (top) and −40 mV (bottom). On the right, mutation of the Caᵥ1.2 channel yielding the Ch14 channel is shown. Modified from Lacinová et al. (1999).
The expressed jellyfish α₁ subunit contained all amino acids identified in IIIS5, IIIS6 and IVS6 that are necessary for high affinity block of the mammalian Caᵥ1.2 channel. However, this channel is neither blocked by isradipine at submicromolar concentrations, nor stimulated by (−)-Bay K 8644.
Testing of the different mutations of the Ca\textsubscript{v}1.2 channel with charged and uncharged DHP analogues (Bangalore et al. 1994; Lacinová et al. 1999b) suggested the presence of an additional site and/or mechanism for interaction of the channel with DHPs. A custom-synthesized dihydropyridine (DHP\textsubscript{ch}), in which the DHP-moiety was separated from the permanently charged head by a spacer chain consisting of ten methylene residues (Baindur et al. 1993; Bangalore et al. 1994) was used in these experiments. Mutations in the IVS6 segment (Ch30) decreased the affinity of the channel for the uncharged DHP isradipine; the charged DHP blocked wild type Ca\textsubscript{v}1.2 and the mutated Ch30 channel with similar, but relatively low affinity at both the hyperpolarized (−80 mV) and depolarized (−40 mV) HPs (Figure 14). DHP\textsubscript{ch} may bind to a different conformation of the channel and interact with different amino acids than the neutral isradipine used in experiments that mapped the DHP site. This suggestion is further supported by analysis of inhibition of the Ch14 channel with DHP\textsubscript{ch} (Figure 14B). In the Ch14 channel, replacement of Thr1061 in the IIIS5 segment by Tyr completely abolished channel block by isradipine at all HPs (Ito et al. 1997). The mutation had similar effect on the Ch14 channel inhibition by DHP\textsubscript{ch} at HP of −80 mV; however, the mutation was without any effect on channel inhibition at HP of −40 mV (Lacinová et al. 1999b and Figure 14B).

The work of several groups suggested that coexpression of β, α\textsubscript{2}δ and γ subunits is required for high affinity binding of DHPs (Mitterdorfer et al. 1994; Wei et al. 1995; Suh-Kim et al. 1996a) and/or for high affinity inhibition of the calcium current by DHPs (Wei et al. 1995; Suh-Kim et al. 1996b). So far, it cannot be decided whether these subunits help to position the α\textsubscript{1} subunit in the membrane, to ensure correct folding of the α\textsubscript{1} subunit, or influence directly the binding site. In contrast to the above results, other groups reported that high affinity binding of DHPs is observed even when only the Ca\textsubscript{v}1.2 subunit was expressed alone.

![Figure 15. A. Amino acid sequences of the cardiac splice variant Ca\textsubscript{v}1.2-a and the smooth muscle Ca\textsubscript{v}1.2-b splice variant of the Ca\textsubscript{v}1.2 channel differ in the four regions (a–d) marked by grey rectangles. B. Dependence of IC\textsubscript{50} for inhibition of cardiac Ca\textsubscript{v}1.2-aCh30 (square) and smooth muscle Ca\textsubscript{v}1.2-bCh30 (circle) splice variants by (+)isradipine on the holding potential. Solid lines are simple connectors of experimental points. C. Left: dependence of IC\textsubscript{50} for inhibition of the chimeric Ca\textsubscript{v}1.2-aCh30 (square) and Ca\textsubscript{v}1.2-bCh30 (circle) channels by (+)isradipine on the holding potential. Solid lines are simple connectors of experimental points. Right: examples of current traces recorded from the Ca\textsubscript{v}1.2-aCh30 and Ca\textsubscript{v}1.2-bCh30 channels in the absence (solid line) and in presence (dashed line) of 1 μmol/l (+)isradipine. To facilitate comparison of current waveforms, traces were normalized to the same amplitude in the bottom part of the panel. D. Left: dependence of IC\textsubscript{50} for inhibition of the chimeric LK7Ch30 channel (circle) by (+)isradipine on the holding potential. Right: in the LK7 channel, the IS6 segment of the smooth muscle Ca\textsubscript{v}1.2-b channel was replaced by the IS6 segment from the cardiac Ca\textsubscript{v}1.2-a channel (region b, see panel A). In addition, mutations of Ch30 channel (Figure 12) were introduced in the IVS6 segment. Modified from Lacinová et al. (1999a).]
Coexpression of the $\beta_3$ subunit did not significantly affect the inhibition of calcium current by isradipine (Lacinová et al. 1995). In the experiments mentioned above, wild type and chimeric channels were always coexpressed with auxiliary subunits. Still, the gain-of-function chimeras failed to reconstruct the voltage dependence of the high affinity channel inhibition by DHPs. Additional channel structures may be involved in this interaction. Analysis of cardiac and smooth muscle splice variants of the Ca$_{v}$1.2 subunit offered an additional insight into this problem (Welling et al. 1993b). It is known that the smooth muscle splice variant is more susceptible to channel inhibition by DHP. Originally, different sensitivities were attributed to the different resting membrane potentials of the two tissues. Molecular cloning of calcium channels, Northern blot and in situ analysis revealed that different splice variants of the L-type channel are expressed in both tissues (Welling et al. 1997). Amino acid sequences of the Ca$_{v}$1.2-a (cardiac) and Ca$_{v}$1.2-b (smooth muscle) splice variants differ only at four sites (Figure 15A). In spite of these structural differences, basic electrophysiological properties of both channels are identical (Lacinová et al. 2000b). The mechanism of the block of channels expressed by isradipine is also identical, but the Ca$_{v}$1.2-b channel is blocked at lower concentrations of the drug at each investigated potential (Figure 15B).

IC$_{50}$ values for isradipine are 32 nmol/l and 8 nmol/l at HP of $-80$ mV, and 10 nmol/l and 1.3 nmol/l at HP of $-50$ mV, for Ca$_{v}$1.2-a and Ca$_{v}$1.2-b, respectively (Lacinová et al. 2000b and Figure 15). The mutations of Ch30 (see Figure 12) affected the channels differently. The voltage dependence of current inhibition, which is related to high affinity interaction with an inactivated channel, was disturbed in the Ca$_{v}$1.2-a, but not in the Ca$_{v}$1.2-b channel (Figure 15C). In contrast, the acceleration of current decay during a depolarizing pulse that reflects low affinity open channel block was not affected (Figure 15C). An in-depth analysis showed that the alternative exon 8a or 8b, which codes for the IS6 segment, is responsible for the difference (Welling et al. 1997; Lacinová et al. 2000b and Figure 15D). The double chimera LK7Ch30, consisting of the Ca$_{v}$1.2-b channel with IS6 segment from the Ca$_{v}$1.2-a channel and mutations of Ch30 in IVS6 segment (see Figure 12) mimicked the properties of the Ca$_{v}$1.2-aCh30 channel (Welling et al. 1997; Lacinová et al. 2000b. and Figure 15D). Similar results were reported by Zühlke and co-workers (1998) and Morel and co-workers (1998), proving that the IS6 segment significantly affects the DHP block. Additional splice variations at the IIIS2 segment and in the intracellular carboxyterminal sequences can also contribute to alterations in DHP affinity (Zühlke et al. 1998).

Contribution of the IS6 segment to the voltage dependence of channel inhibition by DHPs was further investigated in gain-of-function chimeras. The DHP interaction site was reconstructed in the Ca$_{v}$2.3 channel by mutating eight amino acids of the IIIS5, IIIIS6 and IVS6 segments. These mutations yielded the chimeric channel rEC10 (Figure 16A). Study of the voltage dependence of the block of inactivated channels must take into account the voltage dependence of steady-state inactivation of the investigated channels. The Ca$_{v}$2.3 channel as well as the chimeric rEC10 channel inactivate at 50 mV more negative membrane potentials than the
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Figure 16. A. Construction of the chimeric rEC10 and rEC20 channels. In the rEC10 chimera, amino acids of the Ca\textsubscript{v}2.3 sequence were replaced by the corresponding amino acids of the Ca\textsubscript{v}1.2 sequence (bold letters) in the III\textsubscript{S5}, III\textsubscript{S6}, and IV\textsubscript{S6} segments, as indicated. In the rEC20 chimera, besides that, amino acids of the IS6 segment from the Ca\textsubscript{v}1.2 sequence (bold italics) were introduced into the Ca\textsubscript{v}2.3 sequence. Numbering of amino acids is according to the Ca\textsubscript{v}2.3 sequence (Schneider et al. 1994). B. Left: voltage dependence of steady-state inactivation for the Ca\textsubscript{v}1.2 channel (○, \(n = 14\)), chimeric rEC10 channel (▲, \(n = 8\)) and chimeric rEC20 channel (●, \(n = 7\)). Fitting of experimental data by the Boltzmann function resulted in voltages for half-maximal inactivation: \(-19.6 \pm 0.3\) mV, \(-66.6 \pm 0.1\) mV and \(-58.6 \pm 0.2\) mV for the Ca\textsubscript{v}1.2, rEC10 and rEC20 channel, respectively. Right: dependence of IC\textsubscript{50} for inhibition of the channels by (+)isradipine on holding potential. Symbols have the same meaning as in the left panel. IC\textsubscript{50} were calculated by fitting the dose-response curves measured for 7 to 9 cells for each investigated channel by the Hill equation (see Lacinová et al. 1999a).

Ca\textsubscript{v}1.2 channel (Lacinová et al. 1999a and Figure 16B). Therefore, IC\textsubscript{50} for inhibition of the chimeric channel should be evaluated at a more negative HP than IC\textsubscript{50} for inhibition of the wild type Ca\textsubscript{v}1.2 channel. No channels are inactivated at HPs of \(-80\) mV and \(-100\) mV for the Ca\textsubscript{v}1.2 channel and chimeric rEC10 channel,
respectively. IC$_{50}$ for inhibition by (+)isradipine at these HPs were 16 ± 2 nmol/l and 64 ± 10 nmol/l for the Ca$_{v}$1.2 and chimeric rEC10 channels, respectively. Both values decreased in a voltage dependent manner; nevertheless, the slope of the voltage dependence for the rEC10 channel was less steep compared to the slope for the Ca$_{v}$1.2 channel (Figure 16B). Introduction of amino acids of the IS6 segment, which are responsible for increased sensitivity of the smooth muscle Ca$_{v}$1.2-b channel, into the rEC10 channel yielded the gain-of-function chimera rEC20 (Figures 15 and 16). This chimera was: more sensitive to inhibition by (+)isradipine than rEC10 (IC$_{50}$ = 27 ± 4 nmol/l) and voltage dependence of its inhibition by (+)isradipine had a more steep slope close to that of the Ca$_{v}$1.2 channel (Figure 16B). Together with the earlier photoaffinity results (Kalas et al. 1993) these observations further support the suggestion that amino acids of the IS6 segment contribute directly to the DHP-binding pocket.

**Phenylalkylamines and benzothiazepines**

Phenylalkylamines (PAA), such as verapamil, gallopamil or devapamil, block L-type current in a use-dependent manner from the intracellular side of the membrane (Hescheler et al. 1982) and affect binding of DHPs by allosteric interaction (Glossmann and Striessnig 1990). In addition, benzothiazepines (BTZ), such as diltiazem, interact allosterically with binding of DHPs (Glossmann and Striessnig 1990). In contrast to PAA, BTZs label extracellular sites in the linker sequence between IVS5 and IVS6 in the Ca$_{v}$1.1 subunit (Watanabe et al. 1993), in agreement with a recent report that the quaternary 1,5-BTZ DTZ417 blocks the cardiac L-type channel only when applied from the extracellular site (Kurokawa et al. 1997). More recently, it was shown that similar to the PAA devapamil (Catterall and Striessnig 1992), the 1,4-BTZ semotiadil labels a short sequence of the IVS6 segment (Kuniyasu et al. 1998). The PAA verapamil blocks the L-type Ca$_{v}$1.2 Ca$^{2+}$ channel and the non-L-type Ca$_{v}$2.1 and Ca$_{v}$2.3 Ca$^{2+}$ channels at similar concentrations in a state-dependent manner (Cai et al. 1997), whereas diltiazem blocked all three channels at similar concentrations, but only the Ca$_{v}$1.2 Ca$^{2+}$ channel in a state-dependent manner.

Extent of the inhibition of L-type channel by PAA increased, when the extent of VDCC inactivation increased (Sokolov et al. 2001; Hering 2002). Use of Ca$^{2+}$ instead of Ba$^{2+}$ as a charge carrier potentiated inhibition of the L-type Ca$_{v}$1.2 Ca$^{2+}$ channel by both PAA and BTZs, however, this effect was independent of potentiation of Ca-dependent channel inactivation. Potentiation of BTZs and PAA block of Ca$_{v}$1.2 channel depends on the interaction of Ca$^{2+}$ ions with pore glutamates, as demonstrated Dilmac and co-authors (Dilmac et al. 2003, 2004).

Molecular analysis of the Ca$_{v}$1.2 subunit (Schuster et al. 1996; Hockerman et al. 1995, 1997a) showed that the L-type channel specific Ile1175 and the conserved Tyr1174, Phe1186 and Val1187 in IIIS6 and the L-type channel specific Tyr1485, Ala1489 and Ile1492 in IVS6 are necessary to form a high affinity PAA site. In addition, the two glutamates (Glu1140 and Glu1441) in pore regions of repeats III and IV are necessary (amino acid numbering is according to the Ca$_{v}$1.2-b sequence
Figure 17. Amino acids interacting with DHPs (red), PAA (purple), PAAs+BTZs (yellow), DHPs+PAAs (blue) and all three blocker types DHPs+PAAs+BTZs (green) are shown. Amino acids conserved between the DHP-sensitive L-type channels and DHP-insensitive, non-L-type channels are marked by an arrow head. The IS6 segment contributes significantly to high affinity binding of DHP to the smooth muscle L-type channel. Numbering of amino acids is according to the Ca_{v}.1.2-b sequence (Biel et al. 1990).

(Biel et al. 1990; Hockerman et al. 1997a). The effect of mutation of the conserved Tyr1174 depends on the substituent amino acid. Substitution by phenylalanine decreased the affinity for devapamil 18-fold, whereas substitution by alanine increased the affinity 7-fold (Hockerman et al. 1997a). The increased affinity of the Y1174A
mutant is most likely caused by a shift of $-11$ mV of the steady-state inactivation curve. Transfer of the three IVS6 amino acids Tyr1485, Ala1489 and Ile1492 from the Ca$_v$1.2 to the Ca$_v$2.1 subunit introduced PAA and BTZ sensitivity, when measured in a use-dependent protocol (Hering et al. 1996). Furthermore, it was shown that the triple mutation Y1485A, A1489S and I1492A in IVS6 of the Ca$_v$1.2 channel reduced the use-dependent block of the three PAs, devapamil, verapamil and gallopamil, reduced the resting and depolarized blocks of devapamil, but affected poorly the resting and depolarized blocks of verapamil and gallopamil (Johnson et al. 1996).

Together, these results show that the IVS6 segment interacts with various PAs and BTZs. The state-dependent block of the L-type channel is mediated by the same three amino acid residues in IVS6 for diltiazem and devapamil. However, different amino acids are required to allow high affinity interaction at resting state for diltiazem, verapamil and gallopamil. A further problem arises from the finding that DHPs, PAs, and BTZs interact with the same (Tyr1485) or with adjacent (Ile1492 and Ile1493) amino acid side chain. It is difficult to reconcile this close location of interacting site chains with the previously described allosteric modulation of DHP binding by diltiazem or PAs (Glossmann and Striessnig 1990). Amino acids known to contribute to interaction sites for antagonists DHPs, PAs, and BTZs are summarized in the Figure 17.

In addition, for the interaction of agonists DHP, e.g., (–)Bay K 8644 and FLP-64176 with L-type channel are essential amino acids located in H1S5-S6 (Yamaguchi et al. 2003). The replacement of Phe1112 by Ala almost abolished current potentiation by DHP agonist and the replacement of Phe1112 and Ser1115 by alanines turned the potentiation into weak inhibition.

**Inactivation of L-type calcium channels**

HVA channels inactivate by a dual mechanism: voltage-dependent, observable during prolonged depolarization, and calcium-dependent, caused by Ca$^{2+}$ ions entering the cell during channel opening (Brehm and Eckert 1978).

The inactivation mechanism of L-type channels has been studied most extensively. Among the HVA channels, the L-type channels have the most prominent calcium-dependent inactivation. Most authors reported presence of two time constants in the time course of inactivation of L-type channels: a fast one, corresponding to Ca$^{2+}$-dependent inactivation, and a slow one, corresponding to voltage-dependent inactivation. This was found for freshly isolated native cells from smooth muscle (Ganitkevich et al. 1986), ventricular myocytes (Richard et al. 1993; Masaki et al. 1997) and neuronal cells (Cox and Dunlap 1994; Johnson and Byerly 1994) as well as for the Ca$_v$1.2 channel expressed in mammalian cell lines (Zong and Hofmann 1996; Shirokov 1999) or in *Xenopus* oocytes (Bernatczek et al. 1998; Cens et al. 1999). Time constants measured by different authors in native cells vary between 7 ms (Richard et al. 1993) and 50 ms (Ganitkevich et al. 1986) for the fast time constant, and between 65 ms (Masaki et al. 1997) and 400 ms (Johnson and Byerly 1994) for the slow time constant. In expression systems, slightly higher
values between 20 ms (Zong and Hofmann 1996) and 100 ms (Cens et al. 1999) for the fast time constant, and between 160 ms (Zong and Hofmann 1996) and 2000 ms (Cens et al. 1999) for the slow time constant were reported.

**Calcium-dependent inactivation**

While the signal for VACC activation is solely the change of the transmembrane voltage, both the voltage and entering calcium ions serve as a negative feedback signal and cause transition of the channels into a non-conducting inactivated state. The calcium-dependent inactivation of a voltage-operated channel was first described by Brehm and Eckert (1978). Initially it was recognized as a property of L-type channels. Later this inactivation mechanism was described also for N-type (Cox and Dunlap 1994; Shirokov 1999) and P/Q-type channels (Lee A. et al. 1999, 2000). The hallmark of calcium-dependent inactivation of all mentioned HVA channels is a slower inactivation of Ba\(^{2+}\) current compared to inactivation of Ca\(^{2+}\) current. Nevertheless, inactivation of Ba\(^{2+}\) current through L-type channels still has a minor divalent cation-dependent component (Ferreira et al. 1997). Another feature is the dependence of inactivation kinetics on extracellular and intracellular calcium concentrations. R-type channels inactivate with the same kinetics when Ca\(^{2+}\) or Ba\(^{2+}\) is used as a charge carrier, but their inactivation is slowed down when the current is carried by monovalent Na\(^+\) ions (Jouveneau et al. 2000). Therefore, these authors have hypothesized that also the R-type channel is inactivated by a divalent-cations-dependent mechanism.

Another, faster component of calcium-dependent inactivation was defined in cardiac myocytes. This component represents a so-called release-dependent inactivation and is caused by calcium ions released from sarcoplasmic reticulum stores. The time constant of this process is between 6 and 15 ms (Sham et al. 1995; Sham 1997) and corresponds to the fastest time constants observed in native cells (Richard et al. 1993).

Initially, the Ca\(^{2+}\)-dependent inactivation was attributed to calcium binding to the so-called EF-hand motif on the carboxy terminus of the α\(_1\) subunit of the L-type channel (de Leon et al. 1995; Bernatchez et al. 1998). Other authors (Adams and Tanabe 1997; Zhou et al. 1997; Zühlke and Reuter 1998) located a putative calcium binding site into other regions of the carboxy terminus and/or the whole Ca\(_v\)1.2 sequence. Finally, a series of papers from three different laboratories (Peterson et al. 1999; Qin et al. 1999; Zühlke et al. 1999) identified calmodulin (CaM) and its binding to the Ca\(_v\)2.1 channel as a mediator of calcium-dependent inactivation. A similar Ca\(^{2+}\)/CaM-mediated inactivation mechanism was described for the Ca\(_v\)2.1 channel (Lee A. et al. 1999). Ca\(^{2+}\) must be bound to CaM in order to start the process of calcium-dependent inactivation (Zühlke et al. 1999). The EF-hand motif may be involved in the process of transduction of Ca\(^{2+}\)/CaM binding into channel inactivation (Peterson et al. 2000). As there is only one inactivation mechanism, the various fast time constants reported under various experimental conditions most probably reflect variability in the time-dependent concentration of Ca\(^{2+}\) ions in the vicinity of the inner channel mouth.
Voltage-dependent inactivation

While the calcium-dependent inactivation need not be a universal property of all VACCs and has not been proved yet for the Ca_{v}2.3 and Ca_{v}3.x channels, all VACCs undergo a voltage-dependent inactivation. In order to analyze the kinetics of purely voltage-dependent inactivation, the calcium-dependent inactivation must be eliminated. Using Ba^{2+} instead of Ca^{2+} as a permeant ion is not sufficient, as Ferreira et al. (1997) reported charge carrier-dependent channel inactivation caused by Ba^{2+} ions. Replacement of all divalent cations in external solution by monovalent cations eliminates also this inactivation mechanism. However, with Na^{+} as a charge carrier the current inactivation during the depolarizing pulse still has two time constants in both isolated mammalian cardiomyocytes (Boyett et al. 1994; Mitarai et al. 2000) and in expressed Ca_{a}1.2 channel (Lacinová et al. 2000d; Lacinová and Hoffmann 2005). The second, ultra-slow component of voltage-dependent inactivation, has a time constant of several seconds (Boyett et al. 1994; Lacinová et al. 2000d; Lacinová and Hoffmann 2005, but see Mitarai et al. 2000), so that even a 5 s long conditioning pre-pulse to positive membrane potentials was not sufficient to fully inactivate the Na^{+} current through the Ca_{a}1.2 channel (Lacinová et al. 2000d; Lacinová and Hoffmann 2005). From these experiments, we could conclude that the inactivation mechanism of L-type channels may consist of three processes: a fast, Ca^{2+}-dependent process, and two purely voltage-dependent processes.

Neuronal calcium channels

The Ca_{v}2.1 channel

Transcripts of the Ca_{v}2.1 channel are present at high levels in the mammalian brain and in peripheral nervous system (Mori et al. 1991; Starr et al. 1991). Because the Ca_{v}2.1 channel transcripts are expressed in many neurons shown to possess P- and Q-type channels and because the properties of Ca_{v}2.1 channel exhibit similarities with both of these channels (Stea et al. 1994), the Ca_{v}2.1 cDNA is referred to as P/Q-type channel.

The Ca_{v}2.2 channel

The Ca_{v}2.2 channel gene has been cloned exclusively from brain (Dubel et al. 1992; Williams et al. 1992a; Fujita et al. 1993). Expression studies using dysgenic myotubes or Xenopus oocytes revealed that Ca_{v}2.2 channel induced a barium current which is inhibited by low concentrations of ω-conotoxin GVIA (Williams et al. 1992a; Fujita et al. 1993). These results identify the Ca_{v}2.2 channel as the neuronal N-type channel. The Ca_{v}2.2 subunit also binds the ω-conotoxin GVIA with high affinity (Dubel et al. 1992) in extracellular space (Ellinor et al. 1994). Chimeras between the Ca_{v}2.2 and Ca_{v}2.1 subunits indicate that each repeat contributes to the binding pocket with the pore region of repeat III, which is the most important determinant (Ellinor et al. 1994).
The Ca\textsubscript{v}2.3 channel

The sixth gene has been cloned from rat, rabbit and human brain libraries (Niidome et al. 1992; Soong et al. 1993; Schneider et al. 1994; Williams et al. 1994). Initially, this channel was characterized as an LVA T-type channel (Soong et al. 1993). However, later studies showed (Schneider et al. 1994; Williams et al. 1994), that the expressed Ca\textsubscript{v}2.3 channel has the activation and inactivation kinetics of a HVA neuronal channel. The human and rat Ca\textsubscript{v}2.3 currents have some properties in common with the R-type currents observed in the cerebellar granule cells (Ellinor et al. 1993; Randall and Tsien 1997).

Low-voltage-activated calcium channels

Low-voltage-activated (LVA) channels are characterized by threshold membrane potential for activation of macroscopic inward current of about –60 mV, which is below the threshold potential for action potential generation, and is far more negative than for the HVA channels. LVA channels open and inactivate very fast, but deactivate about 10 to 100 times slower than HVA channels. Single channel conductance of LVA channels is very low and is between 5 and 9 pS. For these reasons, they are also called T-type, T for transient (fast inactivation) and tiny (small conductance). LVA channels can be detected in various tissues such as heart, brain, dorsal root ganglia and adrenal gland. In the heart, T-type channels may contribute to generation of action potential in the sino-atrial node, to depolarization of cell membranes and to propagation of the atrio-ventricular calcium action potential. Calcium entry via T-type channel plays a significant role in initiating Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release in the developing heart (Kitchens et al. 2003). The functional role of T-type channels in generating low-threshold spikes and rebound burst-firing has been demonstrated in neurons from the inferior olive, thalamus, hippocampus and neocortex (Huguenard 1996). In the adrenal gland, T-type channels are postulated to be involved in hormone secretion (Cohen et al. 1988). There is also evidence that genetic abnormalities in T-type channel genes give rise to absence epilepsy and cardiomyopathy (Sen and Smith 1994; Tsakiridou et al. 1995; Talley et al. 2000).

Cloning of low-voltage-activated calcium channels

The cloning strategy used to identify the T-type channels is an example of refined search of sequence databases using the motifs common to the family of voltage-gated calcium and sodium channels. Since the degree of homology between the LVA and HVA channels is rather low, standard techniques such as library screening or polymerase chain reaction amplification with degenerate primers are not applicable. The use of different search algorithms on mammalian expressed sequence-tagged cDNAs or on similar sequences of the nematode Caenorhabditis elegans led to identification of several genes, three of which encode LVA channels (Cribbs et al. 1998; Perez-Reyes et al. 1998; Klugbauer et al. 1999a; Lee et al. 1999a; Williams
They were named Ca\textsubscript{v}3.1, Ca\textsubscript{v}3.2, and Ca\textsubscript{v}3.3 channels. The genes for murine Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 channels were mapped to chromosomes 11 (Perez-Reyes et al. 1998) and 17 (Cribbs et al. 1998), respectively. The genes for human Ca\textsubscript{v}3.1, Ca\textsubscript{v}3.2, and Ca\textsubscript{v}3.3 channels were mapped to chromosomes 17 (Perez-Reyes et al. 1998), 16 (Cribbs et al. 1998), and 22 (Mittman et al. 1999), respectively.

Hydrophobicity analysis revealed that T-type channels – like HVA channels – contain four homologous repeats, each consisting of six transmembrane segments (Figure 18). A comparison of the negatively charged residues in the pore loops shows that all T-type channels have a glutamate in repeats I and II and an aspartate in repeats III and IV (EEDD), whereas HVA channels have glutamates in all four repeats (EEEE). The positively charged residues of the HVA S4 voltage sensor are also conserved in the LVA channels (Figure 4). Overall amino acid sequences of the Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 channels exhibit 57% homology and their putative transmembrane segments are 90% identical (Cribbs et al. 2000). The whole amino acid sequence of the Ca\textsubscript{v}3.3 channel is 59.3% identical with the Ca\textsubscript{v}3.1 sequence, and 56.9% identical with the Ca\textsubscript{v}3.2 sequence (Lee et al. 1999a). Its transmembrane segments are only 80% identical with the transmembrane segments of the Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 channels (Lee et al. 1999a). Different splice variants of the Ca\textsubscript{v}3.1 channel have been reported for the rat, mouse and human brain (for review, see Lacinová et al. 2000a). Differentially spliced sequences are found in the intracellular loops connecting repeats II-III and III-IV and at the carboxy terminus.

Tissue distribution of T-type mRNA was analysed by Northern blot and in situ hybridizations. Transcripts of Ca\textsubscript{v}3.1 were identified in rat, human and mouse in the brain and, at lower levels, in the heart (Perez-Reyes et al. 1998; Klugbauer et al. 1999a). Transcripts of Ca\textsubscript{v}3.2 are expressed more ubiquitously. High concentrations of the transcripts were identified in human kidney, liver and heart, and lower levels were present in the brain, placenta, lung, skeletal muscle and pancreas (Cribbs et al. 1998; Williams et al. 1999). Ca\textsubscript{v}3.3 mRNA of 10.5 kb was only found in the
brain on a rat multiple tissue blot. However, smaller fragments were also detectable in the kidney, thymus and liver (Lee et al. 1999a).

*In situ* hybridization analysis of rat and mouse brain revealed that all three mRNAs are differentially expressed (for review see Lacinova 2004). Transcripts of Ca$_{v}$3.1 were found to be widely, but not uniformly, spread in mouse brain (Klugbauer et al. 1999a), with high expression level in the cerebellum, hippocampus, thalamus and olfactory bulb. Talley et al. (1999) found that expression of the three genes was largely complementary in the rat central and peripheral nervous system. Ca$_{v}$3.2 riboprobes hybridized strongest to sensory ganglia, pituitary, dentate gyrus granule neurons and thalamic reticular neurons. The latter contained also Ca$_{v}$3.3 mRNA. Expression was found to be high in the olfactory tubercles for Ca$_{v}$3.3 and Ca$_{v}$3.2, and the subthalamic nucleus for Ca$_{v}$3.3 and Ca$_{v}$3.1.

**Subunit composition of T-type calcium channels**

The activation, inactivation and deactivation kinetics of expressed Ca$_{v}$3.1 and Ca$_{v}$3.2 channels mimics the kinetics observed in native T-type channels (for review, see Lacinová et al. 2000a). Furthermore, the amino acid sequence of all three cloned $\alpha_1$ subunits lacks the AID identified in $\alpha_1$ subunits of HVA channels. The AID domain was shown to be necessary for interaction between HVA channel $\alpha_1$ subunit with a $\beta$ subunit (for review, see Hofmann et al. 1999). Elimination of the four known $\beta$ subunits by transfection of nodus ganglion neurons (Lambert et al. 1997) or mammalian neuronal NG108-15 cells (Leuranguer et al. 1998) with antisense oligonucleotides, or overexpression of the neuronal $\beta_{2a}$ subunit (Wyatt et al. 1998) did not affect the size or voltage-dependence of native T-type current. It was therefore considered as improbable that a $\beta$ subunit could function as an auxiliary subunit in the LVA channel complex. However, recently an increased current amplitude and improved membrane trafficking of all three cloned $\alpha_1$ subunits by $\beta_{1b}$ subunit was described (Dubel et al. 2004), leaving this question open.

In earlier studies, coexpression of the Ca$_{v}$3.1 subunit with the $\alpha_2\delta$-1 or $\alpha_2\delta$-3 subunit did not modulate the T-type current (Lacinová et al. 1999c), or did so only minimally (Dolphin et al. 1999). In contrast, Dubel and co-authors (2004) reported that coexpression of the $\alpha_2\delta$-1 subunit enhanced current through all three Ca$_{v}$3 channels at least 2-fold. Some expression studies support the notion that the $\alpha_2\delta$-2 (Hobom et al. 2000) and the $\gamma$-5 subunit (Klugbauer et al. 2000) may moderately modulate the current through the Ca$_{v}$3.1 channel (see also chapters above). When expressed in *Xenopus* oocytes, $\alpha_2\delta$-2 increased the peak current amplitude of the Ca$_{v}$3.1 channel 1.8-fold (Gao et al. 2000). Recently, minor modulation of gating currents of the Ca$_{v}$3.1 channel by the $\alpha_2\delta$-2 and the $\gamma$-5 subunits was demonstrated (Lacinová and Klugbauer 2004b).

Activation and inactivation kinetics of the expressed Ca$_{v}$3.3 channel is slower than the kinetics of native T-type current (Klöckner et al. 1999; Kozlov et al. 1999; Lee et al. 1999a). This observation together with the reported dependence of channel kinetics on expression system (Lee et al. 1999a) suggests that this LVA channel is regulated by an auxiliary subunit and/or other factors endogenously present
either in HEK 293 cells or in *Xenopus* oocytes. Alternatively, the predominant Ca\(_{\text{v}}\)3.3 subunit may have another carboxy terminus because of the extraordinary short C-terminus and insertion of a repetitive sequence that is not present in the human sequence (Dunham et al. 1999).

**Permeability properties of T-type calcium channels**

Instead of the highly conserved glutamates in all four pore regions, all three cloned T-type channels have glutamates in pore regions I and II, but aspartates in pore regions III and IV. Indirect evidence suggests that the aspartates of pore regions III and IV control the relatively low unit conductance. The expressed Ca\(_{\text{v}}\)3.1 channel has a single-channel conductance of 7.5 pS (Perez-Reyes et al. 1998). For the Ca\(_{\text{v}}\)3.2 channel, a slightly lower conductance of 5.3 pS (Cribbs et al. 1998) and a slightly higher value of 9.1 pS (Williams et al. 1999) have been reported. The expressed Ca\(_{\text{v}}\)3.3 channel has the highest single-channel conductance of 11.0 pS (Lee et al. 1999a; Monteil et al. 2000b). The unit conductances reported for all three channels are 3-fold lower compared to those of the L-type channels. T-type channels, in contrast to HVA channels, are not selective for Ba\(^{2+}\) over Ca\(^{2+}\). The permeability ratios of expressed Ca\(_{\text{v}}\)3.1 channels are Ba\(^{2+}\)/Ca\(^{2+}\) \(\sim\) 0.96 and Sr\(^{2+}\)/Ca\(^{2+}\) \(\sim\) 1.35 (Monteil et al. 2000a). Unlike any of the HVA channels, T-type channels inactivate faster with Ba\(^{2+}\) than Ca\(^{2+}\) as charge carrier (Klöckner et al. 1999; Klugbauer et al. 1999a; Monteil et al. 2000a, and Figure 19).

**Voltage-dependent activation and inactivation of T-type calcium channels**

Gating of T-type channels contrasts in many aspects the gating of L-type channels. When compared with L-type channels, the voltage dependence of T-type channel activation is shifted by 20–30 mV in the hyperpolarized direction, inactivation is rapid and not inherently voltage-dependent, and deactivation is slow.

Parameters of voltage-dependent activation depend on the concentration of charge carrier used, but are not influenced by the choice of Ba\(^{2+}\) or Ca\(^{2+}\) ions (Klöckner et al. 1999; Klugbauer et al. 1999a; for review, see Lacinová et al. 2000a). The membrane potential for half-maximal current peak is approximately −45 mV at physiologically low charge carrier concentrations of 1–2 mmol/l (Perez-Reyes et al. 1998; Klöckner et al. 1999; Kozlov et al. 1999). These values are common for all three Ca\(_{\text{v}}\)3.x channels. Detailed analysis of tail current-voltage relations revealed two components of current activation in the Ca\(_{\text{v}}\)3.1 and Ca\(_{\text{v}}\)3.2 channels. For the Ca\(_{\text{v}}\)3.1 channel, potentials for half-maximal activation voltages (\(V_{0.5\text{act}}\)) were −41.8 mV and −14.7 mV in 2 mmol/l Ca\(^{2+}\) with corresponding slope factors of 5.1 and 11.9 (Monteil et al. 2000a). Similar values of −37.1 mV and 0.0 mV for \(V_{0.5\text{act}}\), and 4.5 mV and 13.7 mV for activation slopes were reported by Lacinová and co-authors (2002). For the Ca\(_{\text{v}}\)3.2 channel, potentials for \(V_{0.5\text{act}}\) were −25.1 mV and +25.5 mV and the corresponding slope factors were 7.5 and 14.7 in 15 mmol/l Ba\(^{2+}\) (Williams et al. 1999).
Voltage-Dependent Calcium Channels

Figure 19. $\text{Ba}^{2+}$ (open symbols) and $\text{Ca}^{2+}$ (filled symbols) currents through an expressed $\text{Ca}_{v}^{3.1}$ channel. Current-voltage relation had identical shapes for both ions (upper left). The time constant of current inactivation was significantly accelerated by $\text{Ba}^{2+}$ ions (upper right). Original current traces are shown below together with averaged current densities. Modified from Klugbauer et al. (1999a). With kind permission of Springer Science and Business Media.
The time course of current activation could be described by a single time constant, which decreases sharply with increasing amplitude of the depolarizing pulse. In case of the Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 channels, this time constant varies from 8–10 ms just above the activation threshold to hundreds of microseconds for pulse amplitudes positive relative to the peak of the current-voltage relationship (Perez-Reyes et al. 1998; Klöckner et al. 1999; Klugbauer et al. 1999a; Williams et al. 1999). In case of the Ca\textsubscript{v}3.3 channel, the activation time constant decreases from approximately 50 to 5 ms with increasing pulse amplitude when calcium channels are expressed in mammalian HEK 293 cells (Klöckner et al. 1999; Kozlov et al. 1999; Lee et al. 1999a). The time constant is two times slower when the channel is expressed in Xenopus oocytes (Lee et al. 1999a). The activation time constants of the Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 channels are independent of the expression system (Perez-Reyes et al. 1998; Klugbauer et al. 1999a; Williams et al. 1999).

The rates of inactivation (Figure 19) and recovery from inactivation of T-type channels are much faster than in any HVA channel. The time course of current decay during a single depolarizing pulse can be fitted by a single exponential. Just above the activation threshold, the time constant is about 50 ms for the expressed Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 channels. With increasing amplitude of the depolarizing pulse, the current decay becomes faster and its time constant saturates at a virtually voltage independent value of 10–15 ms (Cribbs et al. 1998; Perez-Reyes et al. 1998; Klöckner et al. 1999; Klugbauer et al. 1999a; Kozlov et al. 1999; Williams et al. 1999). This time constant is independent of the charge carrier concentration and of the expression system. Curiously, both the Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 channels inactivate faster with Ba\textsuperscript{2+} than Ca\textsuperscript{2+} as a charge carrier (Klöckner et al. 1999; Klugbauer et al. 1999a; Montei et al. 2000a). In spite of the fast inactivation, there is a sustained current component corresponding to about 1–2% of non-inactivated channels (Serrano et al. 1999). Recovery from inactivation caused by a 5 s long pulse to 0 mV is fast and monoexponential, with a time constant of 200 ms at a membrane potential of −100 mV (Klugbauer et al. 1999a). When a 1 s long inactivating pulse was used, in addition to the fast time constant in the range of ≈100 ms, a slow time constant in the range of ≈1 s was identified in Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 channels (Satin and Cribbs 2000). The relative amplitude of the slow time constant was about 20% for the Ca\textsubscript{v}3.1 channel and about 80% for the Ca\textsubscript{v}3.1 channel. The slower apparent recovery from inactivation of the Ca\textsubscript{v}3.1 channel seems to be the only significant difference in electrophysiological profiles of the Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 channel isoforms. Serrano et al. (1999) suggested a model in which the Ca\textsubscript{v}3.2 channel inactivates from any of four closed states and an open state. The rate constants of channel inactivation and recovery in this model are state- but not voltage-dependent. Talavera and collaborators (2003) suggested a similar model with three closed and one open state. In their model, the transitions between the C1-C2-C3-O states and the I1-I2-I3-IO states are voltage-dependent, while inactivation and/or recovery from each closed/open state is a voltage-independent process.

Inactivation properties of the Ca\textsubscript{v}3.3 channel differ from those of the Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.2 channels. When expressed in HEK 293 cells, the channel inactivates
much more slowly, with time constants ranging from about 200 ms at depolarizing pulses just above activation threshold to 50–100 ms at more positive depolarizing pulses (Klöckner et al. 1999; Kozlov et al. 1999; Lee et al. 1999a; Monteil et al. 2000b). The inactivation rate is even slower when the channel is expressed in *Xenopus* oocytes (Lee et al. 1999a). Recovery from the inactivation of the α1I channel is monoexponential, with time constant of 297 ms (Monteil et al. 2000b).

Deactivation of T-type channels is very slow in comparison with the HVA channels. Time courses of tail current decay can be fitted by a single exponential. At extremely hyperpolarized membrane potentials below −100 mV, the time constant of tail current decay reaches a voltage-independent value of 1–2 ms for the CaV3.1 and CaV3.2 channels. At membrane potentials positive to −100 mV, this time constant increases non-linearly (Serrano et al. 1999) and reaches a value of 10–12 ms at −40 mV (Cribbs et al. 1998; Perez-Reyes et al. 1998; Klugbauer et al. 1999a). In other words, current decay and current inactivation of the CaV3.1 and CaV3.2 channels converge to the same voltage-independent rate. The deactivation kinetics of the CaV3.1 channel is independent of the charge carrier (Ba2+/Ca2+; Klugbauer et al. 1999a). Expressed CaV3.3 channels deactivate faster, with a time constant ranging from 0.3–0.5 ms at extremely hyperpolarized membrane potentials to 2 ms at membrane potential of −40 mV (Klöckner et al. 1999; Kozlov et al. 1999; Monteil et al. 2000b).

While the kinetics of expressed CaV3.1 and CaV3.2 channels resemble closely the properties of T-type channels observed in native cells, the activation and inactivation kinetics of expressed CaV3.3 channels was very slow. Chemin and co-authors (2001b) have shown that kinetics of the CaV3.3 channels could be accelerated by expression of the channel in the neuroblastoma/glioma NG 108-15 cell line. To a smaller extent, this effect was observed also when HEK 293 cell line was used as an expression system rather than *Xenopus* oocyte (Chemin et al. 2001b). Because expression of the CaV3.3 channel is predominantly restricted to neuronal tissue, it is possible that this tissue endogenously contains a modulatory factor missing in the *Xenopus* oocyte expression system.

**Pharmacology of recombinant T-type calcium channels**

Cloning of the family of α1 subunits of LVA channels enabled studies of the pharmacology of T-type channels. In native tissues, T-type currents are masked to a considerable extent by HVA currents and have to be dissected using pharmacological and/or biophysical techniques. Published results show a considerable variability (Huguenard 1996), which has been attributed to the putative existence of multiple channel types. This hypothesis was confirmed by isolation of three genes, CaV3.1, CaV3.2 and CaV3.3. Initial studies (Williams et al. 1999; Lacinová et al. 2000c; Perchenet et al. 2000) revealed considerable differences between the CaV3.1 and CaV3.2 channels. The CaV3.2 channel appears to be more sensitive than the CaV3.1 channel to several T-type channel blockers characterized on the native channels. Pharmacology of the CaV3.3 channel has been studied less extensively.
Table 11. Inhibition of expressed LVA calcium channels by inorganic ions

<table>
<thead>
<tr>
<th>Ion</th>
<th>Channel</th>
<th>Charge carrier</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni²⁺</td>
<td>Caᵥ3.1</td>
<td>10 mmol/l Ba²⁺</td>
<td>IC₅₀ = 250 µmol/l</td>
<td>Lee et al. 1999b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 mmol/l Ba²⁺</td>
<td>IC₅₀ = 470 µmol/l</td>
<td>Lacinová et al. 2000c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 mmol/l Ca²⁺</td>
<td>IC₅₀ = 1130 µmol/l</td>
<td>Lacinová et al. 2000c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mmol/l Ca²⁺</td>
<td>IC₅₀ = 133 µmol/l</td>
<td>Monteil et al. 2000a</td>
</tr>
<tr>
<td>Caᵥ3.2</td>
<td></td>
<td>15 mmol/l Ba²⁺</td>
<td>IC₅₀ = 6.6 µmol/l</td>
<td>Williams et al. 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 mmol/l Ba²⁺</td>
<td>IC₅₀ = 12 µmol/l</td>
<td>Lee et al. 1999b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mmol/l Ca²⁺</td>
<td>IC₅₀ (1) = 1.9 µmol/l</td>
<td>Perchenet et al. 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mmol/l Ba²⁺</td>
<td>IC₅₀ (2) = 1350 µmol/l</td>
<td></td>
</tr>
<tr>
<td>Caᵥ3.3</td>
<td></td>
<td>10 mmol/l Ba²⁺</td>
<td>IC₅₀ = 216 µmol/l</td>
<td>Lee et al. 1999b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mmol/l Ca²⁺</td>
<td>IC₅₀ = 184 µmol/l</td>
<td>Monteil et al. 2000b</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>Caᵥ3.1</td>
<td>20 mmol/l Ba²⁺</td>
<td>IC₅₀ = 162 µmol/l</td>
<td>Lacinová et al. 2000c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20 mmol/l Ca²⁺</td>
<td>IC₅₀ = 658 µmol/l</td>
<td>Lacinová et al. 2000c</td>
</tr>
<tr>
<td>Caᵥ3.2</td>
<td></td>
<td>15 mmol/l Ba²⁺</td>
<td>IC₅₀ = 104 µmol/l</td>
<td>Williams et al. 1999</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mmol/l Ca²⁺</td>
<td>IC₅₀ = 218 µmol/l</td>
<td>Perchenet et al. 2000</td>
</tr>
</tbody>
</table>

Block by inorganic cations

The high sensitivity of native T-type current to block by Ni²⁺ was considered to be one of the signatures of this channel. However, IC₅₀ values observed in numerous native tissues varied between 30 µmol/l and 780 µmol/l (reviewed in Huguenard 1996). Experiments with recombinant channels revealed that expressed Caᵥ3.1 and Caᵥ3.3 channels have low affinity to Ni²⁺ (see Table 11). A high affinity block by Ni²⁺ was found only with the expressed Caᵥ3.2 channel. More detailed analyses have shown that this apparent high affinity block may actually consist of high and low affinity sites (see Table 11). This observation is supported by the findings of Lee et al. (1999b) that the Hill coefficient for inhibition of the α₁H channel by Ni²⁺ is significantly lower than one. Interaction of Ni²⁺ with all three cloned T-type channels is complex. The ion shifts the voltage dependence of current activation towards more positive membrane voltages, increases the slope of voltage dependence of current activation and accelerates channel deactivation of the Caᵥ3.1 channel (Lacinová et al. 2000c). Ni²⁺ slows the inactivation time course of the Caᵥ3.2 channel (Lee et al. 1999b). Block by Ni²⁺ is voltage-dependent in all three expressed channels and may be relieved at very positive membrane voltages around +100 mV (Lee et al. 1999b).

Another divalent cation channel blocker, Cd²⁺, blocks Caᵥ3.2 slightly more effectively than Caᵥ3.1 (Table 11). In addition to the block of current amplitude, Cd²⁺ accelerates the time constant of deactivation of the expressed Caᵥ3.1 channel (Lacinová et al. 2000c).

More efficient inorganic blockers, trivalent cations, inhibit the expressed Caᵥ3.1 channel in nanomolar concentrations. The reported IC₅₀ for current carried by 20 mmol/l Ba²⁺ were (in nmol/l): Y³⁺ 28, Er³⁺ 69, Gd³⁺ 87, Ce³⁺ 87, Ho³⁺ 100,
Yb$^{3+}$ 113, Nd$^{3+}$ 148, La$^{3+}$ 184, Sc$^{3+}$ 3946 (Beedle et al. 2002). A somewhat higher IC$_{50}$ value of 700 nmol/l was found for inhibition by La$^{3+}$ when calcium current was carried by 2 mmol/l Ca$^{2+}$ (Lacinová et al. 2002).

**Organic blockers**

Selective inhibition of T-type channels may have clinical importance in cardiovascular diseases (Katz 1999) and some forms of epilepsy (Macdonald and Kelly 1995). An overview of the effects of organic channel blockers on expressed LVA channels, together with the corresponding references, is given in Table 12. The only organic blocker effective at submicromolar concentrations, identified previously for native T-type channels, was mibebradil (for review, see Clozel et al. 1997). Mibebradil also inhibits expressed Ca$_{v}$.3.1, Ca$_{v}$.3.2 and Ca$_{v}$.3.3 channels in nanomolar concentrations. The Ca$_{v}$.3.3 channel has the lowest affinity with IC$_{50}$ of 2.3 µmol/l. Mibebradil was designed as a selective T-type channel antagonist, however, it’s active metabolite inhibits also HVA channels. Huang and co-authors (2004) synthetized nonhydrolyzable analog of mibebradil, NNC 55-0396. This substance appears to be selective T-type channel blocker. In contrast to several reports on native T-type channels (for references see Lacinová et al. 2000a), both channels are resistant to both agonistic and antagonistic DHPs. The charged DHP amlodipine inhibited the Ca$_{v}$.3.2 channel with IC$_{50}$ of 31 µmol/l. Verapamil inhibited the predominantly cardiac Ca$_{v}$.3.2 channel in micromolar concentrations.

The availability of a cloned Ca$_{v}$.3.1 channel enabled identification of the scorpion toxin kurtoxin, which has a high affinity for both the Ca$_{v}$.3.1 and Ca$_{v}$.3.2 channels. Its analog, kurtoxin-like I, is only a weak inhibitor of the Ca$_{v}$.3.3 channel, suggesting selectivity of the kurtoxins family towards Ca$_{v}$.3.1 and Ca$_{v}$.3.2 channels. The Ca$_{v}$.3.2 channel was resistant to ω-Aga IVA, ω-CTx MVIIC, and ω-CgTx GVIA toxins, which are known as inhibitors of neuronal HVA channels. Other Ca$_{v}$.3.x channels were not tested with these toxins. A tetrodotoxin (TTX)-sensitive LVA Ca$^{2+}$ current has been identified in cardiac and neuronal preparations (Aggarwal et al. 1997; Balke et al. 1999). This current was insensitive to low concentrations of Ni$^{2+}$ and its kinetics resembled that of a T-type channel. Both the Ca$_{v}$.3.1 and Ca$_{v}$.3.2 channels are insensitive to TTX and therefore cannot contribute to this type of calcium conductance in native tissues.

It is possible, that at least a part of clinical action of antiepileptics, antipsychotics and anaesthetics is mediated via inhibition of T-type channels. The expressed Ca$_{v}$.3.1 channel has a low sensitivity to the antiepileptic drugs valproate and ethosuximide and is moderately sensitive to phenytoin. Gomora and co-authors (2001) reported similarly a low sensitivity of the human variant of Ca$_{v}$.3.1 channel to ethosuximide. All three LVA channels were moderately sensitive to an active metabolite of methosuximide, α-methyl-α-phenylsuccinimide (MPS). The Ca$_{v}$.3.2 channel is sensitive to ethosuximide. Therefore inhibition of T-type current by phenytoin and ethosuximide may contribute to their clinical action. Several neuroleptics inhibit all three LVA channels in clinically relevant concentrations (Santi et al. 2002). In contrast, Osipenko and co-authors (2003) reported significantly
higher concentrations necessary for T-type current inhibition and full block of inward current could not be reached. Discrepancy may be caused by different expression systems (HEK tsa201 cells in Santi et al. 2002, *Xenopus* oocytes in Osipenko et al. 2003). Nitrous oxide inhibits selectively the Ca\(_{\text{v}}\)3.2, but not the Ca\(_{\text{v}}\)3.1 channel (Todorovic et al. 2001). Whole range anaesthetics inhibits the Ca\(_{\text{v}}\)3.1 channel with IC\(_{50}\) close to their therapeutic plasma concentration (Todorovic et al. 2000). This inhibition may be important part of their clinical action.

The Ca\(_{\text{v}}\)3.2, but not the Ca\(_{\text{v}}\)3.1 channel is potentiated by stimulation of Ca\(^{2+}\)/CaM-dependent protein kinase (Wolfe et al. 2002; Welsby et al. 2003). Furthermore, Ca\(_{\text{v}}\)3.2 channel expressed in *Xenopus* oocytes is potentiated via activation of protein kinase C by phorbol-12-myristate-13-acetate (Park et al. 2003). Imatinib-mesylate, an inhibitor of protein tyrosine kinase (PTK), inhibits expressed Ca\(_{\text{v}}\)3.3 channel with an IC\(_{50}\) of 56.9 µmol/l (Cataldi et al. 2004). Nevertheless, this effect was not related to the inhibition of PTK.

Amiloride is a highly selective T-type channel blocker with an IC\(_{50}\) of 167 µmol/l for the Ca\(_{\text{v}}\)3.2 channel, but with an IC\(_{50}\) > 5 mmol/l for the Ca\(_{\text{v}}\)3.1 channel. T-type current is inhibited by two endogenous substances, arachidonic acid and endocannabinoid anandamide. This modulation may have pathophysiological significance. Anandamide inhibits all three channels in submicromolar concentration (Chemin et al. 2001a). Micromolar concentrations of arachidonic acid inhibit Ca\(_{\text{v}}\)3.1 and Ca\(_{\text{v}}\)3.2 channels (Zhang et al. 2000; Talavera et al. 2004). External acidification from pH 8.2 to 5.5 modulates the activity of the Ca\(_{\text{v}}\)3.2 channel in a complex way (Delisle and Satin 2000). At pH 5.5, current amplitude is inhibited and voltage dependence of both activation and inactivation of the channel is shifted in depolarizing direction. Activation gating is slowed, while deactivation is accelerated. Paradoxically, acidification increases macroscopic slope conductance. Altogether, acidification attenuates the activity of the Ca\(_{\text{v}}\)3.2 channel and could contribute to the protection against abnormal rhythm generation during ischemia.

An overview of effects of organic blockers is given in Table 12.

**Gating of T-type calcium channels**

Opening and closing of a voltage-dependent ion channel is accompanied by movement of the S4 transmembrane segments. The traditional model presupposed movements in outward and inward directions, respectively. Recently, a novel model was suggested for the voltage-dependent K\(^{+}\) channel, in which the S4 segment along with the second part of the S3 segment form a paddle extended from the channel core into the membrane’s fluid interior (Jiang et al. 2003a). When the channel is closed, these paddles are located inside the membrane, near the intracellular surface, and move across the membrane from the inside outward, when the channel opens (Jiang et al. 2003b). In any case, as the S4 segments are rich in positively

Steady-state activation of calcium current cannot be evaluated reliably from a current-voltage relationship. Analysis of voltage dependences of tail current amplitudes revealed two components of voltage dependence of activation of the T-type channel (Williams et al. 1999; Monteil et al. 2000a; Lacinová et al. 2002). Peak tail
**Table 12. Effects of organic blockers on the Ca\textsubscript{v}3.1, Ca\textsubscript{v}3.2, and Ca\textsubscript{v}3.3 channels**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Ca\textsubscript{v}3.1</th>
<th>Ca\textsubscript{v}3.2</th>
<th>Ca\textsubscript{v}3.3</th>
<th>Therapeutic plasma concentration (Flanagan 1998)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular agents</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Mibebradil</td>
<td>IC\textsubscript{50} = 0.39 (\mu)mol/l</td>
<td></td>
<td></td>
<td>0.5–1 (\mu)mol/l</td>
<td>Klugbauer et al. 1999a</td>
</tr>
<tr>
<td>Mibebradil</td>
<td></td>
<td>IC\textsubscript{50} (\approx) 1.2 (\mu)mol/l</td>
<td></td>
<td>0.5–1 (\mu)mol/l</td>
<td>Cribs et al. 1998, Williams et al. 1999, Perchenet et al. 2000, Huang et al. 2004</td>
</tr>
<tr>
<td>NNC 55-0396</td>
<td>IC\textsubscript{50} (\sim) 7 (\mu)mol/l</td>
<td></td>
<td></td>
<td>15 (\mu)mol/l</td>
<td>Perchenet et al. 2000, Williams et al. 1999, Lacinová et al. 2000c, Lacinová et al. 2000c</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>IC\textsubscript{50} = 30.9 (\mu)mol/l</td>
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<td></td>
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</tr>
<tr>
<td>Nimodipine</td>
<td>IC\textsubscript{50} (\geq) 10 (\mu)mol/l</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isradipine</td>
<td>IC\textsubscript{50} (\gg) 1 (\mu)mol/l</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Nifedipine</td>
<td>IC\textsubscript{50} (\gg) 10 (\mu)mol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPK-12 (nifedipine-analogue)</td>
<td>IC\textsubscript{50} = 1.65 (\mu)mol/l</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPK-5 (nifedipine-analogue)</td>
<td>IC\textsubscript{50} = 1.14 (\mu)mol/l</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Bay K 8644</td>
<td>10 (\mu)mol/l min. effect</td>
<td></td>
<td></td>
<td></td>
<td>Williams et al. 1999, Lacinová et al. 2000c</td>
</tr>
<tr>
<td>Verapamil</td>
<td>IC\textsubscript{50} (\gg) 1 (\mu)mol/l</td>
<td></td>
<td></td>
<td>250–800 (\mu)mol/l</td>
<td>Williams et al. 1999, Freeze et al. 2004</td>
</tr>
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</table>

(continued)
**Table 12.** (continued)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Ca$_{v}$ 3.1</th>
<th>Ca$_{v}$ 3.2</th>
<th>Ca$_{v}$ 3.3</th>
<th>Plasma concentration</th>
<th>Reference</th>
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<tr>
<td>Peptide toxins</td>
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<td></td>
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<tr>
<td>Kurtoxin</td>
<td>IC$_{50}$ = 15 nmol/l</td>
<td>IC$_{50}$ = 61 nmol/l</td>
<td>IC$_{50}$ &gt; 700 nmol/l</td>
<td>Chuang et al. 1998</td>
<td></td>
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<tr>
<td>Kurtoxin-like I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Olamendi-Portugal et al. 2002</td>
</tr>
<tr>
<td>$\omega$-Aga IVA</td>
<td>60 nmol/l no effect</td>
<td></td>
<td></td>
<td>Perchenet et al. 2000</td>
<td></td>
</tr>
<tr>
<td>$\omega$-CTx MVIIC</td>
<td>1 $\mu$mol/l no effect</td>
<td></td>
<td></td>
<td>Perchenet et al. 2000</td>
<td></td>
</tr>
<tr>
<td>$\omega$-CgTx GVIA</td>
<td>1 $\mu$mol/l no effect</td>
<td></td>
<td></td>
<td>Perchenet et al. 2000</td>
<td></td>
</tr>
<tr>
<td>TTX</td>
<td>10 $\mu$mol/l no effect</td>
<td></td>
<td></td>
<td>Lacinová et al. 2000c</td>
<td></td>
</tr>
<tr>
<td>TTX</td>
<td>30 $\mu$mol/l no effect</td>
<td></td>
<td></td>
<td>Perchenet et al. 2000</td>
<td></td>
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<tr>
<td>Antiepileptics</td>
<td></td>
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<tr>
<td>Valproate</td>
<td>max. block 10% at 1 mmol/l</td>
<td></td>
<td>300–600 $\mu$mol/l</td>
<td>Lacinová et al. 2000c</td>
<td></td>
</tr>
<tr>
<td>Phenytoin</td>
<td>IC$_{50}$ = 74 $\mu$mol/l</td>
<td></td>
<td>80 $\mu$mol/l</td>
<td>Lacinová et al. 2000c</td>
<td></td>
</tr>
<tr>
<td>Ethosuximide</td>
<td>IC$_{50}$ &lt; 300 $\mu$mol/l</td>
<td>IC$_{50}$ &lt; 300 $\mu$mol/l</td>
<td>700 $\mu$mol/l</td>
<td>Williams et al. 1999</td>
<td></td>
</tr>
<tr>
<td>Ethosuximide</td>
<td>IC$_{50}$ &gt; 3 mmol/l</td>
<td></td>
<td>700 $\mu$mol/l</td>
<td>Lacinová et al. 2000c</td>
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<tr>
<td>MPS</td>
<td>1.95 mmol/l</td>
<td>3.03 mmol/l</td>
<td>1.82 mmol/l</td>
<td>700 $\mu$mol/l</td>
<td>Gomora et al. 2001</td>
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<td>Lamotrigine</td>
<td>inhib. 10% at 100 $\mu$mol/l</td>
<td></td>
<td>no effect</td>
<td>40 $\mu$mol/l</td>
<td>Hainsworth et al. 2003</td>
</tr>
<tr>
<td>Sipatrigine</td>
<td>IC$_{50}$ = 15 $\mu$mol/l</td>
<td>IC$_{50}$ = 15 $\mu$mol/l</td>
<td>IC$_{50}$ = 14 $\mu$mol/l</td>
<td>McNaughton et al. 2000a</td>
<td></td>
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</tbody>
</table>

(continued)
Table 12. (continued)

<table>
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<tr>
<th>Drug</th>
<th>Ca&lt;sub&gt;v&lt;/sub&gt;.1</th>
<th>Ca&lt;sub&gt;v&lt;/sub&gt;.2</th>
<th>Ca&lt;sub&gt;v&lt;/sub&gt;.3</th>
<th>Plasma concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antipsychotics</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Pimozide</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 35 nmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 54 nmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 30 nmol/l</td>
<td>40 nmol/l</td>
<td>Santi et al. 2002</td>
</tr>
<tr>
<td>Pimozide</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 2 µmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 15 µmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 1.6 µmol/l</td>
<td>40 nmol/l</td>
<td>Osipenko et al. 2003</td>
</tr>
<tr>
<td>Penfluoridol</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 95 nmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 64 nmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 72 nmol/l</td>
<td>40 nmol/l</td>
<td>Santi et al. 2002</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 1 µmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 1.5 µmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 35 µmol/l</td>
<td>0.5 µmol/l</td>
<td>Osipenko et al. 2003</td>
</tr>
<tr>
<td>Flunarizine</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ≤ 1 µmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; &gt; 1 µmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 1 µmol/l</td>
<td>0.25 µmol/l</td>
<td>Santi et al. 2002</td>
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<tr>
<td>Fluspirilene</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 12 µmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 7 µmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 12 µmol/l</td>
<td></td>
<td>Osipenko et al. 2003</td>
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<tr>
<td><strong>Anaesthetics</strong></td>
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<tr>
<td>NO₂</td>
<td>not sensitive</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 58%</td>
<td>max. block 66%</td>
<td>50%</td>
<td>Todorovic et al. 2001</td>
</tr>
<tr>
<td>Propofol</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 21 µmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 100 µmol/l</td>
<td>max. block 66%</td>
<td>50 µmol/l</td>
<td>Todorovic et al. 2000</td>
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<tr>
<td>Etonidate</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 161 µmol/l</td>
<td>2 µmol/l</td>
<td>Todorovic et al. 2000</td>
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<td>Octanol</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 160 µmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 10 µmol/l</td>
<td>Todorovic et al. 2000</td>
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<td>Isoflurane</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 277 µmol/l</td>
<td>100 µmol/l</td>
<td>Todorovic et al. 2000</td>
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<tr>
<td>Ketamine</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 1.2 mmol/l</td>
<td>20 µmol/l</td>
<td>Todorovic et al. 2000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thiopental</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 280 µmol/l</td>
<td>20 µmol/l</td>
<td>Todorovic et al. 2000</td>
<td></td>
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</tr>
<tr>
<td>Pentobarbital</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 310 µmol/l</td>
<td>22 µmol/l</td>
<td>Todorovic et al. 2000</td>
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<tr>
<td>Phenobarbital</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 1.5 mmol/l</td>
<td>170 µmol/l</td>
<td>Todorovic et al. 2000</td>
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(continued)
### Table 12. (continued)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Ca&lt;sub&gt;v&lt;/sub&gt;3.1</th>
<th>Ca&lt;sub&gt;v&lt;/sub&gt;3.2</th>
<th>Ca&lt;sub&gt;v&lt;/sub&gt;3.3</th>
<th>Plasma concentration</th>
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<td><strong>Other compounds</strong></td>
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<tr>
<td>Anandamide</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 4.15 µmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 330 µmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; &lt; 10 µmol/l</td>
<td>Q&lt;sub&gt;on&lt;/sub&gt; ≥ 5 mmol/l</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ≤ 10 µmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; = 1.10 µmol/l</td>
<td>230 mmol/l</td>
<td>220 mmol/l</td>
</tr>
<tr>
<td>Amiloride</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; ≥ 5 mmol/l</td>
<td>IC&lt;sub&gt;50&lt;/sub&gt; &lt; 500 nmol/l</td>
<td></td>
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</tr>
</tbody>
</table>

Due to the low level of expression of T-type channels in native tissues, the charge movements are too small to be detected. Cloning of the Ca<sub>v</sub>3.x channel family (reviewed in Lacinová et al. 2000a) enabled to reach a high expression of these channels in heterologous expression systems and, consequently, also analyze the gating currents reflecting their activation.

When calcium current through an expressed Ca<sub>v</sub>3.1 channel was blocked by La<sup>3+</sup>, an asymmetric charge movement could be observed (Lacinová et al. 2002 and Figure 20). The voltage threshold for detection of the gating current was between −70 and −60 mV and the current saturated at about +80 mV. ON-charge movements (Q<sub>on</sub>) observed after the start of depolarizing pulses have a smaller amplitude and slower kinetics than OFF-charge movements (Q<sub>off</sub>) observed after the depolarizing pulses have finished. The voltage dependence of both Q<sub>on</sub> and Q<sub>off</sub> followed a single Boltzmann distribution. V<sub>0.5act</sub> and slopes were +12.9 ± 1.4 mV and 22.4 ± 0.4 mV for Q<sub>on</sub>, and +12.3 ± 0.7 mV and 18.1 ± 0.4 mV for Q<sub>off</sub> (n = 25). Asymmetric currents were detected exclusively in cells expressing the Ca<sub>v</sub>3.1 channel (Lacinová et al. 2002). The amplitudes of Q<sub>off</sub> did not differ significantly from the amplitudes of Q<sub>on</sub> from the same cell (Figure 20C). The charge amplitude increased with the current amplitude. The average amount of charge moved was 0.20 ± 0.02 fC/pA and 0.20 ± 0.01 fC/pA (n = 25) for Q<sub>on</sub> and Q<sub>off</sub>, respectively (Figure 20D).
Figure 20. Voltage dependence of activation of ionic and gating currents of the Ca$_{3.1}$ channel. A. Activation of ionic current was evaluated from the amplitudes of tail currents measured at a constant repolarizing potential of $-100$ mV after depolarization to potentials between $-90$ and $+80$ mV. The length of the depolarizing pulse was adjusted according to time-to-peak of the inward current observed at each potential. An example of tail currents measured according to the described protocol is shown. Depolarization potentials are marked next to each trace. Normalized amplitudes of tail currents (□) could be fitted by a double Boltzmann distribution whose individual components are shown by dashed lines. B. Families of gating currents measured from a cell expressing the Ca$_{3.1}$ channel in the presence of 1 mmol/l La$^{3+}$. The total charge moved during each pulse was calculated by integrating the area under the gating current records. Individual measurements were normalized and averaged. Both $Q_{on}$- and $Q_{off}$-voltage relations were fitted by a single Boltzmann functions (shown as solid lines). Dashed lines demonstrate the two components of current activation as evaluated from the data presented in panel A. C. The maximal value of $Q_{off}$ measured for each individual cell plotted against the maximal $Q_{on}$ value from the same cell (○). Straight line represents unity line. Points above the unity line represent the cells in which $Q_{off}$ was bigger than $Q_{on}$. Points under the unity line represent the cells in which $Q_{off}$ was smaller than $Q_{on}$. Cells with equal $Q_{off}$ and $Q_{on}$ lie on the unity line. D. The maximal $Q_{on}$ or $Q_{off}$ values plotted against current amplitude measured at the peak of the current-voltage relationship ($I_{max}$) in the same cell. The slope of the straight line is 0.20. Modified from Lacinová et al. (2002).
current amplitude was measured at a constant post-pulse repolarization potential of \(-100\) mV following depolarization pulses to voltages between \(-90\) and \(+80\) mV. Conductance was calculated by dividing the peak tail amplitude by the effective driving force \(-100\) mV – \(E_{Ca}\), and normalized to the maximal conductance \((G_{\text{max}})\) measured after the largest prepulse potential \(+80\) mV. When averaged data points were fitted by a double Boltzmann distribution (Figure 20A), 68% of the total activation had a steep slope of \(4.5 \pm 0.4 \) mV and \(V_{0.5\text{act}}\) of \(-37.1 \pm 0.7 \) mV, while activation of 32% of the channels followed a shallow slope of \(13.7 \pm 0.8 \) mV and had \(V_{0.5\text{act}}\) \(0.0 \pm 3.8 \) mV \((n = 11)\). The start of both \(Q_{\text{on}}\) and \(Q_{\text{off}}\) preceded the start of the fast component of current activation by about 10 mV, and the start of the slow component of current activation by more than 30 mV. The fast component of current activation saturated at a membrane potential above \(-10\) mV, when about 30% of gating charge was transferred.

Gating of expressed Ca\(_{v1.2}\) or Ca\(_{v2.2}\) channels preceded channel activation by 30 to 40 mV (Jones et al. 1997; Josephson 1997). The value found for the Ca\(_{v3.1}\) channel was smaller, but similar to the values found for an expressed Ca\(_{v2.3}\) channel (Jones et al. 1998; Qin et al. 1998). These channels are sometimes considered as intermediates between HVA and LVA channels because of their fast kinetics and relatively negative threshold for current activation (reviewed in Hofmann et al. 1999). Voltage dependencies of gating currents measured from native L-type channels (Shirokov et al. 1992) or expressed Ca\(_{v1.2}\) (Josephson 1997), Ca\(_{v2.2}\) (Jones et al. 1997) and Ca\(_{v2.3}\) channels (Olcese et al. 1996; Jones et al. 1998) have a slightly steeper slope (between 15 and 18 mV) than that found for the Ca\(_{v3.1}\) channel (18 to 22 mV, Lacinová et al. 2002). Furthermore, in contrast to Ca\(_{v3.1}\) channels, no difference in slope was found between the \(Q_{\text{on}}\)-V and \(Q_{\text{off}}\)-V relationships in HVA channels. While a single Boltzmann component was found for the Ca\(_{v3.1}\) channel, a second component with a steep slope of about 5–8 mV was reported for the Ca\(_{v2.2}\) (Jones et al. 1999) or Ca\(_{v2.3}\) (Olcese et al. 1996) channel. These differences suggest that the mechanism of transitions between closed, open and inactivated states may differ in the Ca\(_{v3.1}\) channel.

While the current through the Ca\(_{v3.1}\) channel decayed rapidly during the depolarizing pulse due to inactivation and/or deactivation, the amplitude of \(Q_{\text{off}}\) remained constant when the length of the depolarizing pulse was prolonged from 10 ms to 55 ms (Lacinová et al. 2002 and Figure 21). The transition that caused a decay of inward calcium current did not immobilize gating charge. The amplitude and time course of the OFF-charge transient remained unaffected even when the depolarizing pulse was prolonged to 5 s (Figure 21). Immobilization of charge movement coupled to voltage-dependent channel inactivation was reported for native L-type channel (Hadley and Lederer 1991) and for expressed non-L-type Ca\(_{v2.2}\) channel (Jones et al. 1999). The mechanism of T-type channel gating seems to be different. The structural determinants and molecular processes underlying inactivation of LVA channels are largely unknown. Serrano et al. (1999) proposed that the decay of current during a depolarizing pulse represents a mixture of voltage-dependent deactivation and nearly-voltage-independent inactivation. A “ball-and-
Figure 21. Gating current of the Ca\textsubscript{3.1} channel is not immobilized by prolonged depolarization. Currents were activated by depolarizing pulses from the HP of −100 mV to −20 mV (peak of the current-voltage relationship) with lengths increasing from 10 to 55 ms at 0.2 Hz. Charge movement was recorded in the presence of 1 mmol/l La\textsuperscript{3+}. Amplitudes of Q\textsubscript{off} evaluated at the end of each individual pulse were normalized to the Q\textsubscript{off} measured at the end of a 10 ms long pulse, averaged, and plotted against the pulse length (left). An example of charge movements recorded during the described voltage protocol is given in the inset. Charge movement recorded during a 15 ms long depolarizing pulse to −20 mV with (grey) or without (black) a 5 s long conditioning prepulse to 0 mV is shown on the right. Modified from Lacinová et al. (2002).

Chain” mechanism including a highly negatively charged region of 23 amino acids at the amino side of the intracellular carboxy terminus of the Ca\textsubscript{3.1} channel as a putative “ball” was suggested (Staes et al. 2001). Involvement of the IIIS6 segment in channel inactivation was demonstrated by Marksteiner and collaborators (2001). Here we have shown that the process by which a depolarizing pulse transfers Ca\textsubscript{3.1} channels into non-conducting state does not result in a restriction of movement of charged parts of the channel. Our observations support a model in which the process that causes the decay of the Ca\textsubscript{3.1} channel current during the depolarizing step does not involve a voltage-dependent movement of charged parts.

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