Topical Review

Molecular determinants of inactivation in voltage-gated Ca^{2+} channels

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Evolution has created a large family of different classes of voltage-gated Ca^{2+} channels and a variety of additional splice variants with different inactivation properties. Inactivation controls the amount of Ca^{2+} entry during an action potential and is, therefore, believed to play an important role in tissue-specific Ca^{2+} signalling. Furthermore, mutations in a neuronal Ca^{2+} channel ($Ca_v2.1$) that are associated with the aetiology of neurological disorders such as familial hemiplegic migraine and ataxia cause significant changes in the process of channel inactivation. Ca^{2+} channels of a given subtype may inactivate by three different conformational changes: a fast and a slow voltage-dependent inactivation process and in some channel types by an additional Ca^{2+}-dependent inactivation mechanism. Inactivation kinetics of Ca^{2+} channels are determined by the intrinsic properties of their pore-forming α_1-subunits and by interactions with other channel subunits. This review focuses on structural determinants of Ca^{2+} channel inactivation in different parts of Ca^{2+} channel α_1-subunits, including pore-forming transmembrane segments and loops, intracellular domain linkers and the carboxyl terminus. Inactivation is also affected by the interaction of the α_1-subunits with auxiliary β-subunits and intracellular regulator proteins. The evidence shows that pore-forming S6 segments and conformational changes in extra- (pore loop) and intracellular linkers connected to pore-forming segments may play a principal role in the modulation of Ca^{2+} channel inactivation. Structural concepts of Ca^{2+} channel inactivation are discussed.

Ca^{2+} entry during an action potential

Ca^{2+} entry during an action potential initiates and controls multiple cascades of intracellular events which finally result in a variety of cellular functions. These include: impulse generation and propagation, sensory processes, muscle contraction, secretion of hormones and neurotransmitters, cell differentiation and gene expression (Berridge, 1997). Recent interest in the molecular mechanism of voltage-gated Ca^{2+} entry has been stimulated by the discovery that disorders such as familial hemiplegic migraine (FHM), ataxia type 6 and epilepsy are caused by spontaneous mutations in the pore-forming α_1-subunit of neuronal $Ca_v2.1$ (class A) Ca^{2+} channels (see Jen, 1999, for review, and Ertel *et al.* 2000 for new nomenclature of Ca^{2+} channels).

Action potentials are initiated by openings of voltage-gated Na^+ and Ca^{2+} channels induced by membrane depolarizations. The resulting influx of Na^+ and Ca^{2+} ions leads to a further membrane depolarization which in turn initiates more channel openings. This positive feedback mechanism underlies the regenerative process forming the ascending phase of an action potential (for review see Hille, 1992). Simultaneously, membrane depolarization initiates channel closures, a process named inactivation (Fig. 1*A*). The resulting negative feedback restricts the ionic current inflow (jointly with the activation of outward K^+ currents) and determines the action potential duration.

Ca^{2+} channels undergo more than one conformational change during inactivation; the existence of a Ca^{2+}-dependent inactivation mechanism was concluded from the finding that inactivation of Ca^{2+} channels is faster when the charge carrier is Ca^{2+} (compared with Ba^{2+} and other divalent or monovalent ions). This type of inactivation was first discovered in the ciliate *Paramecium* (Brehm & Eckert, 1978), then analysed in $Ca_v1.2$ of myocardial and smooth muscle (Lee *et al.* 1985; Ganitkevich *et al.* 1987) and later also observed in $Ca_v2.1$ (Lee *et al.* 1999; see also Tareilus *et al.* 1994). However, Ba^{2+} currents through Ca^{2+} channels commonly decay with biexponential kinetics suggesting

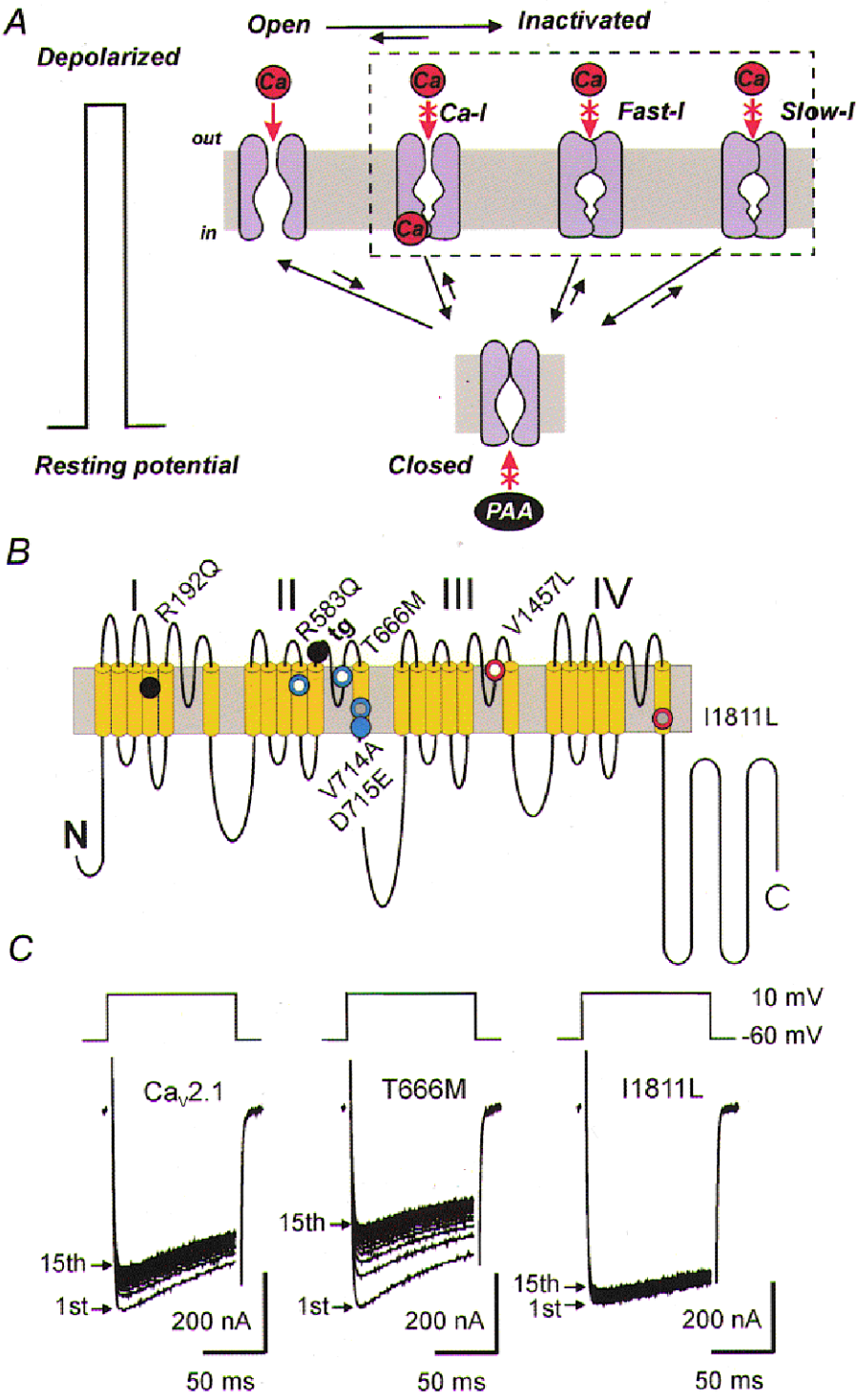


Figure 1. Ca^{2+} channel state transitions during an action potential

A, Ca^{2+} channels reside in different conformational populations: closed (resting) states, activated (open) states and several inactivated states. These populations are closely interconnected and the channel distribution between them is altered by changes in the electric field; membrane depolarization causes Ca^{2+} channels to open and subsequently to close by various inactivation mechanisms. Channel activation (transition from resting to open states) occurs within milliseconds whereas development and recovery from fast (Fast-I) and slow (Slow-I) inactivation occurs within tens of milliseconds or seconds. Ca-I represents the Ca^{2+}-induced inactivated state. The fast and slow inactivated states are closely interrelated. Transitions to inactivation may occur from the open as well as from the resting state (Patil *et al.* 1998). Transitions of open and fast inactivated channels to slow inactivation have been reported (Sokolov *et al.* 2000). Negative shifts of the membrane potential promote transitions of open and/or inactivated channels to the resting state. There is evidence that Ca^{2+} channel blockers such as phenylalkylamines (PAA) access their receptor

that these channels additionally close by fast and slow voltage-dependent inactivation processes.

Ca^{2+} channels dwell for a significantly longer time in an inactivated conformation than in the open state. As illustrated in the schematic diagram in Fig. 1*A* the channels will have to recover from inactivation before being able to open again. Thus, if the subsequent pulses arrive before recovery from inactivation is complete Ca^{2+} entry during spiking will be significantly reduced. Changes in the resting potential affect the steady-state fractions of resting and inactivated channels; at more depolarized voltages a larger fraction of Ca^{2+} channels will reside in one of the inactivated states thereby reducing the number of channels available during an action potential. In summary, Ca^{2+} entry is determined by the membrane potential (driving force and steady-state fractions in different channel conformations), the kinetics of Ca^{2+} channel opening, the kinetics of several inactivation processes and last but not least by the kinetics of recovery from inactivation.

The conformationally distinct Ca^{2+} channel populations (resting, open and inactivated states) are closely interrelated (Fig. 1*A*). In particular, there is evidence that voltage-dependent fast inactivation (Fast-I) of Ca^{2+} channels also occurs from the resting state (Patil *et al.* 1998) and that slow inactivation (Slow-I) in Ca^{2+} channels occurs not only after entry into the Fast-I state but also to a significant extent directly from the open state (Sokolov *et al.* 2000).

The molecular mechanism of Ca^{2+} channel inactivation is much less understood than that of some K^+ and Na^+ channels. Fast inactivation in *Shaker* K^+ channels (N-type inactivation) involves the occlusion of the inner channel mouth by a 'ball region' at the amino (N-) terminus ('ball and chain mechanism'; Armstrong & Bezanilla, 1977; Hoshi *et al.* 1990) while fast inactivation of Na^+ channels occurs predominantly by occlusion of the channel pore by a 'hinge lid' formed by the intracellular linker between domains III and IV of the α_1-subunit (IFM-motif; West *et al.* 1992).

This paper reviews the advances that have been made at the molecular level in the description of the different conformational changes of Ca^{2+} channels during inactivation. Functional studies on chimeric and mutant Ca^{2+} channels over recent years have revealed that the determinants of inactivation in this channel type are less localized than in K^+ and Na^+ channels. Development and recovery from inactivation in Ca^{2+} channels are affected by numerous structural changes in different parts of the α_1-subunit including pore-forming segments, the intracellular loops and the carboxyl (C-) terminus. The inactivation process is also influenced by interactions with intracellular proteins and auxiliary channel subunits, foremost among them being the β-subunit. In view of space constraints, the citations to the literature are selective and focus primarily on general concepts of Ca^{2+} channel inactivation that are illustrated by examples where structural changes in Ca^{2+} channel α_1-subunits induce major changes in inactivation gating.

Inactivation is a hallmark of Ca^{2+} channel subtypes

Evolution has not only created a variety of inactivation mechanisms to control Ca^{2+} entry (Fig. 1*A*) but additionally has produced a large family of voltage-gated Ca^{2+} channels with different inactivation kinetics determined by intrinsic properties of their α_1-subunits as well as by distinct subunit compositions.

High-voltage-activated Ca^{2+} channels are hetero-oligomeric complexes formed by α_1-, β- and α_2/δ-subunits and in some channel classes by an additional γ-subunit.

The α_1-subunit is the pore-forming membrane protein consisting of four homologous repeats (I–IV), each of them composed of six transmembrane segments (S1–S6; Fig. 1*B*). The pore-lining hydrophobic sequence between segments S5 and S6 is called the pore (P-) loop and forms the selectivity filter of the channel (Ellinor *et al.* 1995). The auxiliary β-, α_2/δ- and γ-subunits modulate the activation and inactivation kinetics, expression density, voltage dependence and the pharmacological properties of the α_1-subunit (Hofmann *et al.* 1999). The subunit composition of low-voltage-activated Ca_v3 (T-type Ca^{2+} channels) is less clear. The expression density of the $\alpha_1$3.1-subunit is regulated by the β_{1a}- (Dolphin *et al.* 1999) and α_2/δ-2-subunits (Gao *et al.*

from the intracellular site. Inactivation determinants at the inner channel mouth control PAA sensitivity (Hering *et al.* 1998). *B*, familial hemiplegic migraine (FHM) mutations affecting inactivation gating. Putative folding structure of a $Ca_v2.1$ channel α_1-subunit with indicated FHM mutations accelerating (blue) or slowing (red) the development of inactivation. Mutations inducing a delay in recovery from inactivation (using Ba^{2+} as charge carrier) are marked in white, those accelerating recovery are indicated in grey (see Kraus *et al.* 1998, 2000). Point mutation R192Q (black) did not affect inactivation (Kraus *et al.* 1998). The tottering mutation (*tg*, indicated in black) in the loop between segments IIS5 and IIS6 (P601L; Fletcher *et al.* 1996) reduces (among other changes in gating) channel inactivation (Wakamori *et al.* 1998). *C*, inhibition of Ba^{2+} currents (I_{Ba}) through wild-type $Ca_v2.1$ channels and FHM mutants T666M and I1811L (position I1811 in the human sequence corresponds to position I1819 in the rabbit sequence) expressed in *Xenopus* oocytes during a train of fifteen 100 ms pulses from −60 to 10 mV applied at 1 Hz (reproduced with permission from Kraus *et al.* 1998). Faster I_{Ba} inactivation of mutant T666M was associated with a slower recovery from inactivation resulting in a more pronounced accumulation in an inactivated state during the pulse train. Mutant I1811L displayed a slower inactivation time course. Consequently, less channels accumulated in an inactivated state during the 1 Hz train (compare *A*).

2000). A subtype of a γ-subunit has been shown to accelerate T-type activation and inactivation (Klugbauer *et al.* 2000).

Different classes of voltage-gated Ca^{2+} channels are encoded by different genes ($Ca_v1.1$ (former α_{1S}-subunit), $Ca_v1.2$ (α_{1C}), $Ca_v1.3$ (α_{1D}), $Ca_v1.4$ (α_{1F}), $Ca_v2.1$ (α_{1A}), $Ca_v2.2$ (α_{1B}), $Ca_v2.3$ (α_{1E}), $Ca_v3.1$ (α_{1G}), $Ca_v3.2$ (α_{1H}), $Ca_v3.3$ (α_{1I})) and can be distinguished with respect to their biophysical and pharmacological properties (see Ertel *et al.* 2000 for nomenclature).

The kinetics of inactivation differ significantly between different Ca^{2+} channel classes. Heterologous expression experiments enabled the kinetics of Ca^{2+} channels of similar subunit composition to be compared. Such experiments suggest that Ba^{2+} currents through the Ca_v3 channel family ($Ca_v3.1$–$Ca_v3.3$, also known as T-type channels; Perez-Reyes *et al.* 1999) display the fastest time course of voltage-gated inactivation, followed by $Ca_v2.3$ (possibly R-type; Zhang *et al.* 1994; Spaetgens & Zamponi, 1999), $Ca_v2.2$ (N-type; Hans *et al.* 1999*b*) and $Ca_v2.1$ (P/Q-type; Sather *et al.* 1993) channels. Ba^{2+} currents through Ca_v1 channels (various L-type channels; Williams *et al.* 1992; see Hofmann *et al.* 1999 for review) display the slowest time course of inactivation. More recently, different splice variants of the $\alpha_1$1.2- (Soldatov *et al.* 1998) and $\alpha_1$2.1-subunits (Bourinet *et al.* 1999) with surprisingly different inactivation kinetics have been discovered. The data of Forsythe *et al.* (1998) suggest that different inactivation properties of neuronal $Ca_v2.1$ could be important determinants of synaptic efficacy.

Evidence regarding the molecular mechanism of Ca^{2+} channel inactivation has come from various sources: diseased states associated with altered Ca^{2+} channel inactivation (e.g. FHM mutations, Kraus *et al.* 1998, 2000; and ataxia, Wakamori *et al.* 1998), gain-of-function chimeras, channel splice variants with different inactivation properties, mutational studies directed towards the identification of drug-binding determinants and functional studies analysing the role of auxiliary Ca^{2+} channel subunits and other intracellular proteins.

Mutations associated with familial hemiplegic migraine and ataxia induce changes in inactivation gating

$Ca_v2.1$ channels at presynaptic terminals mediate the release of neurotransmitters (Wu *et al.* 1999). Missense mutations and deletions in the pore-forming $\alpha_1$2.1-subunit of human $Ca_v2.1$ channels are associated with the aetiology of FHM (Ophoff *et al.* 1996; Kraus *et al.* 1998; Hans *et al.* 1999*a*), ataxia type 6 and epilepsy (Fletcher *et al.* 1996; Zhuchenko *et al.* 1997; see Jen, 1999, for review). Moreover, $Ca_v2.1$-deficient mice develop specific characteristics of ataxia and dystonia before dying about 3–4 weeks after birth (Jun *et al.* 1999).

Kraus *et al.* (1998) reported that the introduction of FHM mutations into the rabbit $\alpha_1$2.1-subunit induced significant changes in inactivation gating. Three out of four point mutations either accelerated (T666M, V714A) or slowed (I1811L, corresponds to I1819L in the rabbit sequence) the inactivation of the corresponding Ba^{2+} currents (I_{Ba}). Recovery from fast inactivation was slowed in mutant T666M and accelerated in V714A and I1811L (Fig. 1*B*). Some of the possible pathophysiological consequences of these FHM mutations can be deduced from Fig. 1*A*. On the one hand, an accelerated channel inactivation reduces Ca^{2+} entry during a depolarization (e.g. prominent for T666M, Fig. 1*C*). On the other hand, a delay or facilitation in recovery from an inactivated state will either reduce (T666M) or enhance (I1811L) Ca^{2+} entry during a train of consecutive pulses (see Fig. 1*C* for typical I_{Ba} of mutant channels). Almost identical changes in inactivation gating were later reported by Hans *et al.* (1999*a*) who introduced the same FHM mutations into the human $\alpha_1$2.1-subunit.

Most surprising in our initial study on the functional impact of FHM mutations in *Xenopus* oocytes (Kraus *et al.* 1998) was the finding that changes in the accumulation of inactivated Ca^{2+} channels could be monitored during pulse trains applied at frequencies as low as 1 pulse s^{-1} (Fig. 1*C*). This is far below the spiking frequency in neuronal networks in mammals and more pronounced effects might be expected in patients with FHM. However, the Ca^{2+} 'pulses' during action potentials in the central nervous system at 37 °C are orders of magnitude shorter than the voltage clamp steps illustrated in Fig. 1*C*. The pathophysiological impact of the changed inactivation properties (compared to changes in $Ca_v2.1$ expression level, threshold of activation and single channel conductance; Kraus *et al.* 1998, 2000; Hans *et al.* 1999*a*) remains to be elucidated under more physiological conditions in mammalian cells.

The general concept that FHM mutations modulate Ca^{2+} entry through $Ca_v2.1$ channels by modulating channel inactivation (Fig. 1*A*) was recently confirmed by Kraus *et al.* (2000) who observed similar effects for three newly identified FHM mutations (R583Q, D715E and V1457L (corresponds to V1465L in the rabbit sequence); Fig. 1*B*).

Except for the two mutations in S4 segments, all of the FHM mutations analysed so far are located either in the putative pore-forming S5 and S6 segments or the connecting pore loops (Fig. 1*B*). The charge neutralization in the voltage sensor IIS4 by R583Q also accelerates the time course of channel inactivation and simultaneously delays recovery from inactivation. The pathophysiological mechanism by which FHM results from point mutations that cause either enhanced or reduced Ca^{2+} entry (Fig. 1*C*) remains unclear.

A mutation associated with the pathological state of ataxia (the tottering mutation *tg*, P601L; Fig. 1*B*) is also located in the extracellular linker between segments IIS5 and IIS6. Wakamori *et al.* (1998) reported for Purkinje cells of homozygous ataxic mice carrying the *tg* mutation a reduction in the $Ca_v2.1$ current density, an about 10 mV shift in the threshold of channel activation and inactivation

to more negative voltages and also distinct changes in the inactivation kinetics of Ca^{2+} channel currents. Recent work of Plomp *et al.* (2000) provided the first hints as to how changes in Ca^{2+} channel gating might be associated with ataxia. Plomp and colleagues described an increased $Ca_v2.1$-mediated release of acetylcholine at neuromuscular junctions of homo- and heterozygote *tg*-mutant mice associated with an increased influx of Ca^{2+} at rest. The observed changes in neurotransmitter release could be associated with the leftward shift in the $Ca_v2.1$ activation threshold and/or reduced channel inactivation.

Studying inactivation in gain-of-function chimeras

Different kinetics in different channel classes implied that it should be possible to transfer inactivation determinants from one Ca^{2+} channel class to another. In line with this, Zhang *et al.* (1994) reported the successful transfer of the transient inactivation properties from a marine ray (doe-1) $Ca_v2.3$ channel to a slower inactivating $Ca_v2.1$ channel by transfer of segments IS6 and adjacent intra- and extracellular sequence stretches (Fig. 2*A*).

However, substitution of sequence stretches does not always result in a transfer of the inactivation properties; implantation of part of $Ca_v2.1$ segment IIIS6 and the adjacent extracellular flanking pore loop into $\alpha_1$1.2 produced a chimera with apparently faster inactivation kinetics than the donor $Ca_v2.1$ channel (Tang *et al.* 1993). Moreover, incorporation of a part of domain I of a slowly inactivating $\alpha_1$1.1-subunit into $\alpha_1$1.2 resulted in a chimera with a more transient inactivation than the recipient $Ca_v1.2$ channel (Parent *et al.* 1995).

Most strikingly, transfer of segment IVS6 from a slow inactivating $Ca_v1.1$ to the more rapidly inactivating $Ca_v2.1$ channel resulted in a chimera with significantly faster inactivation properties than native $Ca_v2.1$ (Döring *et al.*

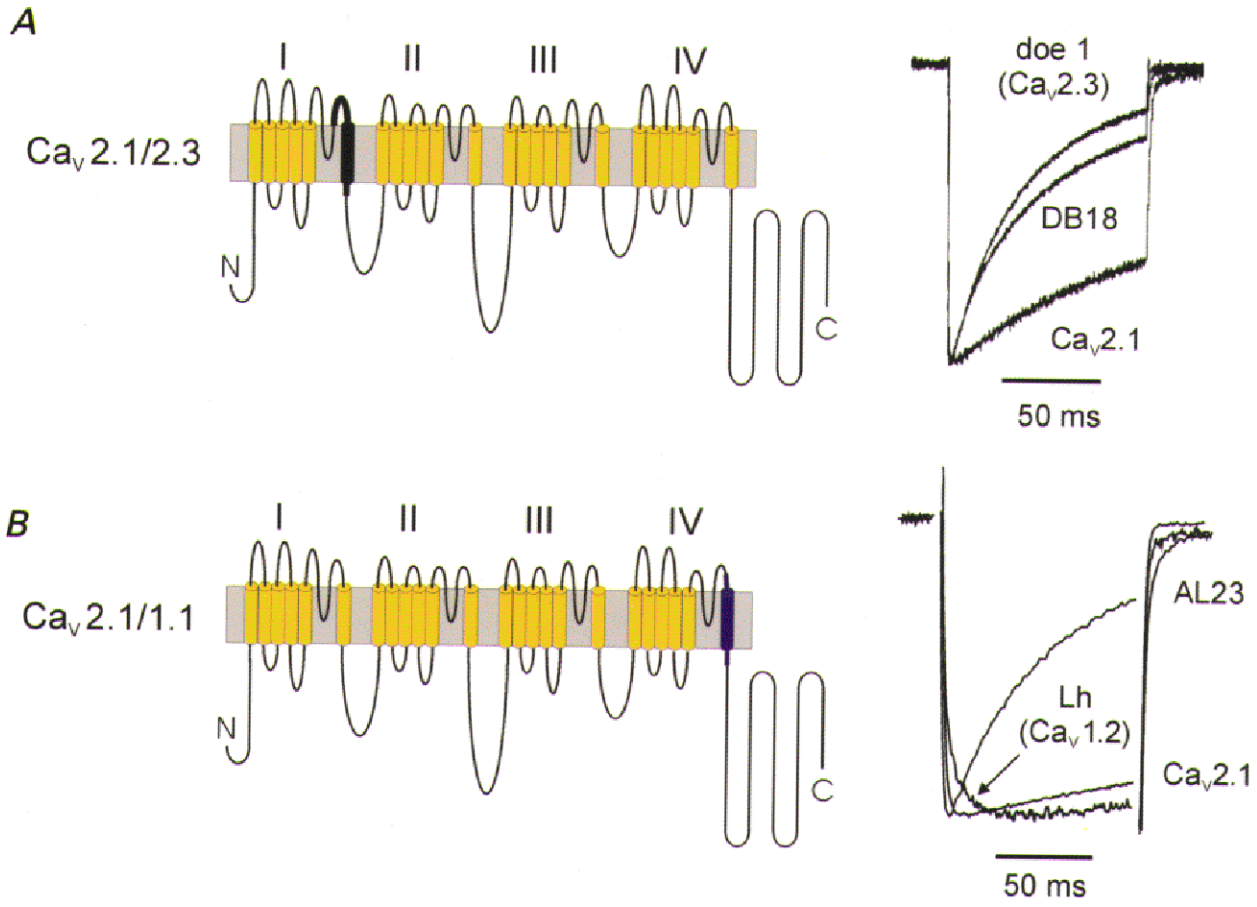


Figure 2. Chimeric channels as tools for studying structure and function of Ca^{2+} channel inactivation

A, a chimeric channel consisting of $\alpha_1$2.1 sequence (yellow) and segment IS6 and adjacent extra- and intracellular sequence stretches from $Ca_v2.3$ (doe-1, black) demonstrates a crucial role of this sequence in inactivation gating (chimera DB18 and current traces (right panel) are reproduced from Zhang *et al.* 1994 with permission from *Nature*; http://www.nature.com/). *B*, $Ca_v2.1/1.1$ chimera consisting of $\alpha_1$2.1 sequence (yellow) and segment IVS6 of L-type sequence (purple, $\alpha_1$1.1 sequence from carp skeletal muscle; Grabner *et al.* 1996). The chimera AL23 inactivated more rapidly than $Ca_v2.1$ and $Ca_v1.m$ channels. A trace of the $Ca_v1.2$ construct (Lh) illustrates the slow inactivation time course of the $Ca_v1.m$ family (data reproduced with permission from Döring *et al.* 1996).

1996; chimera AL23 in Fig. 2*B*) whereas implantation of the $Ca_v1.2$ sequence into segments IIIS5, IIIS6 and the connecting P-loop of $\alpha_1$1.2 almost completely abolished fast inactivation in the resulting chimera (chimera AL20; Hering *et al.* 1996). Switching domains between α_1-subunits of rapidly inactivating $Ca_v2.3$ and a $Ca_v1.2$ produced numerous changes in activation and inactivation gating (Spaetgens & Zamponi, 1999) suggesting, however, that domains II and III contain key inactivation determinants of $Ca_v2.3$ channels. Hans *et al.* (1999*b*) reported that the more transient inactivation kinetics of an $\alpha_1$2.2-subunit were successfully transferred by inserting a sequence stretch between IVS3 and the P-region into an $\alpha_1$2.1-subunit. In the same paper the authors demonstrated that a single point mutation (E1740R) in the P-loop of domain IV significantly accelerated channel inactivation properties and shifted the steady-state inactivation curve suggesting that this amino acid (E1740) plays an essential role in inactivation gating (Fig. 4*A*). Substitution of part of segment IVS5 in an $\alpha_1$1.2-subunit by the equivalent sequence of a fast inactivating Na^+ channel significantly slowed the inactivation kinetics of the resulting construct compared to $\alpha_1$1.2 suggesting a role of the IVS5 segment in inactivation gating (Motoike *et al.* 1999).

Chimeras between Ca^{2+} channels with different inactivation properties represent valuable tools for investigating the structure–activity relationship of inactivation in Ca^{2+} channels. However, attempts to construct Ca^{2+} channel gain-of-function chimeras with respect to inactivation have been less successful than the transfer of drug sensitivity between different channel classes (see Striessnig *et al.* 1998 for review). Voltage-dependent inactivation in Ca^{2+} channels apparently involves structural elements in more than one domain (Fig. 1*B*), and in some cases the transfer of key amino acids from all four domains may be required. Most studies on chimeric channels highlight, nevertheless, the role of pore-forming S6 and S5 segments and adjacent extracellular and intracellular sequence stretches, particularly the S5–S6 linker regions.

Multiple inactivation determinants in pore-forming segments: key determinants at the inner vestibule of the pore

More detailed knowledge regarding the role of single amino acids in the pore-forming S6 segments comes from studies on the molecular mechanism of Ca^{2+} channel block by drugs such as phenylalkylamines (PAAs), 1,4-dihydropyridines (DHPs) and diltiazem (DIL) (Hockerman *et al.* 1997*a*; Striessnig *et al.* 1998). Hockerman *et al.* (1995, 1997*b*) systematically substituted the amino acids in $Ca_v1.2$ segments IIIS6 and IVS6 by alanine to analyse the potential role of pore-forming segments IIIS6 and IVS6 in Ca^{2+} channel inhibition by PAAs and DHPs. The S6 point mutants displayed not only changes in drug sensitivity but also manifold changes in inactivation gating compared to wild-type $Ca_v1.2$ channels (Fig. 3*B* and *C*). Furthermore, replacement of two glutamates in the P-loops in domains II and IV known to form part of the selectivity filter (E709Q, E1419Q; Hockerman *et al.* 1997*a*) caused significant changes in steady-state inactivation (Fig. 3*C*).

We found, by mutating amino acids located in $Ca_v1.2$ and $Ca_v2.1$ segments IIIS6 and IVS6, that Ca^{2+} channel inactivation is particularly sensitive to amino acid substitution in the region of the inner channel mouth. One mutation in L-type segment IVS6 (V1504A; Berjukow *et al.* 1999) and a double mutation in segment IIIS6 of $Ca_v2.1$ (IF1612/1613AA; Sokolov *et al.* 2000) dramatically slowed the time course of fast inactivation (Fig. 3). Amino acid substitutions in segment IVS6 of $Ca_v2.1$ channels have substantial effects on the current decay (I1804Y, F1805M, S1808A, M1811I; Hering *et al.* 1996, 1998). The molecular mechanism by which amino acid substitutions in pore-forming segments affect channel inactivation remains to be elucidated.

Intracellular loops: interaction with β-subunits, G-proteins and syntaxin

Ca^{2+} channel inactivation is sensitive to structural changes in intracellular domain linkers. This has been demonstrated by Adams & Tanabe (1997) for $Ca_v1.2/Ca_v1.1$ chimeras consisting of $\alpha_1$1.1 sequence in linkers between domains I and II and III and IV. A dramatic example of how structural changes in the loop between domains I and II can modulate Ca^{2+} channel inactivation gating was reported by Bourinet *et al.* (1999). By comparing the sequence of the I–II loop and the inactivation properties of two $Ca_v2.1$ splice variants ($\alpha_{1A\text{-}a}$ and $\alpha_{1A\text{-}b}$), these authors discovered that a single valine insertion (V421) into the I–II loop of the $\alpha_{1A\text{-}a}$ splice almost completely removed the fast inactivation thereby converting the kinetic phenotype of Q-type channels into that of slowly inactivating P-type Ca^{2+} channels (Fig. 4*A*). Cens *et al.* (1999) demonstrated that overexpression of the I–II loop of $\alpha_1$2.1 accelerates current kinetics suggesting that the I–II loop may play the role of a ball peptide similar to the ball and chain mechanism in K^+ channels. Cens *et al.* (1999) also hypothesize that Ca^{2+}- and voltage-dependent inactivation share common molecular determinants.

There are several lines of evidence suggesting that Ca^{2+} channel inactivation can be modulated by proteins interacting with intracellular loops. Pragnell *et al.* (1994) identified a consensus sequence for the binding of the Ca^{2+} channel β-subunits in the intracellular loop between domains I and II (Fig. 4). Additional determinants for α_1–β-subunit interaction known to affect inactivation (see above) have been detected in the N- and C-terminus of $Ca_v2.1$ (Walker *et al.* 1998, 1999).

Functional studies confirmed that β-subunit interaction is a key determinant in Ca^{2+} channel inactivation with each β-subunit inducing individual gating effects. β_{2a}-subunit interaction commonly causes the slowest current decay

followed by β_4-, β_{1a}- and the β_3-subunit (Sather *et al.* 1993; Stea *et al.* 1994; De Waard & Campbell, 1995; Sokolov *et al.* 1999). These effects occur irrespective of whether the current is carried by Ba^{2+} or Ca^{2+} ions (Cens *et al.* 1999). Some of the regulatory properties of the β_{2a}-subunit are associated with the palmitoylation of two cysteines at its N-terminus (Chien *et al.* 1996; Qin *et al.* 1999). Recent data of Freise *et al.* (2000) indicate a role for the γ-subunit in inactivation of $Ca_v1.1$. A shift of the steady-state inactivation curve of $Ca_v2.1$ by coexpression of the γ-subunit subtypes γ-2 and γ-4 and an acceleration of the activation and inactivation time course of $Ca_v3.1$ induced by coexpression of the γ-5-subunit was reported by Klugbauer *et al.* (2000).

A motif in the intracellular I–II loop (QXXER; see Dolphin, 1998, for review) plays an essential role in G-protein interactions with the $\alpha_1$2.1-, $\alpha_1$2.2- and $\alpha_1$2.3-subunits (De Waard *et al.* 1997). Single amino acid substitutions in this motif have pronounced effects on inactivation gating: substitution of an arginine in the G-protein-interaction motif of $\alpha_1$2.1 by glutamate (QQIE**R**→QQIE**E**; see Fig. 4*A*) reduced channel inactivation, the reverse mutation in $\alpha_1$1.2 (QQLE**E**→QQLE**R**) accelerated the Ba^{2+} current decay (Herlitze *et al.* 1997).

Further support for the hypothesis that protein interactions with intracellular loops play an essential role in Ca^{2+} channel inactivation comes from Bezprozvanny *et al.* (1995) who reported that coexpression of syntaxin with $Ca_v2.2$ or $Ca_v2.1$ channels in *Xenopus* oocytes enhances voltage-dependent inactivation (see Catterall, 1999, for review). The evidence shows that SNARE protein interaction with a synaptic protein interaction site (Synprint; Fig. 4) on the domain II–III linker of $Ca_v2.1$ and $Ca_v2.2$ channels represents a further mechanism by which Ca^{2+} channel inactivation can be determined by structural changes in intracellular domain linkers (Bezprozvanny *et al.* 1995; Zhong *et al.* 1999; Degtiar *et al.* 2000).

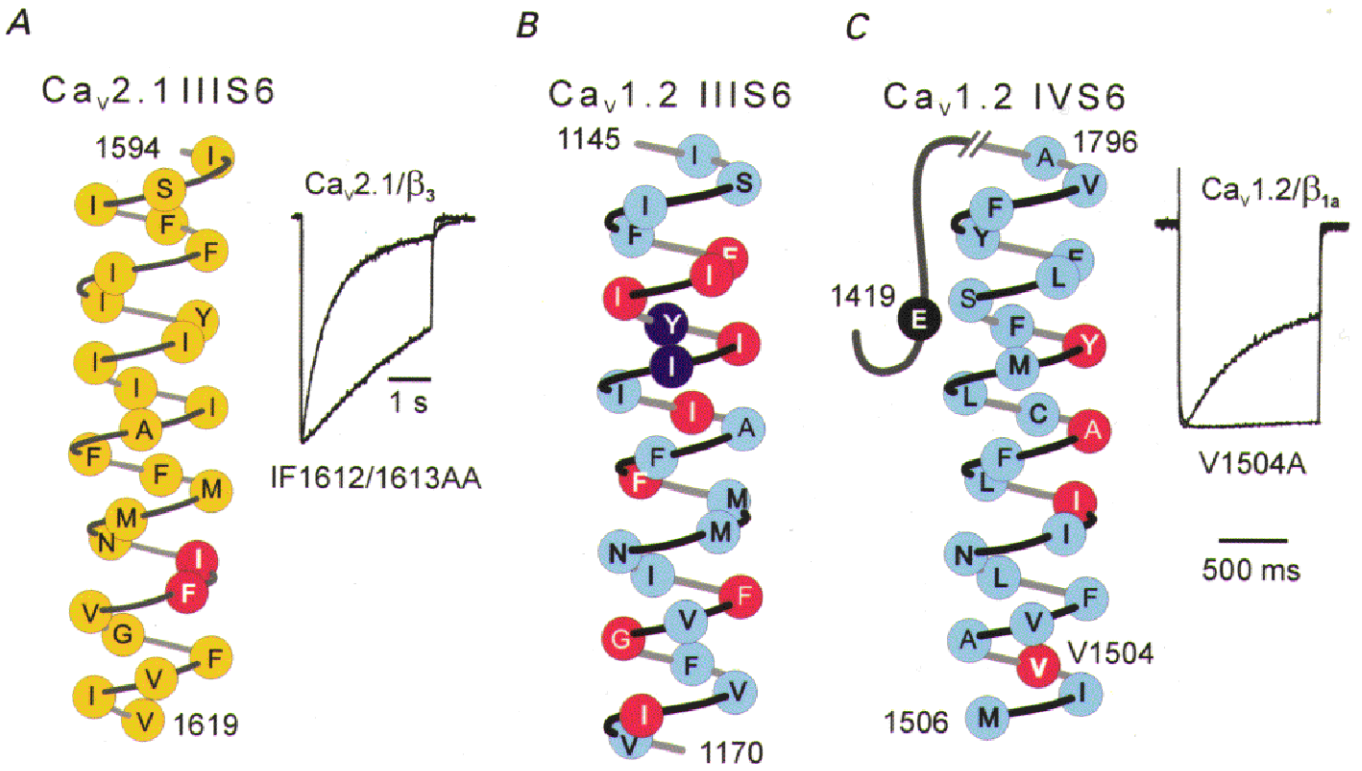


Figure 3. Multiple inactivation determinants in pore-forming segments IIIS6 and IVS6

A, inactivation determinants in segment IIIS6 of a $Ca_v2.1$ channel (Sokolov *et al.* 2000). Substitution of two amino acids located close to the inner channel mouth (indicated in red) substantially slowed inactivation (current traces from Sokolov *et al.* 2000). *B*, inactivation determinants in segment IIIS6 of $Ca_v1.2$. Alanine substitutions for the indicated amino acids shifted the inactivation curves either to more depolarized voltages (> 5 mV, red) or in the hyperpolarizing direction (> 5 mV, purple) (unpublished data supplied by Drs G. Hockerman and T. Scheuer). *C*, inactivation determinants in $Ca_v1.2$ segment IVS6. Substitution of a single valine by alanine (V1504A) almost completely abolished fast inactivation (Ba^{2+} current traces reproduced with permission from Berjukow *et al.* 1999). The inactivation curve of the triple $Ca_v1.2$ mutant lacking the high-affinity PAA determinants in segment IVS6 (YAI; Hockerman *et al.* 1995) was shifted to the right. Substitution of glutamate in position E1419Q (black) induced a 6 mV shift of the inactivation curve to more positive potentials (Hockerman *et al.* 1997*a*).

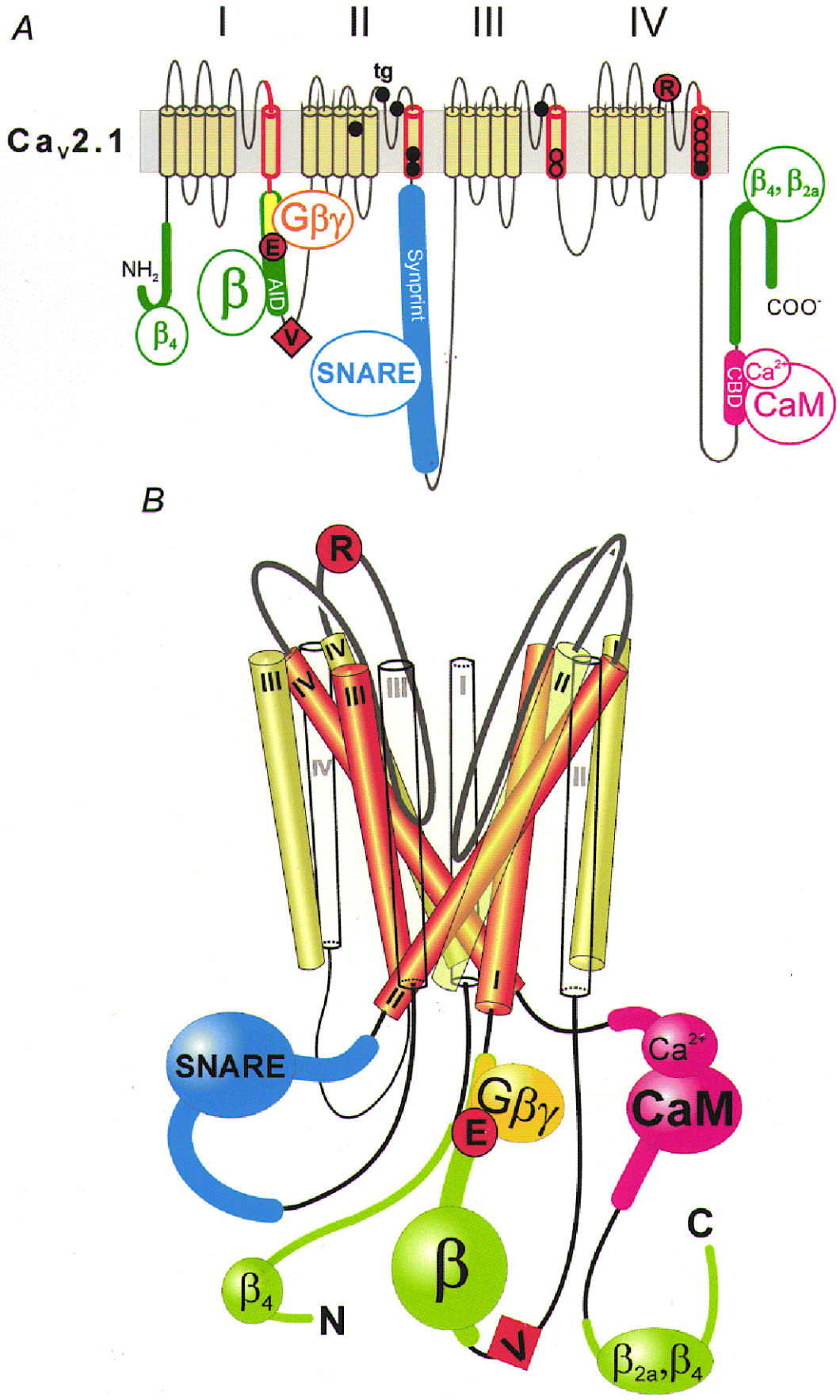


Figure 4. For legend see facing page.

The C-terminus: $Ca_v1.2$ splice variants and Ca^{2+}-dependent inactivation

The first evidence for a crucial role of the C-terminus in Ca^{2+} channel inactivation was reported by Soldatov *et al.* (1997) who identified a number of $Ca_v1.2$ channel splice variants. Coexpression of six $Ca_v1.2$ splice variants with auxiliary α_2/δ- and β_1-subunits in *Xenopus* oocytes resulted in currents with significantly different inactivation kinetics. Two of the isoforms ($\alpha_{1C,72}$ and $\alpha_{1C,86}$) contained C-terminal insertion sequences due to alternative splicing of exons 40–43. Corresponding Ba^{2+} currents displayed significantly faster inactivation kinetics. Another splice variant with alterations at the inner channel mouth in segment IS6 also displayed changes in inactivation kinetics (Soldatov *et al.* 1997, 1998).

Ca^{2+}-dependent inactivation was first suggested to be caused by Ca^{2+} binding to a specific motif (EF-hand) at the C-terminus of $Ca_v1.2$ (De Leon *et al.* 1995). This sequence is analogous to the EF-hand motif known from Ca^{2+}-binding proteins such as calmodulin (CaM) and parvalbumin and, except in $Ca_v1.3$, to a certain degree conserved in the C-terminus of all known Ca^{2+} channel α_1-subunits. It is called EF-hand because the Ca^{2+}-binding site represents a helix-loop-helix motif where the two helices in the original structure of parvalbumin were labelled as E and F. Ca^{2+}-dependent inactivation of $Ca_v1.2$ can be eliminated if the EF-hand motif in $\alpha_1$1.2 is replaced by corresponding residues of $\alpha_1$2.3 (De Leon *et al.* 1995). However, the conformational changes during Ca^{2+}-dependent inactivation are more complex. Bernatchez *et al.* (1998) revealed that a residue that is conserved within the EF-hand motif of Ca_v1 (E1537) contributes to voltage-dependent inactivation and hypothesized that this part of the intracellular C-terminus may play a role as an inactivation ball in voltage-dependent inactivation. Moreover, several groups have recently demonstrated that the ubiquitous Ca^{2+}-binding protein CaM is the main sensor for Ca^{2+}. CaM binds to the C-terminus of different Ca^{2+} channel classes thereby mediating Ca^{2+}-dependent inactivation (Qin *et al.* 1999; Zuhlke *et al.* 1999; Peterson *et al.* 1999; Lee *et al.* 1999). It appears that the EF-hand motif and the consensus CaM-binding motif (including the crucial amino acids isoleucine and glutamine, so called 'IQ'-motif; Zuhlke *et al.* 1999) that are located close to each other near the inner channel mouth at the C-terminus of $Ca_v1.2$ have a complementary role in Ca^{2+}-dependent inactivation. Peterson *et al.* (2000) suggest that the EF-hand may support the transduction of the conformational changes induced by the Ca^{2+}–CaM-binding step onto the $\alpha_1$1.2. The CaM-binding domain on $Ca_v2.1$ (CBD; Lee *et al.* 1999) is illustrated in Fig. 4*A*.

Determinants of slow inactivation in Ca^{2+} channels

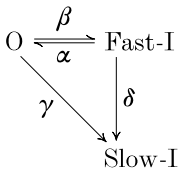
Evidence for slow inactivation in Ca^{2+} channels comes from the slow phase in channel recovery kinetics after a sustained membrane depolarization. Interestingly, use-dependent Ca^{2+} channel blockers such as PAAs, DIL and mibefradil induce a slow component in recovery from inactivation suggesting that these drugs promote a conformation resembling the slow inactivated state (Hering *et al.* 1997; Aczél *et al.* 1998; Berjukow *et al.* 1999). A role for syntaxin as a physiological modulator of the slow inactivation of $Ca_v2.2$ was recently reported by Degtiar *et al.* (2000).

We have analysed the kinetics of the slow inactivation of $\alpha_1$2.1 expressed together with β_{1a}-, β_{2a}-, β_3- or β_4-subunits. Our data clearly demonstrate that $Ca_v2.1$ channels proceed to the slow inactivated state from the fast inactivated state as well as directly from the open state. A reduction of fast voltage-dependent inactivation by coexpression of $\alpha_1$2.1

Figure 4. Molecular determinants of $Ca_v2.1$ channel inactivation

A, inactivation of $Ca_v2.1$ channels is affected by mutations in the $\alpha_1$2.1-subunit and proteins interacting with intracellular loops. The scheme highlights crucial segments and the location of some key point mutations: segment IS6 and adjacent intra- and extracellular sequences (red; Zhang *et al.* 1994); mutations in the putative pore-lining region of segments IIIS6 and IVS6 (red circles; Hering *et al.* 1996, 1998; Sokolov *et al.* 2000); point mutations associated with FHM, shown as black circles (see Kraus *et al.* 1998, 2000, for functional studies); point mutations in the intracellular I–II loop (G-protein-interaction motif (yellow): QQIE**R**→QQIE**E**; Herlitze *et al.* 1997) and the IVS5–IVS6 linker (R1740; Hans *et al.* 1999*b*), represented by large red circles; the additional valine of a $Ca_v2.1$ splice variant converting fast Q-type into slow P-type kinetics (V421; Bourinet *et al.* 1999), symbolized as a red square; the α-subunit interaction domain (AID) in the I–II loop and additional β-subunit interaction motifs on N- and C-terminal regions of $\alpha_1$2.1, shown in green (Pragnell *et al.* 1994; Walker *et al.* 1998, 1999); SNARE protein interaction with the Synprint site on the domain II–III linker (Bezprozvanny *et al.* 1995; Rettig *et al.* 1996; Zhong *et al.* 1999), shown in blue; and the calmodulin (CaM)-binding domain (CBD; Lee *et al.* 1999) in the C-terminus, shown in pink. *B*, hypothetical scheme of the structural organization of Ca^{2+} channel segments S1 (white), S5 (green) and S6 (red) and the interaction with intracellular proteins. Analogous to the crystal structure of the KcsA channel (Doyle *et al.* 1998), the pore-forming S6 segments are arranged as an inverted 'teepee' (see also Hering *et al.* 1998). The loop interactions with intracellular proteins and the location of some crucial inactivation determinants are indicated.

with β_{2a} increased channel state transitions into slow inactivation:



with α, β, γ and δ representing the rate constants for transitions between the open (O), fast inactivated (Fast-I) and slow inactivated (Slow-I) states. Thus, under certain circumstances open channels are even more willing to enter the slow inactivated channel conformation than channels in the fast inactivated state (i.e. $\gamma > \delta$) (Sokolov *et al.* 2000). Tissue-specific expression patterns of different β-subunits or changes in the subunit assembly during development appear, therefore, as significant determinants of fast *and* slow inactivation in $Ca_v2.1$ channels. Further functional studies are required to identify the molecular determinants of slow inactivation in Ca^{2+} channels and to estimate the relative impacts of slow inactivation, fast inactivation of open channels and closed-state-dependent inactivation (Patil *et al.* 1998) on Ca^{2+} entry during trains of action potential-like stimuli under physiological conditions.

Inactivation determinants affect drug binding in the pore region

Some of the residues involved in inactivation modulate Ca^{2+} channel block by PAAs, DIL and DHPs. This led us to propose a 'teepee'-dissociation model where mutations in the inner pore regions modulate inactivation gating by affecting the positions of S6 segments. This in turn modulates intracellular access, trapping or binding of PAAs and DIL in the channel pore (Hering *et al.* 1998; Berjukow *et al.* 1999). Indirect evidence for an inactivation mechanism involving conformational changes in the putative bundle-crossing region of S6 segments comes from recent experiments by Sokolov *et al.* (1999) demonstrating that the PAA sensitivity of a triple $Ca_v2.1$ mutant ($\alpha_{1A\text{-}PAA}$) is substantially influenced by a mutation in the I–II linker and by β-subunit interaction. The putative α-subunit interaction domain (AID) in the I–II linker and the mutation Arg387Glu are located far away from the putative drug-binding domain in the channel pore of the mutant $\alpha_1$2.1-subunit. In particular, the β_{2a} interaction slowed channel inactivation and correspondingly reduced the channel block by (−)gallopamil in a similar manner and to a comparable extent as previously reported for mutations close to the inner channel mouth in segment IIIS6 (Hering *et al.* 1997, 1998). Therefore, structural changes in intracellular linkers connected to S6 segments and S6 mutations might control the PAA sensitivity by similar mechanisms. Like PAAs and DIL, DHPs appear to bind in the pore region of Ca_v1 (key binding determinants are located on pore-forming segments IIIS5, IIIS6, IVS6 and connected P-loops; see Striessnig *et al.* 1998). Substitution of distinct inactivation determinants on pore-forming segment IVS6 destabilizes a DHP-induced inactivated channel conformation suggesting a synergism between intrinsic channel inactivation and DHP-induced inactivation (Berjukow *et al.* 2000).

Conclusions and outlook

The molecular determinants of inactivation are more widely spread over the Ca^{2+} channel protein than those in Na^+ and K^+ channels (Fig. 4). β interaction with different domains on the α_1-subunit, point mutations in S6 segments (particularly the putative bundle-crossing region near the inner channel mouth; Fig. 4*A*), protein interactions with intracellular domain linkers, structural changes in the C-terminus induced by Ca^{2+}–CaM interaction, alternative splicing or point mutations in the EF-hand motif all have pronounced affects on inactivation gating of Ca^{2+} channels.

Several hypotheses can be put forward for how these structural changes in different parts of the α_1-subunits affect inactivation. The first hypothesis is that distortions of the putative 'teepee' structure of pore-forming S6 segments play a key role in inactivation gating. This assumption is in line with studies analysing the interaction of 'inactivation-deficient' Ca^{2+} channel mutants with PAAs, DIL and DHPs (Hering *et al.* 1998; Sokolov *et al.* 1999; Berjukow *et al.* 2000). Hence, structural changes in pore-forming S5 and S6 segments, domain linkers, P-loops and the C-terminus may all affect the orientation of drug-binding determinants in the channel pore thereby modulating drug sensitivity (Fig. 4). An alternative view could be that domain linkers (particular the I–II linker) and the C-terminus in $Ca_v1.2$ and $Ca_v2.1$ may form part of separate inactivation gates occluding the inner channel mouth (Bernatchez *et al.* 1998; Cens *et al.* 1999; Peterson *et al.* 2000; Stotz *et al.* 2000). Finally, the most complicated case where a receptor structure for an inactivation gate at the inner channel mouth is determined by the orientation of S6 segments also cannot be excluded. Nevertheless, at present it is more attractive to think that structural changes in the intracellular loops and C-terminus of Ca^{2+} channels affect the orientation of pore-forming segments rather than modulating a variety of distinct inactivation gates (Fig. 4).

In conclusion, it appears that a number of crucial questions remain. What is the structural basis of the β-subunit modulation of Ca^{2+} channel inactivation? What is the molecular mechanism underlying the numerous changes in inactivation produced by point mutations in pore-forming segments? Are structural changes in the pore region and β-subunit modulation interdependent? How do the different inactivation mechanisms in Ca^{2+} channels finally modulate the interaction of Ca^{2+} channel α_1-subunits with blockers such as PAAs, DIL and DHPs? Have the fast and slow voltage-dependent inactivation and the Ca^{2+}-dependent inactivation common structural determinants? We anticipate that detailed functional studies of the different inactivation mechanisms (Fast-I, Slow-I, Ca-I; Fig. 1*A*) on Ca^{2+} channels expressed in recombinant systems combined with new structural information will provide the answers.

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