Voltage and Calcium Use the Same Molecular Determinants to Inactivate Calcium Channels*

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During sustained depolarization, voltage-gated Ca\(^{2+}\) channels progressively undergo a transition to a non-conducting, inactivated state, preventing Ca\(^{2+}\) overload of the cell. This transition can be triggered either by the membrane potential (voltage-dependent inactivation) or by the consecutive entry of Ca\(^{2+}\) (Ca\(^{2+}\)-dependent inactivation), depending on the type of Ca\(^{2+}\) channel. These two types of inactivation are suspected to arise from distinct underlying mechanisms, relying on specific molecular sequences of the different pore-forming Ca\(^{2+}\) channel subunits. Here we report that the voltage-dependent inactivation (of the \(\alpha_{1A}\) Ca\(^{2+}\) channel) and the Ca\(^{2+}\)-dependent inactivation (of the \(\alpha_{1C}\) Ca\(^{2+}\) channel) are similarly influenced by Ca\(^{2+}\) channel \(\beta\) subunits. The same molecular determinants of the \(\beta\) subunit, and therefore the same subunit interactions, influence both types of inactivation. These results strongly suggest that the voltage and the Ca\(^{2+}\)-dependent transitions leading to channel inactivation use homologous structures of the different \(\alpha_{1}\) subunits and occur through the same molecular process. A model of inactivation taking into account these new data is presented.

Calcium channels formed integral membrane proteins through which Ca\(^{2+}\) ions can flow into the cells during membrane depolarization, thereby activating many fundamental physiological functions such as contraction, synaptic transmission, or gene activation (1, 2). According to their biophysical and pharmacological properties, they have been classified into L, N, P, Q, R, and T type Ca\(^{2+}\) channels (2–6). All these types are able to respond to membrane depolarization by opening a pore, selective for Ca\(^{2+}\) and other divalent cations, and thus generating an inward current into the cells (2, 7). During sustained depolarizations, all of these Ca\(^{2+}\) currents progressively undergo a voltage-dependent inactivation with specific kinetics, primarily regulated by the membrane potential (2, 3, 8, 9). Of particular interest in the case of the L-type voltage-gated Ca\(^{2+}\) channels is the existence of a second type of inactivation driven by the intracellular Ca\(^{2+}\) concentration (10–12). In this case, Ca\(^{2+}\) ions entering into the cell through calcium channels can bind to a specific site located close to the inner mouth of the channel and promote the so-called Ca\(^{2+}\)-dependent inactivation (12–15). This type of inactivation, as opposed to the voltage-dependent inactivation, is not recorded when Ba\(^{2+}\) or Sr\(^{2+}\) permeate the channel (2, 4, 11, 12). While voltage-dependent inactivation has been found in all types of Ca\(^{2+}\) channels, the Ca\(^{2+}\)-dependent inactivation seems to be specific to the L-type, dihydropyridine-sensitive, Ca\(^{2+}\) channels and has been suggested to arise from a completely different mechanism (5, 12, 15–19).

It is strongly believed that all types of functionally characterized Ca\(^{2+}\) channels possess at least three different subunits: \(\alpha_{1}\), the pore-forming subunit, and \(\alpha_{2}\) and \(\beta\), two regulatory subunits (20–26). The biophysical and pharmacological properties of a Ca\(^{2+}\) channel are primarily driven by the \(\alpha_{1}\) subunit (13, 27–31), for which 10 genes (named class A, B, C, D, E, F, G, H, I, and S) have been identified (23, 25, 26, 32–34). The \(\beta\) subunit (four genes identified: \(\beta_{1}\)–4) and, to a lesser extent, the \(\alpha_{2}\) subunit (only one gene) seemed to have only a regulatory role on these properties (8, 35–42). The \(\alpha_{1}\) subunit is an integral membrane protein organized in four homologous domains, each containing six \(\alpha\)-helical membrane-spanning segments (23, 25, 26). It has been shown that in the kinetics of the voltage-dependent inactivation between two different \(\alpha_{1}\) subunits (encoding class E and A calcium channels, respectively) were due to the segment S6 of the first domain (I-S6) (30), suggesting a role for important and delimited molecular structures in the process of inactivation as was the case for K\(^{+}\) channels (43). On the other hand, a typical Ca\(^{2+}\) binding site (44) and other regions involved in the Ca\(^{2+}\)-dependent inactivation (45, 46) have recently been located in the carboxyl-terminal tail of the \(\alpha_{1}\) subunit. The identification of two different structures regulating voltage and Ca\(^{2+}\)-dependent inactivation supports the hypothesis of different underlying mechanisms for these two types of inactivation. However, donation of this Ca\(^{2+}\) binding site (EF-hand motif) to the \(\alpha_{1E}\) subunit gave chimerical Ca\(^{2+}\) channels that did support Ca\(^{2+}\)-dependent inactivation, whereas the parental \(\alpha_{1E}\) subunit did not. The fact that Ca\(^{2+}\)-dependent inactivation can be transplanted by the sole donation of a Ca\(^{2+}\) binding site presupposes that the molecular backbone responsible for the closure of the channel during sustained depolarization may be shared by voltage- and Ca\(^{2+}\)-dependent inactivation and would therefore be present on all types of Ca\(^{2+}\) channel.

To test this hypothesis, we have compared the regulation of inactivation of the class A and class C Ca\(^{2+}\) channels by different \(\beta\) subunits in conditions where pure voltage and Ca\(^{2+}\)-dependent mechanisms drove inactivation, respectively. We found that voltage and Ca\(^{2+}\)-dependent inactivations were similarly regulated by the four \(\beta\) subunits. Moreover, deleted or chimerical forms of the \(\beta\) subunit that altered the regulation of the voltage-dependent inactivation also modified the Ca\(^{2+}\)-dependent inactivation. We proposed a scheme for the inactivation mechanism and suggested that common distal steps shared by Ca\(^{2+}\)- and voltage-dependent inactivations use ho-
FIG. 1. Regulation of voltage- and Ca\(^{2+}\)-dependent inactivation by β subunits. A and B, Ba\(^{2+}\) and Ca\(^{2+}\) currents recorded from oocytes injected with the \(\alpha_{1A}, \alpha_{1B}, \alpha_{1C}, \) or \(\alpha_{1E}\) Ca\(^{2+}\) channel subunits together with the auxiliary \(\beta_1\) (A) or \(\beta_2\) (B) subunit. Inactivation \((I_2/I_1)\) was quantified by dividing the current at the end of a 400-ms-long depolarization \((I_2)\) by the peak current \((I_1)\) and is displayed as a bar graph for these different subunit combinations in the presence of external Ba\(^{2+}\) and Ca\(^{2+}\) (10 mM). Corresponding scaled traces are displayed at the bottom of each combination. Currents were recorded during 400-ms-long depolarizations to +10 mV from a holding potential of -100 mV. \(\alpha_{1A}\) plus \(\beta_i\) (\(n = 6\)), \(\alpha_{1A}\)
Fig. 2. Effect of the β subunits on the kinetics of inactivation of the α_{1A} and α_{1C} Ca^{2+} channels. Top, inactivation was quantified as the ratio of the noninactivating current at the end of the pulse over peak amplitude (I2/I1). D p polarizations of 2.5 s were used to maximize measurements of current inactivation. Dashed lines, superimposed fit of the two exponential components Tau1 and Tau2 necessary to describe current inactivation kinetics. I2/I1. Noninactivating currents calculated for different subunit combinations having either a voltage-dependent inactivation (α_{1A}, barium) or a Ca^{2+}-dependent inactivation (α_{1C}, calcium). Note that the β_b subunit decreased both the voltage-dependent (α_{1A}β_b subunit recorded in the presence of Ba^{2+}) and the Ca^{2+}-dependent (α_{1C}β_b subunit recorded in the presence of Ca^{2+}) inactivation (n = 8, 10, 6, 7, and 7 for α_{1A}, α_{1A}β_b plus β_α, α_{1C}β_b plus β_β, α_{1A}β_b plus β_δ and α_{1C}β_b plus β_γ, respectively; n = 6, 9, 7, 2, and 4 for α_{1C}β_b plus β_δ, α_{1C}β_b plus β_α, α_{1C}β_b plus β_γ, respectively). * α_{1C}β_b plus β_α significantly different from α_{1A}β_b alone; # α_{1C}β_b plus β_γ significantly different from α_{1C}β_b plus β_δ (see “Results and Discussion”). Tau1, fast time constant of voltage-dependent inactivation (α_{1A} or recorded using Ba^{2+}) and Ca^{2+}-dependent (α_{1C}) inactivation recorded using Ca^{2+}). Note that, in both cases, the fast time constant was accelerated by expression of the different β subunits. Tau2, only the β_b subunit increased the slow time constant of both the voltage-dependent (α_{1A}) and the Ca^{2+}-dependent (α_{1C}) inactivations. Currents were recorded during depolarization at +10 mV of 2.5 s (n = 7, 12, 4, 8, and 7 for α_{1A}, α_{1A}β_b plus β_α, α_{1A}β_b plus β_δ, α_{1A}β_b plus β_δ, and α_{1C}β_b plus β_γ, respectively; n = 3, 9, 8, 3, and 3 for α_{1C}β_b, α_{1C}β_b plus β_δ, α_{1C}β_b plus β_γ, and α_{1C}β_b plus β_δ, respectively.

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**COMMON STEPS IN VOLTAGE AND Ca^{2+}-DEPENDENT INACTIVATIONS**

**EXPERIMENTAL PROCEDURES**

**Preparation of Truncated and Chimerical Forms of the β Subunits—**The following calcium channel subunits were used: α_{1A} (47), α_{1B} (48), α_{2A} (49), α_{2C} (50), β_2 (40), β_4 (38), β_2 (37), and β_3 (36). All of these subunit cDNAs were inserted into the PM2 expression vector (51). Ndel-β2 was obtained by PCR. The sense primer was engineered to possess an EcoRI site (italic type), a start codon (boldface type), and the 748–769 sequence (underlined, accession number M80545) of the β_b subunit (5′-GGAAATTCATGGAGGAA-
CATGGGCTACAGCACATG-3′). In the reverse primer, an XhoI site was added at the 5′-end of the 2190–2168 sequence of β_b (5′-
GCTCTAGATCAT-
TGGCGAGTGATACATC-3′). The PCR product was checked and purified on agarose gel. The chimera were obtained by a PCR strategy as described (52). The N-terminal fragments were amplified using the following primers: for β_b, sense primer, 5′-GGAAATTCAGCCCCCTTGAAG-
GATC-3′ (representing EcoRI plus positions 114–133 of β_b) and reverse primer, 5′-CAGCAGCGAGAGGCTGTCTAGTTTGACCGGGCTTG-3′ (representing positions 576–559 of β_b, accession number X61394, plus positions 747–731 of β_b); for β_4, sense primer same as β_b sense primer and reverse primer, 5′-TATGGACCTGCTGAGGCATAGGACACCCGTACT-
C-3′ (representing positions 235–249 of β_4) and reverse primer, 5′-TATGGACCTGCTGAGGCATAGGACACCCGTACT-
C-3′ (representing EcoRI plus positions 67–84 of β_b) and reverse primer, 5′-T-
AGGATCTGCGAACCCTGTACAGGCGTGGTGT-3′ (representing positions 424–440 of β_b) plus positions 217–234 of β_b). The C-terminal fragments were amplified using the following primers: for β_b, sense primer, 5′-CAGCAGCGAGTGATACATC-3′ (representing EcoRI plus positions 731–747 of β_b) and reverse primer, 5′-GCTCTAGATCATGGAGGAA-
CATGGGCTACAGCACATG-3′. The PCR product was checked and purified on agarose gel. The chimera were obtained by a PCR strategy as described (52). The N-terminal fragments were amplified using the following primers: for β_b, sense primer, 5′-GGAAATTCAGCCCCCTTGAAG-
GATC-3′ (representing EcoRI plus positions 114–133 of β_b) and reverse primer, 5′-CAGCAGCGAGAGGCTGTCTAGTTTGACCGGGCTTG-3′ (representing positions 576–559 of β_b, accession number X61394, plus positions 747–731 of β_b); for β_4, sense primer same as β_b sense primer and reverse primer, 5′-TATGGACCTGCTGAGGCATAGGACACCCGTACT-
C-3′ (representing positions 235–249 of β_4) and reverse primer, 5′-T-
AGGATCTGCGAACCCTGTACAGGCGTGGTGT-3′ (representing positions 424–440 of β_b) plus positions 217–234 of β_b). The C-terminal fragments were amplified using the following primers: for β_b, sense primer, 5′-CAGCAGCGAGTGATACATC-3′ (representing EcoRI plus positions 731–747 of β_b) and reverse primer, 5′-GCTCTAGATCATGGAGGAA-
CATGGGCTACAGCACATG-3′.
Mutations on the β subunit have the same effects on the two types of inactivation. A, schematic representation of the truncated and chimeric forms of the β subunit used to study the regulation of inactivation. B, effect of these constructions on voltage and Ca\textsuperscript{2+}-dependent inactivation. Oocytes were injected with the α\textsubscript{1A} or the α\textsubscript{1C} subunits in combination with the β\textsubscript{1} (n = 12 and 7, respectively), β\textsubscript{2} (n = 6 and 7), Ndel-β\textsubscript{2} (n = 4 and 11), β\textsubscript{ch1} (n = 3 and 9), β\textsubscript{ch4} (n = 6 and 3), and β\textsubscript{ch5} (n = 5 and 4) subunits. Currents were recorded during depolarization at +10 mV during 2.5 s, and traces were scaled. Note that the presence of the N-terminal sequence of the β\textsubscript{2} subunit governed the kinetics of both types of inactivation. C, bar graph, averaged ratio I\textsubscript{2}/I\textsubscript{1} of the noninactivating α\textsubscript{1A} and α\textsubscript{1C} currents for the six combinations of β subunit tested. β\textsubscript{1} and β\textsubscript{2} were the same values as in Fig. 2. Each subunit has the same effect on the two types of inactivation. An asterisk represents a statistical difference between α\textsubscript{1A} plus β\textsubscript{ch1} and the constructs.

All PCR products were separated on 1% agarose gel, cut out, and purified. A second PCR was performed using these products to produce the final chimeras. Ndel-β2 and β chimeras were finally digested using EcoRI and XbaI, subcloned into pBluescript (Stratagene) for sequencing (DiDeoxy Terminator technology; Applied Biosystems), and subsequently subcloned into pMT2 for injection and expression.

I-II and III-IV loops of the α\textsubscript{1A} and α\textsubscript{1C} subunits were produced by PCR, subcloned into pBluescript, and in vitro transcribed (T7 m-MESSAGE m-Machine; Ambion) for oocyte injection (at an mRNA concentration of 1 μg/μl). Correct translation and molecular weight of these loops

FIG. 3. Mutations on the β subunit have the same effects on the two types of inactivation. A, schematic representation of the truncated and chimeric forms of the β subunit used to study the regulation of inactivation. B, effect of these constructions on voltage and Ca\textsuperscript{2+}-dependent inactivation. Oocytes were injected with the α\textsubscript{1A} or the α\textsubscript{1C} subunits in combination with the β\textsubscript{1} (n = 12 and 7, respectively), β\textsubscript{2} (n = 6 and 7), Ndel-β\textsubscript{2} (n = 4 and 11), β\textsubscript{ch1} (n = 3 and 9), β\textsubscript{ch4} (n = 6 and 3), and β\textsubscript{ch5} (n = 5 and 4) subunits. Currents were recorded during depolarization at +10 mV during 2.5 s, and traces were scaled. Note that the presence of the N-terminal sequence of the β\textsubscript{2} subunit governed the kinetics of both types of inactivation. C, bar graph, averaged ratio I\textsubscript{2}/I\textsubscript{1} of the noninactivating α\textsubscript{1A} and α\textsubscript{1C} currents for the six combinations of β subunit tested. β\textsubscript{1} and β\textsubscript{2} were the same values as in Fig. 2. Each subunit has the same effect on the two types of inactivation. An asterisk represents a statistical difference between α\textsubscript{1A} plus β\textsubscript{ch1} and the constructs.

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were checked by *in vitro* translation (T7-TNT coupled reticulocyte lysate system from Promega).

*Xenopus* oocyte preparation and injection (5–10 nl of α1, α1 plus β, or α1 plus α2δ plus β cDNAs at ~0.3 ng/ul) were performed as described elsewhere (42, 55). Oocytes were then incubated for 2–7 days at 19 °C under gentle agitation before recording.

**Electrophysiological Recordings**—Whole cell Ba2+ and Ca2+ currents were recorded under two-electrode voltage clamp using the GeneClamp 500 amplifier (Axon Instruments, Burlingame, CA). Current and voltage electrodes (less than 1 megohm) were filled with 2.8 M CsCl, 10 mM BAPTA, pH 7.2, with CsOH. Ca2+ and Ba2+ current recordings were performed after addition of BAPTA (one or two 40–70 ms injections at 1 bar of 100 mM BAPTA free acid (Sigma), 10 mM CaOH, 10 mM HEPES, pH 7.2, using solutions of the following composition: 10 mM BaOH/CaOH, 20 mM tetraethylammonium hydroxide, 50 mM N-methyl-D-glucamine, 2 mM CsOH, 10 mM HEPES, pH 7.2, with methanesulfonic acid. Ca2+ and Ba2+ current amplitudes were usually in the range of 1–5 μA, except for the α1C subunit expressed alone (70–250 nA) as reported by others (35). Expression of the α2δ subunit was sometimes performed but did not significantly modify either the kinetics of the two types of inactivation or the effects of overexpressing intracellular I-IV and III-IV loops. All of the results mentioned here were obtained without α2δ.

Currents were filtered and digitized using a DMA-Tecmar labmaster and subsequently stored on an IFC 468 personal computer by using version 6.02 of the pClamp software (Axon Instruments). Ba2+ or Ca2+ currents recorded during a typical test pulse from −80 mV to +10 mV of 2.5 s duration were well fitted using a biexponential function: \(i(t) = (A1^* \exp(-(t - V/Tau1)) + A2^* \exp(-(t - V/Tau2))) + C\), where \(t\) is the time; \(K\) is the zero time; \(A1^*\) and \(A2^*\) are the amplitudes and \(K\) is the conditional depolarization (inactivation curve) or the membrane potential used to record current (current-voltage curve), and \(C\) is the proportion of noninactivating current. All values are presented as mean ± S.D. Student’s *t* test was used at the 0.05 confidence level to test the significance of the difference between two means.

**RESULTS AND DISCUSSION**

When expressed in *Xenopus* oocytes, each class of Ca2+ channels displays only one type of inactivation, which can be driven either by membrane potential or change in intracellular Ca2+ concentration (Fig. 1, A and B). Current kinetics of the α1Aα2δβ1 and α1E Ca2+ channel, co-expressed with the ancillary Ca2+ channel β1 or β2 subunits, were identical when using either Ca2+ or Ba2+ as charge carriers, as shown by the superimposed scaled traces seen in Fig. 1, A and B. These kinetics were only modulated by the amplitude of the depolarization, as expected for a voltage-dependent inactivation (not shown) (41, 54, 55). In contrast, Ca2+ currents recorded from oocytes injected with the α1C subunit inactivated markedly faster than the corresponding Ba2+ currents (Fig. 1A). This behavior, typical of the so-called Ca2+-dependent mechanism of inactivation (13, 56–58), was present whether the β1 or the β2 subunit was co-expressed. Despite differences in their initiating events, voltage- and Ca2+-dependent inactivations were nevertheless both sensitive to the co-expression of the ancillary β subunits. Co-expression of the α1A subunit with the β1 or β2 subunit, for example, led to fast or slowly decaying Ba2+ currents, respectively (Fig. 1C, left). These differences in current kinetics were similar, in the case of the α1A subunit (but also α1B and α1G), using either 10 mM Ca2+ or Ba2+ as the charge carrier (see Fig. 1, A and B). Fast and slowly inactivating Ca2+ currents were also recorded upon co-expression of the β1 or β2 subunit with the α1C subunit (Fig. 1C, right). In this case, however, the use of 10 mM Ba2+ as the charge carrier completely blocked current inactivation for these two subunit combinations, further demonstrating the absolute requirement of Ca2+ for α1C inactivation (Fig. 1A and B). To understand the molecular mechanisms underlying these two different types of inactivation, the α1A and α1C subunits were chosen as prototype Ca2+ channels with pure voltage and Ca2+-dependent inactivations, respectively. Averaged data using long depolarizations and extended to the co-expression of the four known β subunits with these two α1 subunits are shown in Fig. 2. When compared with the corresponding α1 subunit expressed alone, voltage- and Ca2+-dependent inactivations were significantly accelerated upon co-expression of the β2 subunits (Fig. 2, α1A (top left) and α1C (top right); *p* < 0.05). The same effect was found upon co-expression of the β2 with the β1, α1C subunit, without significant difference when compared with α1A plus β1. Co-expression of the β2 subunit, however, slowed both the voltage and the Ca2+-dependent inactivation when expressed with the α1A or the α1C subunit, respectively (significantly different from α1A plus β1). A kinetic analysis of these current decays confirmed and extended this observation. Voltage- as well as Ca2+-dependent inactivation should be well approximated by a biexponential decay. The fast time constant (Tau1, Fig. 2) of inactivation of the α1A or α1C subunits was always accelerated upon co-expression of each of the β subunits (no statistical differences between the four β subunits). The slow time constant (Tau2, Fig. 2) was either decreased (β1, β2, and β4) or increased (β2), for these two α1 subunits, after
co-expression of a β subunit, and it was thus responsible for the marked slowing of inactivation recorded with the β2 subunit (together with an increase in the relative amplitude of the slow component in both cases; not shown). Taken together, these data demonstrate that β subunits modified the same kinetic components of the voltage- and Ca2+-dependent inactivations and thus suggest that they affect the same molecular processes on the two α subunits. Because protein-protein interactions between the α1 and the β subunits are the basis of these regulations (8, 41, 42, 59–63) and because protein-protein interactions between the α1 and the β subunits are the basis of these modifications of the α1 subunit (Fig. 3, Ndel-β). This deleted β subunit had exactly the same effect on the α1C Ca2+-dependent inactivation, which became as fast as β3 (see Fig. 3, Ndel-β2, right). When this deleted sequence was inserted at a homologous position in the β1 subunit (βch1; see Fig. 3, top, for construction and traces), Ba2+ currents recorded from oocytes expressing α1α plus βch1 subunits displayed the slow inactivation kinetics typically recorded with the β2 subunit. A similar slowing of the Ca2+-dependent inactivation was recorded when this chimera was expressed with the α1C subunit (Fig. 3, βch1, right). These data suggest that the presence of the amino-terminal end of the β2 subunit is responsible for the slow current decays in both voltage and Ca2+-dependent inactivation. This was further confirmed by the use of chimera βch4 and βch5 (see Fig. 3, top, for constructions). Co-expression of βch4 (where the β subunit had its first variable domain (64), corresponding to amino acids 0–58, replaced by the homologous amino acids of the β2 subunit) decreased the voltage-dependent inactivation (α1A) as
well as the Ca\(^{2+}\)-dependent inactivation (\(\alpha_{1C}\)). In both cases, inactivation became as slow as in the case of co-expression of the full-length \(\beta_2\) subunit (Fig. 3, \(\beta ch4\) traces). The reverse chimera \(\beta ch5\) (where the \(\beta_2\) subunit has its first domain, corresponding to amino acids 1–16, replaced by the homologous domain of \(\beta_1\)) had opposite effects and induced fast-inactivating currents comparable with those recorded with the \(\beta_1\) subunit (or \(\text{NdeI}-\beta_2\)), irrespective of the \(\alpha_1\) subunit and therefore of the mode of inactivation. All of these data, summarized at the bottom of Fig. 3, clearly show that any change in the structure of the \(\beta\) subunit affecting the voltage-dependent inactivation also modified the Ca\(^{2+}\)-dependent inactivation and suggest that the same mechanism underlies these two distinct phenomena.

Voltage- as well as Ca\(^{2+}\)-dependent inactivations are known to be intrinsic properties of the \(\alpha_1\) subunit. However, until now, only voltage-dependent inactivation was known to be regulated by auxiliary \(\beta\) subunits (8, 41, 42, 51, 65). We show here the presence of two kinetic components of inactivation (characterized by \(\text{Tau1}\) and \(\text{Tau2}\)) in both \(\alpha_{1A}\) and \(\alpha_{1C}\)-directed currents. These two components are both either voltage-dependent (\(\alpha_{1A}\) inactivation) or Ca\(^{2+}\)-dependent (\(\alpha_{1C}\) inactivation; see Fig. 2), suggesting that the two types of inactivation each have two different underlying mechanisms. Our results emphasize the fact that a given \(\beta\) subunit regulates, in the same way, each of these two components, independently of the mode of inactivation (voltage- or Ca\(^{2+}\)-dependent), and therefore the type of \(\alpha_1\) subunit. To take into account these new data, we propose a new scheme of inactivation where voltage and Ca\(^{2+}\) inactivate these two different channels by using a “ball and chain” mechanism, with blocking particles and binding sites encoded by homologous sequences on the \(\alpha_{1A}\) and \(\alpha_{1C}\) subunits and therefore sensitive to the same molecular interactions with the \(\beta\) subunit (see Fig. 4). Binding of the particle to its binding site would ensure channel inactivation, as in the case of potassium channels (43). In the case of voltage-dependent inactivation, this binding is not ion-sensitive and can occur with either Ba\(^{2+}\) or Ca\(^{2+}\) as charge carriers, voltage-dependence being due to state-dependent changes in the mobility of the particle and/or the accessibility of the binding site. The Ca\(^{2+}\)-dependent mechanism of inactivation is essentially the same, except that accessibility to, or functionality of, the binding site needs the fixation of a Ca\(^{2+}\) ion to a site located near the inner mouth of the channel (44) (Fig. 4, right). In our scheme, this fixation would produce a conformational modification of the binding site into a high affinity state, therefore allowing binding of the ball and inactivation of the channel. The sensitivity of this newly formed inactivated state to the continuous presence of a bound Ca\(^{2+}\) ion on the \(\alpha_1\) subunit might explain the differences in the immobilization of channel gating charges between voltage- and Ca\(^{2+}\)-dependent inactivation (56). In this scheme, regulation of inactivation by \(\beta\) subunits occurs mainly through their N-terminal tail, where essential palmitoylation sites have just been identified (66–68), and would be due to modifications in the mobility of the inactivating particle by bound auxiliary subunits (Fig. 4). Accordingly, this region of the \(\beta_2\) subunit has also been shown to influence voltage-dependent inactivation of the \(\alpha_{1A}\) Ca\(^{2+}\) channel (68–70). Therefore, the I-II loop, connecting domains I and II of the pore-forming \(\alpha_1\) subunit represents an attractive candidate for the blocking particle, since it possesses the \(\alpha_1\) interaction domain sequence, responsible for the interaction with the \(\beta\) subunit (60), and multiple mutations in this domain affect inactivation (71, 72). Accordingly, co-expression of an excess of free \(\alpha_{1A}\) I-II loop with the slowly inactivating \(\alpha_{1A}\) plus \(\beta_2\) Ca\(^{2+}\) channels significantly accelerated voltage-dependent inactivation (\(\alpha_{1A}\)I-II; Fig. 5, A and B). Similar effects on the voltage-dependent inactivation of the \(\alpha_{1A}\) plus \(\beta_2\) Ca\(^{2+}\) channels were also recorded after co-expression of the I-II loop from \(\alpha_{1C}\) (\(\alpha_{1C}\)I-II; Fig. 5, A and B) but not with the III-IV loop from \(\alpha_{1A}\) (\(\alpha_{1A}\)III-IV), suggesting that the \(\alpha_{1C}\)I-II loop can promote voltage-dependent inactivation of the \(\alpha_{1A}\) Ca\(^{2+}\) channel. Indeed, similar changes were obtained when steady state inactivation was studied, with smaller noninactivating current and hyperpolarizing shift of the half-inactivation potentials obtained with I-II loops but not the III-IV loop (Fig. 5C; mean values for \(V_{1/2}\) and \(R\) as follows: \(\text{H}_2\text{O} - 12 \pm 7\) mV and 0.57 \(\pm\) 0.08 \(n = 12\); \(\alpha_{1A}\)I-II, \(-13 \pm 7\) mV and 0.61 \(\pm\) 0.13 \(n = 15\); \(\alpha_{1A}\)I-I, \(-22 \pm 5\) mV and 0.35 \(\pm\) 0.13 \(n = 11\); \(\alpha_{1C}\)I-I, \(-16 \pm 7\) mV and 0.41 \(\pm\) 0.09 \(n = 7\)). Co-expression of these loops did not affect significantly (or slightly hyperpolarized; see Fig. 5C) the voltage dependence of activation and the average current amplitude (not shown). These latter data confirmed a direct effect on inactivation rather than a displacement of the \(\beta_2\) subunit from \(\alpha_{1A}\) by an excess of free \(\alpha_1\) interaction domain, which would be expected to depolarize the current-voltage curve (mean activation potentials were as follows: \(\text{H}_2\text{O} - 8 \pm 3\) mV \(n = 12\); \(\alpha_{1A}\)III-IV, \(-11 \pm 6\) mV \(n = 15\); \(\alpha_{1A}\)I-I, \(-15 \pm 6\) mV \(n = 11\); \(\alpha_{1C}\)I-I, \(-7 \pm 12\) mV \(n = 7\)) and depress current amplitude (62). Possible molecular determinants for the binding site include the carboxyl end of the \(\alpha_1\) subunit, where an EF-hand Ca\(^{2+}\) binding motif and other sequences, important for Ca\(^{2+}\) dependent inactivation, have been identified (44–46, 72, 73). Supporting our scheme, transfer of this domain to the \(\alpha_{1B}\) subunit confers Ca\(^{2+}\)-dependent inactivation (44), while \(\alpha_{1C}\) chimera subunits harboring a carboxyl-terminal tail originating from a non-Ca\(^{2+}\)-sensitive \(\alpha_1\) subunit lost their Ca\(^{2+}\)-sensitive inactivation while preserving voltage-dependent inactivation (72).

The presence of the two kinetic components (\(\text{Tau1}\) and \(\text{Tau2}\)) can be best explained by the existence of an additional blocking particle instead of multiple binding sites, since (i) fast and slow inactivation have distinct regulation by \(\beta\) subunits and (ii) the two components are either voltage- (\(\alpha_{1A}\)) or Ca\(^{2+}\)- (\(\alpha_{1C}\)) dependent, as expected for a unique binding site. Differences in the proportion of each component between voltage- and Ca\(^{2+}\)-dependent inactivation may be due to sequence variation between the \(\alpha_1\) subunits. This scheme also disregards any participation of phosphatases in the process of Ca\(^{2+}\)-dependent inactivation, since the intracellular Ca\(^{2+}\) concentration was sufficiently buffered to inhibit Ca\(^{2+}\)-dependent enzyme, and ATP\(\gamma\)S, as well as okadaic acid, had no effect either on Ca\(^{2+}\) current amplitude or on kinetics (74, 75). In conclusion, we show that voltage and Ca\(^{2+}\) use homologous structure of related \(\alpha_1\) subunits to inactivate Ca\(^{2+}\) channels, and we provide evidence for a direct channel block by the intracellular I-II loop of the \(\alpha_1\) subunit. The use of similar molecular determinants for the two types of inactivation suggests that voltage-dependent inactivation has been modified during evolution to provide the structural basis for the Ca\(^{2+}\)-mediated inactivation, therefore preventing cellular Ca\(^{2+}\) overload.

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Common Steps in Voltage and Ca<sup>2+</sup>-dependent Inactivations
