Subunit regulation of the neuronal $\alpha_{1A}$ Ca$^{2+}$ channel expressed in *Xenopus* oocytes

Michel De Waard and Kevin P. Campbell*

*Howard Hughes Medical Institute, Department of Physiology and Biophysics, University of Iowa, College of Medicine, Iowa City, IA 52242, USA*

1. Voltage-dependent Ca$^{2+}$ channels are multi-protein complexes composed of at least three subunits: $\alpha_1$, $\alpha_2\delta$ and $\beta$. Ba$^{2+}$ currents were recorded in *Xenopus* oocytes expressing the neuronal $\alpha_{1A}$ Ca$^{2+}$ channel, using the two-electrode voltage-clamp technique. Various subunit combinations were studied: $\alpha_{1A}$, $\alpha_{1A}\alpha_2\delta_b$, $\alpha_{1A}\beta$ or $\alpha_{1A}\alpha_2\delta_b\beta$.

2. The $\alpha_{1A}$ subunit alone directs the expression of functional Ca$^{2+}$ channels. It carries all the properties of the channel: gating, permeability, voltage dependence of activation and inactivation, and pharmacology. The $\alpha_{1A}$ channel is activated by low voltages when physiological concentrations of the permeant cation are used. Both ancillary subunits $\alpha_2\delta$ and $\beta$ induced considerable changes in the biophysical properties of the $\alpha_{1A}$ current. The subunit specificity of the changes in current properties was analysed for all four $\beta$ gene products by coexpressing $\beta_{1b}$, $\beta_{2a}$, $\beta_{3}$ and $\beta_{4}$.

3. All $\beta$ subunits induce a stimulation in the current amplitude, a change in inactivation kinetics, and two hyperpolarizing shifts – one in the voltage dependence of activation and a second in the voltage dependence of steady-state inactivation. The most significant difference in regulation among $\beta$ subunits is the induction of variable rate constants of current inactivation. Rates of inactivation were induced in the following order (fastest to slowest): $\beta_3 > \beta_{1b} = \beta_4 > \beta_{2a}$.

4. The $\alpha_2\delta_b$ subunit does not modify the properties of $\alpha_{1A}$ Ca$^{2+}$ channels in the absence of $\beta$ subunits. However, this subunit increases the $\beta$-induced stimulation in current amplitude and also regulates the $\beta$-induced change in inactivation kinetics.

5. Of all the subunit combinations tested, Ca$^{2+}$ channels that included a $\beta$ subunit were the most prone to decrease in activity. It is concluded that $\beta$ subunits are the primary target for the inhibitory mechanisms involved in Ca$^{2+}$ channel run-down.

6. Both $\alpha_2\delta_b$ and $\beta_{1b}$ subunits slightly modified the sensitivity of the $\alpha_{1A}$ subunit to the snail peptide $\omega$-conotoxin MVIIC.

7. The subunit-induced changes in properties of the $\alpha_{1A}$ channel are surprisingly similar to changes reported for other $\alpha_1$ subunits. These modifications in channel activity should therefore represent important functional landmarks in the on-going characterization of subunit–subunit interactions.

Voltage-dependent Ca$^{2+}$ channels are well characterized on the basis of their biophysical and pharmacological properties. At least three types of high-voltage-activated Ca$^{2+}$ channels, termed L-, N- and P-type, have been differentiated by their sensitivities to various neurotoxins and organic Ca$^{2+}$ channel modulators (Miller, 1992). The purified rabbit skeletal muscle L-type channel is composed of four subunits: the $\alpha_1$ pore-forming subunit, which contains the binding sites for all C$^{2+}$ channel modulators affecting this channel type; the $\alpha_2\delta$ subunit, a disulphide-linked dimer; the transmembrane $\gamma$ subunit and the intracellular $\beta$ subunit (Takahashi, Seagar, Jones, Reber & Catterall, 1987). The primary structures of all the subunits composing this L-type Ca$^{2+}$ channel were deduced by cloning (Tanabe *et al.* 1987; Ellis *et al.* 1988; Ruth *et al.* 1989; Jay *et al.* 1990). By using these cDNA sequences a
probes, it was found that as many as six different genes coded for each \( \alpha_1, \beta \) and \( \alpha_2\delta \) subunit (Tanabe et al. 1987; Ellis et al. 1988; Mikami et al. 1989; Ruth et al. 1989; Hui, Ellinor, Krizanova, Wang, Diebold & Schwartz, 1991; Mori et al. 1991; Dubel et al. 1992; Niidome, Kim, Friedrich & Mori, 1992; Perez-Reyes et al. 1992; Castellano, Wei, Birnbaumer & Perez-Reyes, 1993a, b). Also, additional molecular diversity arises from alternative splicing of transcripts of these distinct genes. In spite of this remarkable diversity, all these subunits have conserved structural features. \( \beta \) subunits are all capable of binding to a single amino acid motif that is present on all \( \alpha_1 \) subunits cloned so far (Pragnell, De Waard, Mori, Tanabe, Snutch & Campbell, 1994). It was also found that the \( \alpha_2\delta \) subunit is conserved in a wide range of organs (Ellis et al. 1988; Morton & Froehner, 1989). For instance, cloning and sequencing of a cDNA encoding a rat brain \( \alpha_2\delta \) subunit shows 95% amino acid identity with an alternative splice variant expressed in skeletal muscle (Kim, Kim, Lee, King & Chin, 1992). More recently, the brain N-type Ca\(^{2+} \) channel was also purified (Witcher et al. 1993) and reconstituted into bilayers (De Waard, Witcher & Campbell, 1994). Despite their differing cellular functions, the N-type Ca\(^{2+} \) channel bears remarkable structural and functional homologies with the L-type channel. This channel is composed of structurally similar subunits (\( \alpha_1, \alpha_2\delta, \beta \) for the N-type versus \( \alpha \), \( \alpha_2\delta, \beta_2 \) for the L-type) although it differs by the presence of a fourth subunit (a 95 kDa protein versus \( \gamma \) subunit in the L-type channel). Expression experiments provide further evidence that the core subunit composition of all voltage-dependent Ca\(^{2+} \) channels could be minimally described by \( \alpha_2\delta\beta\gamma \). Coexpression of various \( \beta \) subunits with all classes of \( \alpha_1 \) subunits (S, A, B, C, D and E if one adopts the most recent acknowledged nomenclature; Birnbaumer et al. 1994) results in an enhanced current amplitude (Mori et al. 1991; Hullin et al. 1992; Williams et al. 1992a, b; Ellinor et al. 1993) and/or altered voltage dependence and kinetics of the resulting Ca\(^{2+} \) channels (Lacerda et al. 1991; Singer, Biel, Lotan, Flockerzi, Hofmann & Dascal, 1991; Varadi, Lory, Schultz, Varadi & Schwartz, 1991; Soong, Stea, Hodson, Dubel, Vincent & Snutch, 1993). There are actually fewer experimental data illustrating the regulation of Ca\(^{2+} \) channel currents by the \( \alpha_2\delta \) subunit. However, it has been demonstrated that this subunit contributes to a normalization of the activation and inactivation kinetics of the \( \alpha_1C \) subunit (Table 2). Altogether, these observations prompted us to determine how various \( \alpha_1 \) subunits correlate among each other by isolating common functional features. Conserved subunit regulation among \( \alpha_1 \) subunits should provide some insights into the structural determinants involved in subunit–subunit interactions of Ca\(^{2+} \) channels. To reach this goal, we chose to study the subunit regulation of the \( \alpha_1C \) Ca\(^{2+} \) channel since the characterization of this neuronal channel is still very fragmentary. *Xenopus* oocytes were used because (1) these cells have been an expression system of choice for Ca\(^{2+} \) channels and (2) they express only low levels of endogenous voltage-gated Na\(^+ \), K\(^+ \) and Ca\(^{2+} \) channels (Dascal, 1987). This report is therefore the first detailed description of the biophysical and pharmacological properties of the \( \alpha_1A \) subunit expressed by itself. Comparisons between the properties of \( \alpha_2\delta \) Ca\(^{2+} \) channels on the one hand and \( \alpha_2\delta\beta \) or \( \alpha_2\delta\beta\gamma \) Ca\(^{2+} \) channels on the other demonstrate that contrary to an initial report by Mori et al. (1991), but consistent with the expression of other \( \alpha_1 \) subunits, both ancillary subunits \( \alpha_2\delta \) and \( \beta \) have dramatic effects on the activity of the \( \alpha_1 \) Ca\(^{2+} \) channel.

**METHODS**

**Preparation of Xenopus oocytes**

*Mature Xenopus laevis* female frogs were purchased from NASCO (WI, USA). The animals were maintained under a 12 h light–12 h dark cycle at 16 °C. To harvest oocytes, the frogs were anaesthetized to full immobility with 0.03% ethyl p-amino-benzoate (Sigma) and their ovaries were surgically removed. The incision created was sutured immediately after removal of the oocytes and the animals returned to the tank following recovery from surgery. They were then allowed to recover for at least 2 months before being reused. Follicle membranes from isolated oocytes were enzymatically digested for 2 h with 2 mg ml\(^{-1} \) of collagenase (Type IA, Sigma) in Ca\(^{2+}\)-free Earth’s solution (Table 1). After defolliculation, oocytes at stages V and VI were isolated and washed several times with Ca\(^{2+}\)-free and subsequently standard Earth’s solution (Table 1). The oocytes were then incubated overnight at 18 °C in Earth’s solution before RNA injections.

**cRNA synthesis and injection into oocytes**

*In vitro* transcription was performed in a volume of 25 µl containing the following (mM): 40 Tris-HCl, 50 NaCl, 8 MgCl\(_2\), 2.5 spermidine, 30 dithiotreitol, 0.4 ribonucleotides, 0.3 Capanalog m\(^7\)G(5')ppp(5')G, 1 µg of linearized and proteinase K-treated DNA template, and 10 units of T7 RNA polymerase (Stratagene, La Jolla, CA, USA; for the synthesis of the \( \alpha_2\delta \) and \( \beta \) subunits) or 20 units of SP6 RNA polymerase (Promega, Madison, WI, USA; for the synthesis of the \( \alpha_1A \) subunit) (pH 7.5). The mixture was incubated at 37 °C for 60 min, then resupplemented for an additional 30 min with 10 or 20 units of T7 or SP6 RNA polymerase, respectively. At the end of the reaction, the cRNA template was digested for 10 min with 10 units of DNase I. After phenol/chloroform extraction and ethanol precipitation, the cRNA product was resuspended in 150 mM KCl. Fifty nanolitres of various *in vitro* transcribed RNA mixtures (0.5 µg µl\(^{-1} \) \( \alpha_1\delta \), 0.5 µg µl\(^{-1} \) \( \alpha_2\delta \), and/or 0.5 µg µl\(^{-1} \) \( \beta \)) were injected per oocyte. After injection, the oocytes were maintained for 4-6 days at 16 °C in defined nutrient oocyte medium (Eppig & Dumont, 1976) supplemented with 50 µg ml\(^{-1} \) gentamycin. Only oocytes expressing Ba\(^{2+} \) currents higher than 50 nA were included in the analysis. When present, endogenous Ba\(^{2+} \) currents never exceeded 10 nA (n = 8). These currents were not significantly stimulated by expression of the \( \alpha_2\delta \) subunit (n = 7) and reached, on average, less than 60 nA upon expression of \( \beta \) subunits (n=10) or \( \alpha_2\delta\beta \) subunit combinations (n = 5).
Electrophysiological recordings and data analysis

The experiments were performed at room temperature (20–22 °C) using the Ba²⁺ solutions listed in Table 1. Two-electrode voltage clamp was performed using a Dagan TEV-200 amplifier (Dagan Instruments, Minneapolis, MN, USA). For bath applications of ω-CgTX MVIC, 1 mg ml⁻¹ of cytochrome c was added to the external Ba²⁺ solution to saturate non-specific binding sites. Control experiments demonstrate that cytochrome c has no effect on channel activity at this concentration (data not shown). Microelectrodes were filled with 3 M KCl and had resistances between 0.5 and 2 MΩ. The bath solution was clamped to a reference potential of 0 mV. The membrane current was permanently monitored and we considered only the oocytes for reference potential of 0 mV. The membrane current was analysed using pCLAMP version 5·5 (Axon Instruments). Fits of I–V curves were obtained using an activation curve of a Boltzmann type:

\[ I_{Ba} = \frac{[g(V_t-E)]}{[1 + \exp(-(V_t-V_{1/2})/k)]}, \]

where \( V_t \) represents the test potential, \( g \) the maximum conductance, \( E \) the apparent reversal potential, \( V_{1/2} \) the potential of half-activation, and \( k \) the range of potential responsible for an e-fold change around \( V_{1/2} \). The steady-state inactivation curves were also described by a Boltzmann-type equation:

\[ I_{Ba} = \frac{[1 + \exp((V-V_{1/2})/k)]}{1}, \]

where the current amplitude \( I_{Ba} \) has decreased to a half-amplitude at \( V_t \) with an e-fold change over \( k \) mV. Steady-state inactivation protocols lasted 7 min. For the analysis of the activation and inactivation data, cells that had significant run-down were discarded. Run-down was assessed by the irreversible decrease in current amplitude with time. All values are means ± S.E.M. Our analyses are based on recordings from a total of \( n = 206 \) oocytes.

cDNA clones

cDNA clones used for expression were pSPCBI-2 (α₁A subunit from Mori et al. 1991; gene bank accession number X57477), α₁βδδ, (T. P. Snutch, unpublished sequence; accession number M86621), β₁₁ (Pregnell, Sakamoto, Jay & Campbell, 1991; accession number X61394), β₁ (Hullin et al. 1992; accession number X64297), β₂ (Dr D. R. Witcher, unpublished sequence; accession number M88751) and β₁ (Castellano et al. 1993b; accession number L02315).

RESULTS

The α₁A subunit directs the expression of functional Ca²⁺ channels, the properties of which are not regulated by the α₁βδδ subunit

Expression of α₁ subunits generally results in low current density (Mori et al. 1991; Singer et al. 1991; Stea et al. 1993; Tomlinson, Stea, Bourinet, Charnet, Nargeot & Snutch, 1993). In many cases, the current densities are in fact so low that accurate biophysical descriptions of α₁ Ca²⁺ channels cannot be performed unless β subunits are coexpressed (Mori et al. 1991; Williams et al. 1992b; Ellinor et al. 1993). We increased the expression levels of the α₁A subunit by selecting Xenopus frog donors that yielded oocytes capable of high expression levels. To saturate translation levels, these cells were injected at cRNA concentrations higher than previously reported for this channel (Mori et al. 1991; Sather, Tanabe, Zhang, Mori, Adams & Tsien, 1993). Since high concentrations of permeant ion increase the resolution of Ca²⁺ current, the recordings were also performed with 40 mM external Ba²⁺ as the charge carrier unless otherwise stated. We also estimated the peak level of Ca²⁺ channel expression at 4–6 days after cRNA injection.

Under these optimized conditions, we found that expression of the α₁A subunit results in a very significant inward current. The maximum mean current amplitude was reached at 20 mV with \( I_{Ba} \) of \( -269 \pm 66 \) nA (\( n = 4 \)). This was much larger than the mean endogenous Ca²⁺ current (< 10 nA). This rather high level of expression therefore allowed a more systematic characterization of α₁A Ca²⁺ channels in the absence of the auxiliary subunits.

Table 1. Composition of the solutions used in this study

<table>
<thead>
<tr>
<th>Solution</th>
<th>Na⁺</th>
<th>K⁺</th>
<th>Ca²⁺</th>
<th>Ba²⁺</th>
<th>Mg²⁺</th>
<th>Cl⁻</th>
<th>SO₄²⁻</th>
<th>HCO₃⁻</th>
<th>HEPES</th>
<th>Niflumic acid</th>
</tr>
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<tbody>
<tr>
<td>BS (Ca²⁺ free)</td>
<td>90</td>
<td>4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0·82</td>
<td>89</td>
<td>0·82</td>
<td>2·4</td>
<td>15</td>
</tr>
<tr>
<td>BS (standard)</td>
<td>90</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>0·82</td>
<td>89</td>
<td>0·82</td>
<td>2·4</td>
<td>15</td>
<td>–</td>
</tr>
<tr>
<td>Ba-40</td>
<td>50</td>
<td>2</td>
<td>40</td>
<td>–</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Ba-30</td>
<td>60</td>
<td>2</td>
<td>30</td>
<td>–</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Ba-20</td>
<td>70</td>
<td>2</td>
<td>20</td>
<td>–</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Ba-10</td>
<td>80</td>
<td>2</td>
<td>10</td>
<td>–</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Ba-5</td>
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<td>2</td>
<td>5</td>
<td>–</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Ba-2</td>
<td>90</td>
<td>2</td>
<td>2</td>
<td>–</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>1</td>
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<tr>
<td>Ba-1</td>
<td>93</td>
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<td>1</td>
<td>–</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Ba-0</td>
<td>93</td>
<td>2</td>
<td>0</td>
<td>–</td>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>1</td>
</tr>
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</table>

All concentrations are in mM. The solutions were titrated to pH 7·4 with NaOH (Barth’s solution, BS) or with methanesulphonic acid (barium solutions). EGTA (0·1 mM) was added to Ba-0 to effectively buffer all free divalent cations. Niflumic acid was obtained from Sigma.
Activation of the \( a_{1A} \) Ca\(^{2+} \) channel current started at 
\(-20\) mV, experimentally peaked at \(+20\) mV and reversed at 
\(53\) mV (Fig. 1A). A fit of the data with a modified 
Boltzmann equation estimated the peak current more 
precisely at \(17.5 \pm 0.9\) mV. Ba\(^{2+} \) current activated quickly 
and reached peak amplitude in \(9 \pm 1\) ms \((n = 4)\) at \(20\) mV 
(data not shown). Coexpression of the \( a_{2b} \delta \) subunit with the 
\( a_{1A} \) Ca\(^{2+} \) channel did not significantly modify the mean 
current amplitude or the voltage dependence of activation. 
The maximal inward current of \( a_{1A} \delta a_{2b} \) was of a similar 
amplitude to the \( a_{1A} \) Ca\(^{2+} \) channel with \(-289 \pm 141\) nA at 
\(20\) mV. Also, the current peaked at about \(17.6 \pm 0.6\) mV 
\((n = 16)\), which was similar to the peak for \( a_{1A} \) Ca\(^{2+} \) 
channels.

The voltage dependence of the steady-state inactivation of 
\( a_{1A} \) Ca\(^{2+} \) channels was also determined. The data show that 
the inactivation of this channel occurred at fairly 
depolarized values over a \(40\) mV interval. The mid-point of 
inactivation occurred at \(-46 \pm 1.2\) mV \((n = 4)\) at 
\(20\) mV. In contrast, the ratio of 
the fast inactivating component, were carried by the 
\( a_{1A} \) Ca\(^{2+} \) channel. As for \( a_{1A} \) Ca\(^{2+} \) channels, 
the estimated half-inactivation potential of \( a_{1A}a_{2b} \delta \) 
channels was \(-42 \pm 2\) mV \((n = 4)\).

During sustained membrane depolarization, the current 
amplitude of \( a_{1A} \) Ca\(^{2+} \) channels progressively decayed as a 
result of the inactivation of the channel. We found that the 
time course of this inactivation consisted of two kinetic 
components. At \(20\) mV, for instance, the first inactivating 
component of \( a_{1A} \) Ca\(^{2+} \) channels follows a fast 
time constant, \(\tau_1\), of \(27 \pm 0.7\) ms \((n = 4)\) that constitutes 30\% of 
the total inactivating current. The second inactivating 
component was represented by a slower time constant, \(\tau_2\), 
of \(355 \pm 16\) ms and constituted the remaining 70\% of 
the inactivating current (Fig. 1C). The slow inactivating 
component was the most voltage sensitive. The time 
constant of inactivation increased by a factor of 3-1 
between depolarizing pulses of 0 and 50 mV. In comparison, 
the fast inactivating component increased by 1.4-fold over 
the same range of potentials. In contrast, the ratio of 
current amplitude of these components was only slightly 
voltage dependent (not shown). The fast inactivating 
component, which equaled 32\% of the total inactivating 
current at 0 mV, still represented 25\% of the current at 
\(40\) mV. Again, as for the voltage dependence of activation 
and inactivation, the expression of the \( a_{2b} \delta \) subunit did not 
modify the inactivation kinetics of the \( a_{1A} \) Ca\(^{2+} \) channel.

We ensured that both components, and in particular the 
fast inactivating current, were carried by the \( a_{1A} \) Ca\(^{2+} \) 
channel. Several experimental observations demonstrated 
that this component was indeed not supported by 
endogenous Ca\(^{2+} \) channels normally present in oocytes. (1) 
In the absence of \( \beta \) and \( a_{2b} \delta \) subunits, the endogenous 
current was less than 10 nA in every case \((n=8)\). In 
comparison, the fast component of the inactivating current 
of the \( a_{1A} \) Ca\(^{2+} \) channel reached an estimated current 
amplitude of 90 nA. (2) The ratio of fast and slow current 
amplitudes remained constant from oocyte to oocyte and 
was independent of the total current amplitude recorded.

This would not be expected if slow and fast components 
were carried by different Ca\(^{2+} \) channels. (3) Both 
components were irreversibly blocked by \(\alpha_{-CgTX}\) MVIIIC 
(not shown). This twenty-six amino acid toxin, isolated 
from the marine snail \( Conus magus \) (Hillyard et al. 1992), is 
a potent and complete blocker of the currents carried by the 
\( a_{1A}a_{2b}\delta \) Ca\(^{2+} \) channel (Sather et al. 1993) and is not known 

\section*{\( \beta \) subunits induce four major modifications of the 
biophysical properties of the \( a_{1A} \) channel, two of which 
are modulated by the \( a_{2b} \delta \) subunit}

As previously done for the \( a_{2b} \delta \) subunit, we monitored the 
effects of \( \beta \) subunits on the amplitude, voltage dependence 
and kinetics of \( a_{1A} \) Ca\(^{2+} \) channel currents. We found that \( \beta \) 
subunits modified all of these properties. We also 
demonstrated that the \( a_{2b} \delta \) subunit regulated two of the 
four major modifications induced by \( \beta \) subunits.

Expression of the \( \beta_{1b} \) subunit resulted in a hyperpolarizing 
shift in the voltage dependence of \( a_{1A} \) Ca\(^{2+} \) channel 
activation (Fig. 2Aa). For instance, the estimated peak 
current of \( a_{1A}a_{2b}\beta_{1b} \) occurred at \(-0.5 \pm 1.4\) mV \((n = 11)\) 
which, when compared with \( a_{1A} \) Ca\(^{2+} \) channels, 
corresponded to a \(17 \pm 1\) mV shift towards hyperpolarized 
potentials. Overall, the shift in peak current was due to a 
lower threshold potential \((-30\) instead of \(-20\) mV) and a 
steeper voltage dependence \((k=3.8 \pm 0.3\) instead of 
\(7.6 \pm 0.3\) mV for the \( a_{1A} \) Ca\(^{2+} \) channels). Expression of 
both \( a_{2b} \delta \) and \( \beta_{1b} \) subunits did not modify the amplitude of 
this hyperpolarizing shift. On average, the shift induced by 
\( \beta_{1b} \), equaled \(15 \pm 3\) mV in the presence of \( a_{2b} \delta \) 
\((n = 13, \text{Fig. 2Ab})\). Interestingly, all \( \beta \) subunits induced 
hypermolarizing shifts in the current—voltage relationship 
of \( a_{1A}a_{2b}\delta\) with, however, some minor quantitative differences 
(Fig.2Ab). The mean shifts in peak values were 
\(7.5 \pm 2.3\) mV \((a_{1A}a_{2b}\delta\beta_{2a}, n = 8)\), \(9.5 \pm 2.2\) mV 
\((a_{1A}a_{2b}\delta\beta_{3}, n = 8)\) and \(17.5 \pm 2\) mV 
\((a_{1A}a_{2b}\delta\beta_{4}, n = 9)\). We classified the \( \beta \) subunits according to their abilities to shift the 
voltage dependence of activation with \(\beta_{1} = \beta_{2a} > \beta_{1b} > \beta_{2a} \).

Expression of \( \beta \) subunits also resulted in a considerable 
stimulation of the peak current of the \( a_{1A} \) Ca\(^{2+} \) channel.
The maximum current amplitude of α1β1b Ca2+ channel was \(-1955 \pm 230\) nA \((n = 11)\) at 0 mV (Fig. 2Ba). This corresponds to a mean β1b stimulation of \((7.3 \pm 1.2)\)-fold \((n = 15)\). Although the α2δ subunit did not itself affect the current amplitude of α1 Ca2+ channels (mean current amplitudes, \(-269\) and \(-289\) nA for α1 and α1α2δb Ca2+ channels, respectively), expression of α2δ did increase the β1b stimulation further by a factor of 2.5 and brought the

Figure 1. Properties of α1A current and absence of regulation by the α2δ subunit

A, voltage dependence of activation and effect of α2δ. Aa, trace examples of Ba2+ current activation at -10, 0 and 20 mV test pulses. Holding potential is -90 mV. Ab, average current-voltage relationship for α1 (n = 4, ○, continuous line) and α1α2δb currents (n = 16, O, dashed line). Test pulses were delivered by 10 mV increments. Error bars are S.E.M. Fits of the average activation data yield \(g = 9.9\) or \(8.8\) µS, \(E = 52.8\) or \(56.6\) mV, \(V_\text{50} = 7.4\) or \(5.5\) mV and \(k = 7.7\) or \(6.9\) mV without or with α2δ coexpression, respectively. B, voltage dependence of inactivation and effect of α2δb. Ba, trace examples of α1A Ba2+ current at holding potentials of -80, -60 and -40 mV to show the reduction in current amplitude by steady-state inactivation. Bb, average steady-state inactivation curve for α1A (○, continuous line) and α1α2δb currents (O, dashed line). Peak current amplitudes were normalized to the maximum current amplitude reached during the protocol. Normalized current amplitudes were then averaged and plotted as a function of holding potential. Fits to the average data yield \(V_\text{50} = -46\) or \(-42\) mV and \(k = 8.3\) or \(6.1\) mV without or with α2δ coexpression, respectively. Data are the means ± S.E.M. of \(n = 5\) (-α2δ) and \(n = 4\) (+α2δ) oocytes. C, kinetics of inactivation and effect of α2δb. Ca, trace example at 30 mV test potential and decomposition of the inactivating current into two components (dashed line).Cb, plot of the two time constants \(\tau_1\) and \(\tau_2\) as a function of test potential for α1A (filled symbols, continuous lines) or for α1α2δb inactivating currents (open symbols, dashed lines).
final mean current to \(-5272 \pm 762\) nA (n = 13). This \(\alpha_2 \delta_\beta\) potentiation provided a total stimulation of \((18.2 \pm 6.6)\)-fold by \(\beta_1\). The stimulation in current amplitude and the potentiation in stimulation by the \(\alpha_2 \delta_\beta\) subunit could be observed upon expression of any of the four \(\beta\) subunits. However, the \(\beta\) subunits differed in their ability to stimulate the current. The mean stimulations were \(5.38 \pm 2.1\) (\(\beta_2\)), \(5.48 \pm 2.1\) (\(\beta_3\)) and \(19.3 \pm 7.8\) (\(\beta_4\)). We therefore also classified the \(\beta\) subunits according to their stimulation efficiencies with \(\beta_1 = \beta_1 > \beta_3 = \beta_2\). As a result of the shift in voltage dependence of activation, the stimulation of current amplitude induced by \(\beta\) subunits was larger at hyperpolarized potentials (Fig. 2B). For instance, expression of both \(\beta_1\) and \(\alpha_2 \delta_\beta\) resulted in an \((87 \pm 42)\)-fold stimulation of \(\alpha_1 \delta\) current amplitude when measured at 0 mV. In contrast, when the current was measured at 30 mV, this stimulation was on average six times smaller with a \((14 \pm 5)\)-fold enhancement in amplitude. A lower but

Figure 2. Regulation of \(\alpha_1 \delta\) currents by four \(\beta\) subunits and modulation by \(\alpha_2 \delta_\beta\) subunit

A, \(\beta_1\) subunits shift the current–voltage relationship towards hyperpolarized potentials. \(\alpha\), normalized mean current–voltage relationship for \(\alpha_1 \delta\) (dashed line, same data as Fig. 1A) and \(\alpha_1 \beta\) (n = 11, solid line). Fit of the activation data yields \(g = 39.1 \mu S, E = 56\) mV, \(V_0 = -9.9\) mV and \(k = 4.2\) mV for \(\alpha_1 \beta\) currents. The peak current amplitudes and the curve generated by the fit were normalized to the maximal current amplitude reached during the protocol of activation. \(\beta\), mean hyperpolarizing shifts in current–voltage relationship by \(\alpha_2 \delta_\beta\) (same data as in Fig. 1A) and by various \(\alpha_2 \delta_\beta \beta\) subunit combinations. Fits to the mean activation data yield \(g = 105.6 \ (\alpha_1 \beta_1 \beta_1 \beta_1, n = 13), 35.1 \ (\alpha_1 \alpha_2 \beta_1 \beta_1, n = 8), 34.8 \ (\alpha_1 \alpha_2 \beta_1 \beta_1, n = 8) \) and 106 \(\mu S\) \(\alpha_1 \alpha_2 \beta_1 \beta_1\) and \(\alpha_1 \alpha_2 \beta_1 \beta_1\) and 59.4 mV \(\alpha_1 \alpha_2 \beta_1 \beta_1\) and \(\alpha_1 \alpha_2 \beta_1 \beta_1\) and \(-9.8\) mV \(\alpha_1 \alpha_2 \beta_1 \beta_1\) and \(-9.8\) mV \(\alpha_1 \alpha_2 \beta_1 \beta_1\) and \(k = 5.3\) \(\alpha_1 \alpha_2 \beta_1 \beta_1, 5.1\) \(\alpha_1 \alpha_2 \beta_1 \beta_1\) and \(3.8\) mV \(\alpha_1 \alpha_2 \beta_1 \beta_1\). B, the current amplitude stimulation by ancillary subunits is more pronounced at hyperpolarized potentials. \(\beta\), maximum current amplitude of the \(\alpha_1 \delta\) Ca\(^{2+}\) channel in various subunit combinations. \(\beta\), \(\alpha_1 \delta\) current amplitude stimulation provided by the expression of \(\alpha_1 \delta\) subunit with (■) or without (●) the coexpression of \(\alpha_2 \delta_\beta\) subunit as a function of depolarization. Number of oocytes studied are \(n = 4\) \(\alpha_1 \delta\), \(n = 16\) \(\alpha_1 \alpha_2 \beta_1 \beta_1\), \(n = 11\) \(\alpha_1 \alpha_2 \beta_1 \beta_1\) and \(n = 13\) \(\alpha_1 \alpha_2 \beta_1 \beta_1\).
similar reduction in stimulation efficiency was also observed with stronger depolarizations in the absence of the α2δ subunit.

The voltage dependence of inactivation of the α1A Ca2+ channel was also shifted towards more negative potentials by the association of β subunits. At a holding potential of –55 mV more than 50% of the α1Aβ1b channels were inactivated, whereas only a small fraction of α1A channels were inactivated at a similar potential (compare Fig. 3Aa with Fig. 1B).

α1Aβ1b Ca2+ channels had a mean mid-point of inactivation of –61·2 ± 1·2 mV (n = 9) which, compared with –46 mV for the α1A Ca2+ channel, corresponded to a hyperpolarizing shift of 15 ± 2 mV (Fig. 3A). Expression of the α2δb subunit did not modify the direction and amplitude of the β-induced shift in inactivation (Fig. 3B). Overall, the expression of β1b subunit resulted in a 17 ± 4 mV shift from a mid-point of inactivation of –42 (α1Aα2δb) to –59·5 ± 2·8 mV (n = 13, α1Aα2δbβ1b). These shifts in steady-state inactivation were observed for all four β subunits with mid-points of inactivation of –56·6 ± 2 mV for β2a (n = 7), –58·6 ± 1·9 mV for β3 (n = 6) and –65·5 ± 1·3 mV for β1 (n = 7). Again, the β subunits could be classified according to their ability to shift the voltage dependence of inactivation with β3 > β1b = β3 = β2a.

The inactivation kinetics of the α1A Ca2+ channel were also modified by the β subunits or by α2δb–β subunit combinations. For all four β subunits studied, the kinetics of α1A Ca2+ channel currents were converted from a biexponential to a monoexponential decay (Fig. 4). In marked contrast with the biexponential decay of the α1A subunit, α1Aβ1b Ca2+ channels inactivated along a single

Figure 3. β subunits regulate the voltage dependence of inactivation
Aa, trace examples of α1Aβ1b Ca2+ channel inactivation at –80, –55 and –40 mV holding potentials. Test potential is 10 mV. Ab, normalized mean steady-state inactivation curve for α1A currents (dashed line, same data as in Fig. 1B) and α1Aβ1b currents (continuous line). Fit to the mean α1Aβ1b data yields $V_{1/2} = –61.3$ mV and $k = 9$ mV for α1Aβ1b currents (n = 9). B, all β subunits induced similar shifts in the voltage dependence of inactivation. The amplitudes of these shifts were not modified by the α2δ subunit.

Baa, trace examples of α1Aα2δbβ1b current at holding potentials of –90, –55 and –40 mV to show steady-state inactivation. Test potential was 10 mV. Baba, normalized mean steady-state inactivation curve for currents (dashed line, same data as in Fig. 1B) and α1Aα2δb coexpressed with various β subunits (different symbols, continuous lines). Fitting parameters are $V_{1/2} = –58.9$ (β1b, n = 9), –57 (β2a, n = 7), –58·6 (β3, n = 6) and –65·6 mV (β4, n = 7), and $k = 10·5$ (β1b), 10·3 (β2a), 6·7 (β3) and 7 mV (β4) for mean data. ○, +β1b; △, +β2a; ●, +β3; □, +β4.
inactivating component with a time constant of 490 ± 27 ms at 10 mV (n = 11). At the same potential, the inactivation of the α1A Ca2+ channel was characterized by two time constants of 31 ± 1 and 498 ± 39 ms (n = 4). Although the inactivation kinetics of the α1A Ca2+ channel were not regulated by α2δ in the absence of β subunits, we found that expression of α2δ influenced the rate by which the current inactivated in the presence of β subunits. At 10 mV, the decay of α1Aα2δβ1b Ca2+ channel current occurred with a time constant, τ, of 211 ± 19 ms (n = 14). This is therefore 2·3 times faster than the decay of α1Aβ1b Ca2+ channels at the same potential. All four β subunits were equally able to induce a monoexponential decay of either α1A or α1Aα2δ Ca2+ channel currents. However the various β subunits induced different time constants of inactivation. For instance, we measured the following τ values for each β subunit at 30 mV: 214 ± 18 ms (n = 14) for α1Aα2δβ1b, 533 ± 28 ms (n = 8) for α1Aα2δβ2a, 127 ± 6 ms (n = 8) for α1Aα2δβ3 and 243 ± 24 ms (n = 8) for α1Aα2δβ4 Ca2+ channels. The rank order of β subunits with respect to the speed of inactivation was therefore β3 > β1b = β4 > β2a. The time constants of all these channels were sensitive to the extent of depolarization. The time constants were minimal for potentials superior to 0 mV that maximally activated the channels.

Ancillary subunits affect the rate of run-down but not the toxin sensitivity of the α1A Ca2+ channel

We have examined the effects of α2δ and β subunit expression on the potency of ω-CgTX MVIIIC to inhibit Ca2+ channel currents by studying the blocking time course of the toxin (Fig. 5). The recordings were performed in 2 mM extracellular Ba2+ since the potency of ω-CgTX MVIIIC is inhibited by increases in divalent cation concentration (not shown). We found that the blocking time course of 2 µM ω-CgTX MVIIIC apparently differed slightly depending on the subunit composition. In all cases, the block of Ba2+ currents at this toxin concentration would have been completed if followed out to their steady-state values. Control experiments showed that the differences in blocking time course were mostly due to differences in rate of run-down of the various Ca2+ channel complexes. The block of α1Aα2δβ1b Ca2+ channel current was fast with a 97·8 ± 0·7% block within 10 min of the toxin application. This speed of action was partly due to the increased sensitivity of the current to run-down. In contrast, α1Aα2δβ2a Ca2+ channel currents, which are less efficiently blocked by ω-CgTX MVIIIC (72 ± 7% block in 10 min), also failed to demonstrate obvious signs of run-down. However, slight

Figure 4. β subunits differentially regulate the inactivation kinetics of α1A currents

A, mean time constants of α1A current inactivation upon coexpression of β1b, subunit with (●, continuous line, n = 14) or without α2δ (○, dashed line, n = 11) as a function of test potential. Inset, example of α1Aα2δβ1b current at 30 mV and monoexponential fit to the inactivating current (thick line). B, C and D, same as in A for α1Aα2δβ2a (▲, n = 8), α1Aα2δβ3 (◇, n = 8) and α1Aα2δβ4 (■, n = 9), respectively.
contributions of both α2δb and β subunits to the toxin sensitivity cannot be ruled out since run-down did not account for all the observed changes in the blocking effect of the toxin. For instance, a 10 min application of ω-CgTX MVIIC resulted in a 98% block of a1Aα2δbβ1b current and a lower 81.3 ± 3.2% block of a1Aβ1b current, despite respective run-down values of 39.6 ± 9.8 and 57.3 ± 4.1% suggesting a facilitatory action of α2δb on the toxin block. Overall, our data suggest that inhibitory mechanisms, responsible for the decrease of channel activity during run-down, may be acting on β subunits. The fact that β subunits are required for high functional expression of voltage-dependent Ca2+ channels is consistent with this observation. The data also demonstrate that a1A is the only receptor for ω-GgTX MVIIC and that β and α2δb subunits may slightly regulate the blocking properties of this toxin at 2 µM in accordance with the important changes in the biophysical properties of the current.

The a1A Ca2+ channel can be activated by weak depolarizations if the recordings are performed with low divalent cation concentrations

It has recently been suggested that the a1B subunit is a low-voltage-activated channel (Soong et al. 1993). However, the voltage dependence of this Ca2+ channel has been recorded with low divalent cation concentrations as a source of permeant ion. By screening negative charges at the surface of the plasma membrane, changes in divalent cation concentrations are known to affect the transmembrane potential sensed by a Ca2+ channel. Therefore, accurate comparison of the voltage dependence of various cloned a1 subunits has been made difficult by the experimental use of different extracellular Ba2+ concentrations. In order to normalize for the experimental conditions, we examined to what extent the peak of the current–voltage relationship could be modified by changes in divalent cation concentrations. Figure 6 reports the normalized peak current of the a1Aα2δbβ1b Ca2+ channel in various Ba2+ concentrations. We found that very significant current amplitudes could be recorded despite large reductions in the extracellular Ba2+ concentration. The current amplitude was maximal at 40 mM Ba2+. Reductions in current amplitude occurred at concentrations below 30 mM, and the estimated half-current amplitude occurred around 3 mM Ba2+.

As expected, the reduction in the extracellular Ba2+ concentration resulted in hyperpolarizing shifts of the current–voltage relationship of activation (Fig. 7). At 2 mM Ba2+, for instance, the peak current was reached at –23 mV which can be directly compared with a peak at

![Figure 5](image-url)
6 mV in 40 mM Ba²⁺. Thus, reducing the permeant cation to near physiological concentrations was enough to provoke a hyperpolarizing shift of 29 mV. We estimated that at 4 mM Ba²⁺, the peak current occurs at a potential of −14·2 mV which is comparable to a potential of −10 mV for α₁E currents under similar experimental conditions (Soong et al. 1993).

We conclude, therefore, that α₁A cannot be distinguished by its voltage dependence from the α₁E subunit and that, contrary to an initial report (Mori et al. 1991), the α₁A subunit is also more likely to behave as a low-voltage-activated channel in a more physiological divalent cation concentration.

**DISCUSSION**

The α₁A subunit alone is sufficient to direct the expression of functional Ca²⁺ channels

The low level of expression of the α₁A Ca²⁺ channel in the absence of β subunits has been a serious limitation to its functional characterization (Mori et al. 1991). Also, because so little expression could be observed in the absence of auxiliary subunits, their effects on the functional properties of the α₁A subunit have not been qualitatively and quantitatively well assessed. Our data demonstrate for the first time that α₁A Ca²⁺ channels can give rise to significant levels of expression on their own. The α₁A subunit carries the main properties of this neuronal Ca²⁺ channel, such as ionic permeability, gating, voltage sensitivity and pharmacology. Probably the most striking observation is that RNA coding for this subunit causes the appearance of Ba²⁺ current that displays not one, but two kinetic components. Since these two components have similar pharmacological and biophysical properties, we can rule out the possibility that they both originate from two distinct Ca²⁺ channel conductances. Instead, the data suggest that a single α₁ molecule can display a complex macroscopic behaviour. It also remains possible that differential processing and modulation of a fraction of these molecules favours the appearance of a population of channels with different functional properties. In the study presented here, a better description of the properties of α₁A Ca²⁺ channels in the absence of auxiliary subunits has been a key determinant in the understanding of the regulatory contribution of these auxiliary subunits themselves. Our findings illustrate that two of the ancillary subunits (α₂δ and β), which are the most likely to associate with α₁A directly interact with the channel and regulate the current activities in specific ways. We analysed the differences in current properties introduced by β, α₂δ or a combination of both subunit types and used these differences as an index of the functional contribution of each of these auxiliary subunits.

**Auxiliary β subunits of voltage-gated Ca²⁺ channels regulate the properties of the α₁A subunit: implications for the underlying molecular mechanisms.**

Of the two subunits that are the most likely to assemble with the α₁A subunit in vivo, β subunits have the most dramatic regulatory properties. We report four primary functional modifications that are well conserved among β subunits with, however, some minor quantitative differences. The most obvious regulation by β subunits is the dramatic increase in α₁A current amplitude. The mechanism underlying this stimulation is best understood for the α₁C Ca²⁺ channel for which comparable data are available. The

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**Figure 6. Relation between Ba²⁺ concentration and current amplitude**

A, trace examples of α₁Aβδβ1b currents in various Ba²⁺ concentrations. Traces represent the peak of current–voltage relationship at each Ba²⁺ concentration. B, mean current increase as a function of the extracellular Ba²⁺ concentration ([Ba²⁺]₀). Current amplitudes were normalized to the peak current obtained at 40 mM Ba²⁺. The data represent the mean of n = 4 experiments (± S.E.M.). The data are fitted with a hyperbolic function $I_{\text{Ba}} = I_{\text{max}} [\text{Ba}^{2+}] / ([\text{Ba}^{2+}]_0 + [\text{Ba}^{2+}])$, where $I_{\text{Ba}}$ is the mean normalized current amplitude, $I_{\text{max}}$ maximum normalized current ($I_{\text{max}} = 1·025$), $[\text{Ba}^{2+}]_0$ the external Ba²⁺ concentration and $[\text{Ba}^{2+}]$, the Ba²⁺ concentration responsible for half-maximal current amplitude ($[\text{Ba}^{2+}]_0 = 2·9$ mM).
cardiac \( a_{1c} \) subunit is also the only channel for which a direct comparison between current density and dihydropyridine binding sites was reported. It was demonstrated that the increase in current triggered by \( \beta \) subunits can be correlated with an increase in the number of dihydropyridine binding sites (Perez-Reyes et al. 1992; Castellano et al. 1993b). Since dihydropyridines bind on the \( a_{1c} \) subunit, the increase both in the current and in the number of dihydropyridine binding sites would suggest that \( \beta \) subunits act by increasing the number of channels present at the plasma membrane. However, gating charge measurements (Neely, Wei, Olcese, Birnbaumer & Stefani, 1993) and immunoblot analysis (Nishimura, Takeshima, Hofmann, Flockerzi & Imoto, 1993) demonstrated instead that the number of \( a_{1c} \) subunits expressed at the plasma membrane was in fact not altered by the \( \beta \) subunits. To reconcile what appear to be contradictory results, it was hypothesized that \( \beta \) subunits act primarily by inducing important conformational changes in the pore-forming subunit. These changes in \( a_{1} \) subunit conformation would then in turn be responsible for an increase not only in the opening probability of the channel, but also in the accessibility of the drug to its site. Although no binding data are available for the \( a_{1a} \) subunit, our data suggest that

![Figure 7](image-url)

**Figure 7. The current–voltage relationship of \( a_{1a}a_{2\delta}b_{1\beta} \) currents is a function of the extracellular divalent cation concentration**

A, trace examples of \( a_{1a}a_{2\delta}b_{1\beta} \) currents with 30 and 5 mM Ba\(^{2+}\) and 0 mM Ba\(^{2+}\), 93 mM Na\(^{+}\) in the extracellular solution. In 0 mM Ba\(^{2+}\), extracellular Na\(^{+}\) and intracellular K\(^{+}\) were the charge carriers. With decreasing Ba\(^{2+}\) concentrations, the osmolarity of the solution was kept constant by adjusting the NaOH concentration (Table 1). B, average normalized current–voltage relationship for oocytes injected with \( a_{1a}a_{2\delta}b_{1\beta} \) 30 (\( n = 6 \)), 5 (\( n = 2 \)) and 0 mM (\( n = 3, 93 \) mM Na\(^{+}\)) extracellular Ba\(^{2+}\) (continuous lines). The dashed line represents the current–voltage relation in 40 mM Ba\(^{2+}\). Fit of the activation data yields \( g = 94, 68.6 \) and 130 \( \mu \)S, \( E = 55, 50 \) and 9 mV, \( V_0 = -7, -27 \) and -35 mV, and \( k = 5.3, 5.7 \) and 5.1 mV for 30, 5 and 0 mM Ba\(^{2+}\), respectively. Note the hyperpolarizing shift in reversal potential when Na\(^{+}\) and K\(^{+}\) are the charge carriers. C, mean potential of peak current as a function of the extracellular Ba\(^{2+}\) concentration (\( n = 37 \) oocytes studied in total). The data were best described with a hyperbolic function: \( V_{\text{peak}} = \frac{(V_{\text{max}} \, [\text{Ba}^{2+}])}{([\text{Ba}^{2+}]) + V_0} \) where \( V_{\text{max}} \) of 38.7 mV was the maximum shift in peak potential induced by Ba\(^{2+}\), \( [\text{Ba}^{2+}] \) of 11.1 mM was the Ba\(^{2+}\) concentration that induced a half-maximal shift in peak potential, and \( V_0 = -24.5 \) mV was the peak potential of \( a_{1a}a_{2\delta}b_{1\beta} \) in the absence of Ba\(^{2+}\). Any point above this curve has a lower (−) voltage sensitivity than the \( a_{1a} \) channel. The open circle represents the potential of the peak current of the \( a_{1b} \) subunit at 4 mM Ba\(^{2+}\) (data from Soong et al. 1993).
the blocking potency, and therefore probably the affinity of ω-CgTX MVIIC for its binding site on this structurally related subunit, is only slightly modified by the expression of ancillary subunits. These findings suggest that the binding site to this toxin is not greatly affected by the β-induced conformational changes in the α1A subunit. These observations are also consistent with the fact that ω-CgTX MVIIC binds to a different region on the α1 molecule from the dihydropyridines.

All β subunits trigger significant changes in the voltage dependence of the α1A Ca2+ channel. The voltage dependence of both activation and inactivation are affected. The amplitude of the changes observed were either very similar among β subunits (inactivation) or differed slightly (activation). Since for all β subunits these shifts were directed towards more negative potentials, the molecular contribution of these subunits should be very similar. The functional equivalence between these subunits is, however, not complete if one considers the regulation of the inactivation kinetics. The β2a subunit induces a very slow inactivation of the current whereas the βC subunit induces the fastest inactivation kinetics. Interestingly, although the various β subunits differ in their regulation of the time constant of inactivation, there is not a strong difference in the amplitude of the hyperpolarizing shift of the voltage-dependent inactivation process. These observations strongly suggest that the control of the inactivation kinetics and of the voltage dependence of inactivation of Ca2+ channels occurs by different mechanisms and molecular interactions.

Which β subunit is normally associated with the α1A Ca2+ channel in neurons?

Because all β subunits seem to be capable of normalizing the properties of α1A, it is difficult to infer from expression experiments which β subunit associates with this neuronal Ca2+ channel in situ. Our observations suggest, however, that the β1 subunit has the most prominent effects on the properties of this channel. Firstly, our findings show that for most regulation, β subunits differ in the amplitude of their effects such that generally β4 > β1b > β3 > β2a (Table 2). Since expression of the β1 subunit induces the greatest shift in current amplitude and also the most significant shift in voltage dependence of inactivation, β1 is the most likely β subunit associated with α1A. Secondly, both α1A and β1 subunits have been found to be highly expressed in the cerebellum, as assessed by ‘Northern blot’ analysis (Starr, Prystay & Snutch, 1991; Castellano et al. 1993b) increasing the likelihood of their association in vivo.

Although β subunits contribute to a larger calcium influx, they also impart to the channel an increased sensitivity to cell inhibitory regulation

Our data show that the increase in current amplitude and the hyperpolarizing shift in activation would both account for an increase in calcium influx. However, since these two primary regulations do not occur independently of each other, the stimulation of Ca2+ current by β subunits is even more dramatic. This is particularly true at hyperpolarized potentials where β subunits would dramatically increase the amount of Ca2+ influx during an action potential. A consequence of the stimulatory function of β subunits is also an increased need for inhibitory cellular mechanisms capable of controlling the Ca2+ influx into the cell. Remarkably, cell inhibitory mechanisms can be favoured by two additional modes of β subunit regulation. For instance, the hyperpolarizing shift in the voltage dependence of steady-state inactivation should clearly increase the sensitivity of the channel to inactivation by cell depolarization. Such a mechanism would represent a simple and efficient mechanism of calcium entry limitation. Also, by a process that is still not well understood, we found that β subunits increase the sensitivity of Ca2+ channels to rundown. Of course, rundown represents a more definitive cell inhibitory mechanism of the channel activity.

Association of β subunits with the α1A Ca2+ channel is essential to uncover the functional importance of the αδ subunit

None of the four biophysical properties of the α1A Ca2+ channel studied were modified by αδ in the absence of β subunits. Although RNA coding for an endogenous αδ subunit has been detected in oocytes (Singer-Lahat, Lotan, Itagaki, Schwartz & Dascal, 1992), it is unlikely that the αδ protein is present in sufficient amounts to associate with the α1A subunit. Although the presence of an endogenous αδ could have explained the observed lack of regulation of the α1A channel by the exogenous αδ subunit, this possibility is remote because (1) native oocytes show only low levels of endogenous Ca2+ channel activity and (2) injection of exogenous αδ cRNA enhances the expression of exogenous α1C cRNA (Singer et al. 1991). Also, we found that the exogenous αδb subunit modulates the β-induced Ca2+ current stimulation and inactivation kinetics changes. Interaction sites of αδ subunits have not been mapped yet in Ca2+ channels. However, despite the fact that the functional contributions of αδ in the presence of β subunits (current amplitude stimulation and change in inactivation kinetics) are of the same nature as those induced by β subunits alone, direct αδ–β interactions are unlikely to explain the data. It has been shown that β subunits are entirely cytoplasmic and the membrane topology of αδ predicts that despite some hydrophobic segments this subunit is mostly extracellular (Jay, Sharp, Kahl, Vedvick, Harpold & Campbell, 1991). Instead, it seems more likely that the αδ subunit is unable to interact with α1A unless α1 – β interactions have first occurred (Pragnell et al. 1994). This suggests that α1–β subunit interactions could favour the occurrence of α1–αδ interactions via the same conformational changes that are required for enhanced current amplitude (Neely et al. 1993). In the presence of β
Table 2. Comparison of the functional effects of $\alpha_2\delta_b$ and $\beta$ subunits on the properties of two cloned $\alpha_1$ subunits

<table>
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<td>Regulation of drug potency by $\beta$ or $\alpha_2\delta$</td>
<td>Effect of $\omega$-CgTX MVIC modified by $\alpha_2\delta_b$ or $\beta_{1b}$</td>
<td>Bay K 8644 stimulation or nifedipine inhibition not affected by $\beta_3, \beta_4$ or $\alpha_2\delta^{(b, c, f)}$</td>
</tr>
<tr>
<td>Increase in number of drug binding sites by $\beta$ or $\alpha_2\delta$</td>
<td>n.a.</td>
<td>Increased number of dihydro--pyridine binding sites by $\beta_2$ and $\beta_4^{(a, b)}$</td>
</tr>
<tr>
<td>Change in drug affinity by $\beta$ or $\alpha_2\delta$</td>
<td>n.a.</td>
<td>Not modified by $\beta$ subunits $(\beta_2)^{(a)}$</td>
</tr>
</tbody>
</table>

$a$ Perez-Beyes et al. 1992; $b$ Castellano et al. 1993b; $c$ Tomlinson et al. 1993; $d$ Wei et al. 1991; $e$ Mikami et al. 1989; $f$ Castellano et al. 1993a; n.a., not applicable.
subunits, these α-αβδ interactions could in turn either directly regulate the α1A properties or indirectly influence the regulatory contribution of β subunits.

**Do auxiliary subunits regulate the same set of properties in all α1 subunits?**

We have underlined the functional similarities of all β subunits by demonstrating that they were able to shift the voltage dependence of activation and inactivation, to stimulate the current amplitude and to induce a monoexponential decay of the inactivation kinetics. We have also demonstrated that the increase in current and the changes in inactivation kinetics are also modulated by the α2δ subunit. To get some insights into the possible molecular mechanisms underlying subunit regulation in Ca2+ channels, we have examined the type and extent of regulation previously observed with structurally unrelated α1 subunits. A very striking observation is that the subunit regulation processes observed with the α1A Ca2+ channel have also been described for different α1 Ca2+ channels. The stimulation in current amplitude by β subunits and the potentiation by the α2δ subunit have been observed for all α1 subunits (Mori et al. 1991; Hullin et al. 1992; Williams et al. 1992a,b; Ellinor et al. 1993) with the exception of the α1E subunit (Soong et al. 1993). Table 2 summarizes and compares both α2δ and β subunit regulation of two functionally and structurally different α1 subunits. As for the α1A Ca2+ channel, β subunits also increase the current amplitude, shift the voltage dependence of activation towards hyperpolarized potentials and modify the inactivation kinetics of the α1C Ca2+ channel. It is interesting to observe that the order in which β subunits are classified, with respect to the rate constant of inactivation, is identical for α1A, α1C (Table 2) and Doe-1 Ca2+ channels (Ellinor et al. 1993). Also similar to events in the α1A channel, β subunits shift the steady-state inactivation of α1B Ca2+ channels 20 mV towards more negative potentials (Stea et al. 1993). Altogether, these results suggest that the regulation of the functional properties of various α1 Ca2+ channels by β subunits occurs via a set of conserved interaction sites. These α1–β interaction sites should also have identical functional consequences. One such interaction site has recently been reported with the identification of an amino acid motif that is present on the cytoplasmic linker between repeat I and II of every α1 subunits. This repeat is essential to the current stimulation, the changes in voltage dependence and the modulation of inactivation kinetics (Pragnell et al. 1994). There is evidence to believe that, besides this primary interaction, additional sites may exist between these two subunits. The fact that β subunits do not regulate all α1 subunits in a similar manner is consistent with this interpretation, β subunits do not shift the voltage dependence of steady-state inactivation of the α1C subunit, nor affect the voltage dependence of activation of α1B Ca2+ channels (Stea et al. 1993). Neither do they affect the activation kinetics of the α1A channel. These observations appear to suggest that the mere existence of secondary α1–β interaction sites is determined by the primary sequence of each α1 subunit. The near complete functional equivalence of β subunits stands in contradiction with the apparent specificity of α1–β interactions that has clearly been illustrated in two purified voltage-gated Ca2+ channels. Indeed, it remains to be understood why the α1B subunit of the N-type Ca2+ channel or the α1S subunit of the skeletal muscle dihydropyridine receptor, associate with the β3 (Witcher et al. 1993) and the β1a (Ruth et al. 1989) subunits, respectively.

**Not all functional properties of native Ca2+ channels can be compared with properties of cloned Ca2+ channel subunits**

With the increasing number of Ca2+ channel α1 subunits being cloned, there is a natural propensity to correlate functionally these molecules to their native counterparts. Expression of the α1 Ca2+ channel with various auxiliary subunits demonstrates the risks of interpretation involved in such an exercise. It is generally assumed that native Ca2+ channels are a complex of four subunits as illustrated by the subunit identification of the purified skeletal muscle L-type and neuronal N-type Ca2+ channels (Takahashi et al. 1987; Witcher et al. 1993). However, data about the subunit composition of other native Ca2+ channels are still lacking. Also, it cannot be ruled out that a significant fraction of each channel subtype may also exist as incomplete protein complexes in the plasma membrane (i.e. α1, α1α2δ, α1β and α1α2δβ Ca2+ channels). Although this question will ultimately be resolved by a better knowledge of the cellular processing of Ca2+ channels, it raises the intriguing possibility that part of the biophysical variability of each native Ca2+ channel may also arise from a variability in their subunit composition. Besides differences in pharmacological properties, gating, permeability and voltage dependence of activation and inactivation have all been used as critical determinants for the biophysical classification of various Ca2+ channel subtypes (Bean, 1989). However, the expression data show, for example, that multiple inactivating components can be detected in the kinetics of α1α or α1α2δ Ca2+ channels despite the clear molecular homogeneity of these channel populations. These results demonstrate that the kinetics of inactivation cannot be used as a reliable criterion of Ca2+ channel identification. This is also further illustrated by the fact that the number and rate constants of the inactivating components are under the strict control of the associating subunits. Not only do the β subunits change the number of inactivating components from two to one, but they also differentially affect the rate constant of inactivation of the α1A Ca2+ channel. Also, since different β subunits affect the voltage dependence of both activation and inactivation processes to various extents, these parameters do not represent ideal tools of channel identification and classification. Finally, much confusion has arisen concerning the voltage dependence of various cloned α1 subunits. Our data demonstrate that despite the fact
that α1A (Mori et al. 1991) and α1E (Soong et al. 1993) subunits were initially classified as high- and low-voltage-activated Ca\(^{2+}\) channels, respectively, there are in reality no differences in the voltage sensitivity of these two channels. Overall expression data illustrate the risks involved in discriminating native Ca\(^{2+}\) channels by their biophysical properties. The findings also suggest that the functional comparison between the cloned α1A subunit and the native P-type Ca\(^{2+}\) channel (Sather et al. 1993) should await the data on the purification and the subunit identification of this neuronal Ca\(^{2+}\) channel.


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