Regulation of Maximal Open Probability Is a Separable Function of Cavβ Subunit in L-type Ca2+ Channel, Dependent on NH2 Terminus of α1C (Cav1.2α)

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β subunits (Cavβ) increase macroscopic currents of voltage-dependent Ca2+ channels (VDCC) by increasing surface expression and modulating their gating, causing a leftward shift in conductance–voltage (G-V) curve and increasing the maximal open probability, Po,max. In L-type Cav1.2 channels, the Cavβ-induced increase in macroscopic current crucially depends on the initial segment of the cytosolic NH2 terminus (NT) of the Cav1.2α (α1C) subunit. This segment, which we term the “NT inhibitory (NTI) module,” potently inhibits long-NT (cardiac) isoform of α1C that features an initial segment of 46 amino acid residues (aa); removal of NTI module greatly increases macroscopic currents. It is not known whether an NTI module exists in the short-NT (smooth muscle/brain type) α1C isoform with a 16-aa initial segment. We addressed this question, and the molecular mechanism of NTI module action, by expressing subunits of Cav1.2 in Xenopus oocytes. NT deletions and chimeras identified aa 1–20 of the long-NT as necessary and sufficient to perform NTI module functions. Coexpression of βm subunit reproducibly modulated function and surface expression of α1C, despite the presence of measurable amounts of an endogenous Cavβ in Xenopus oocytes. Coexpressed βm increased surface expression of α1C approximately twofold (as demonstrated by two independent immunohistochemical methods), shifted the G-V curve by ∼14 mV, and increased Po,max 2.8–3.8-fold. Neither the surface expression of the channel without Cavβ nor βm-induced increase in surface expression or the shift in G-V curve depended on the presence of the NTI module. In contrast, the increase in Po,max was completely absent in the short-NT isoform and in mutants of long-NT α1C lacking the NTI module. We conclude that regulation of Po,max is a discrete, separable function of Cavβ. In Cav1.2, this action of Cavβ depends on NT of α1C and is α1C isoform specific.

INTRODUCTION

Voltage-dependent Ca2+ channels are grouped into three families, Cav1–Cav3 (Ertel et al., 2000). The main structural component of all Cav channels is the α1 subunit that bears the archetypal features of a voltage-dependent channel, with four membrane-spanning domains and a large cytosolic domain comprising the NH2- and COOH-terminal parts of the protein (NT and CT, respectively), and three large intracellular loops, L1–L3, connecting the membrane-spanning domains (Fig. 1 A). In addition, members of Cav1 and Cav2 families also contain at least two auxiliary subunits, β (Cavβ1–Cavβ4) and δ (Isom et al., 1994; Varadi et al., 1995; De Waard et al., 1996; Birnbaumer et al., 1998; Walker and De Waard, 1998; Striessnig, 1999; Catterall, 2000). The αδ subunit regulates channel expression and trafficking to the plasma membrane (PM) (Shistik et al., 1995; Yasuda et al., 2004; Canti et al., 2005), increases the open probability (Po) of α1C (Shistik et al., 1995), and regulates some pharmacological properties of the channel (De Waard et al., 1996). Cavβ subunits are modular MAGUK-type proteins with an SH3-like and a guanylate kinase (GK)-like domain (Chen et al., 2004; McGee et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004). The latter binds with high affinity to a conserved AID (α-interaction domain) motif within the first intracellular loop (L1) of α1 (Pragnell et al., 1994). The β subunits profoundly modulate the properties of voltage-dependent Ca2+ channels. The most prominent effect is a great increase in the magnitude of macroscopic Ca2+ currents, caused by the expression of Cavβ on top of α1 or α1C and αδ in most heterologous expression systems (Mori et al., 1991; Singer et al., 1991; Varadi et al., 1991; Williams et al., 1992; Castellano et al., 1993; Lory et al., 1993), or by the expression of Cavβ in cardiac cells (Wei et al., 2000; Colecraft et al., 2002). Accordingly, depletion or elimination of endogenous Cavβ subunits by knockdown/knockout strategies greatly reduces voltage-dependent Ca2+ currents in various excitable cells (Gregg et al., 1996; Strube et al., 1996; Leuranguer et al., 1998; Namkung et al., 1998; Chu et al., 2004).

Extensive studies in heterologous expression systems revealed a multitude of effects of β subunits on...
biosynthesis and gating of VDCCs. First, Caβ increases the surface expression of α1, by improving its trafficking from the ER to the PM (Chien et al., 1995; Brice et al., 1997; Tareilus et al., 1997; Gao et al., 1999), probably by relieving an ER retention signal (Bichet et al., 2000). Second, Caβ regulates several gating properties of VDCCs. The extent of regulation varies among subtypes and isoforms of Caβ and Caα1. The most prominent changes in macroscopic currents include hyperpolarizing (leftward) shifts in current–voltage (IV) (or conductance–voltage, G-V) and steady-state inactivation curves, an increase in the rate of activation, and changes in the kinetics of voltage-dependent inactivation (for reviews see Birnbaumer et al., 1998; Stotz and Zamponi, 2001; Dolphin, 2003). The shift in G-V curve reflects an improved coupling between gating charge movement and channel opening (Neely et al., 1993, 2004). On the single channel level, coexpression of Caβ increases the open probability, Po, without changing the single channel conductance (Wakamori et al., 1993; Shistik et al., 1995; Costantin et al., 1998).

The overall increase in macroscopic Ca2+ channel currents caused by heterologous expression of Caβ results both from increased surface expression, and from changes in gating that lead to increased Po. Regulation of trafficking by Caβ is clearly separable from modulation of gating; changes in trafficking and gating occur on distinct time and Caβ concentration scales, and mutations that disrupt the high-affinity interaction between AID and Caβ disrupt colocalization of α1 and β in the PM and membrane targeting of α1, but spare some or all of the β-induced changes in gating parameters. Thus, the high-affinity binding of Caβ to AID is obligatory for the regulation of trafficking but not gating (Yamaguchi et al., 1998; Canti et al., 1999, 2001; Gerster et al., 1999; Hullin et al., 2003; McGee et al., 2004; Leroy et al., 2005; Maltez et al., 2005). The SH2-GK domain interaction may also play an important role in regulating trafficking (Takahashi et al., 2005). Among gating effects of Caβ, at least one, the regulation of kinetics of voltage-dependent inactivation (VDI), is separable from the others. A palmitoylated isoform of β2a (usually designated simply as β2a) decelerates the VDI of several Caα, whereas all other β subunits, including a nonpalmitoylated isoform of β2a (np-β2a, of rabbit, and its human orthologue β2b), accelerate VDI. At the same time, the enhanced trafficking of α1 and the hyperpolarizing shift in G-V curve are independent of palmitoylation of Caβ (Olcese et al., 1994; Chien et al., 1996; Qin et al., 1996, 1998; Gao et al., 1999). The separability of different effects of Caβ implies that they are determined by distinct molecular interactions between different parts of β and/or α1. Some actions of β may rely upon low-affinity interactions of β (the SH2 domain in particular) with regions outside the AID in α1, either in L1 or in the CT in some types of α1 (Tareilus et al., 1997; Walker et al., 1999; Takahashi et al., 2004; Maltez et al., 2005). At present, it is unclear whether gating effects of Caβ other than change in kinetics are also separable, and what is the molecular basis of separate effects of Caβ on different gating parameters.

It is notable that cells most widely used for heterologous expression of Ca2+ channels, Xenopus oocytes and human embryonic kidney (HEK) cells, contain small but measurable amounts of an endogenous Caβ protein that undoubtedly aids the “β-less” channels to reach the PM (Tareilus et al., 1997; Canti et al., 2001; Leroy et al., 2005). Arguably, the presence of a minimal amount of endogenous Caβ may be obligatory for surface expression of at least some subtypes of Ca1 and Ca2 channels (Tareilus et al., 1997; Leroy et al., 2005). An absence of such endogenous Caβ may explain the reports that in some cell lines transfected with α1C alone or even with α1C+α2δ, no functional Ca2+ channel expression is observed (Gao et al., 1999; Harry et al., 2004; Kobrinsky et al., 2004). The presence of the endogenous Caβ, which is permissive for trafficking of α1 to PM, does not impair the ability of coexpressed or exogenously added Caβ protein to modulate the biophysical properties of the channel (Tareilus et al., 1997; Yamaguchi et al., 1998; Garcia et al., 2002). Therefore, it has been proposed that the endogenous β only “chaperones” α1, helping it to leave the ER without staying with it in the PM; the added exogenous β then binds to α1 and modulates the gating (the single Caβ-binding model). An alternative multiple Caβ-binding model contends that the endogenous β remains irreversibly bound to AID, and additional β subunit(s) modulate channel gating by interacting with other parts of α1 (discussed by Birnbaumer et al., 1998; Jones, 2002; Dolphin, 2003). A recent study that used a β3 subunit tethered to the end of α1C strongly supports a functional 1:1 α1β stoichiometry (Dalton et al., 2005); unfortunately, not all functions of β were fully recovered by the tethered β, leaving this fundamental issue open for argument.

Unfortunately, the exact extent of regulation of different Caα channels by various Caβ is still debated (discussed in Yasuda et al., 2004), and the contribution of different mechanisms to the increase in whole-cell current has not yet been precisely assessed. The variations are aggravated by apparent inconsistencies between results obtained in different cells and the use of different isoforms of Caα1 and Caαβ. To properly understand the molecular principles underlying the different actions of Caβ, one needs to reliably monitor both the surface expression of α1 and a defined set of gating parameters, in a well characterized system. In this report, we implemented such an approach to analyze the mechanism of regulation of Ca1.2 (α1C), the L-type channel present in most excitable tissues, by β2a. The study focused on the parameters of channel activity that lead to changes in the magnitude of the macroscopic Ba2+ current (Iba),
leaving the regulation of inactivation out of scope. We have previously found that in the cardiac (long-NT) isoform of α\textsubscript{1C}, the first 46 aa of the cytosolic NT constitute an NH\textsubscript{2}-terminal inhibitory (NTI) module whose removal greatly increases the macroscopic current and the P\textsubscript{o}. The presence of the NTI module is also crucial for β\textsubscript{2a}-induced increase in I\textsubscript{Ba} via the long-NT isoform α\textsubscript{1C} in Xenopus oocytes. We therefore proposed that the β subunit acts, in part, by functionally counteracting the inhibitory effect of this module (Shistik et al., 1998; Ivanina et al., 2000). Here we demonstrate that the long-NT initial segment selectively regulates a single action of β\textsubscript{2a}: the elevation of P\textsubscript{o,max}. This modulation is absent in deletion mutants lacking the initial NT segment, and in a short-NT isoform of α\textsubscript{1C} (smooth muscle/brain subtype), in which the initial 46 aa encoded by exon 1a are replaced by a partially homologous stretch of 16 aa encoded by exon 1. Other effects of β (trafficking, G-V curve shift) are preserved. This is the first report on a discrete regulation by Ca\textsubscript{1,β} of P\textsubscript{o,max} in Ca\textsubscript{1,2}. These findings bear upon the isoform-specific physiological properties of the L-type Ca\textsuperscript{2+} channel and upon the physiological role of Ca\textsubscript{1,β} in different tissues.

**MATERIALS AND METHODS**

DNA Constructs and mRNA

cDNAs of rabbit heart α\textsubscript{1C} (X15539), rabbit heart npβ\textsubscript{2a} (L06110) (here termed β\textsubscript{2b}), skeletal muscle α\textsubscript{1S} (P13806) subunits, and the α\textsubscript{1C}, NH\textsubscript{2}-terminal truncation mutants α\textsubscript{1C}\textsubscript{Δ5, α1CΔ20, α1CΔ46, and α1CΔ139 were prepared and used as described previously (Shistik et al., 1998). The mutants Δ6-20, Δ21-46, T\textsubscript{YKP}A/ Y\textsubscript{PYP}DA; the first two amino acids SR were added in order to create an XbaI restriction site in the corresponding cDNA sequence) has been inserted into the extracellular loop after the S5 transmembrane segment of α\textsubscript{1C}-nt, between amino acids Q713 and T714, by two consecutive PCRs. The Δ21-46-HA and NT\textsubscript{C1C}-HA were then produced by standard subcloning procedures. All mutations and PCR products were verified by nucleotide sequencing at the Tel Aviv University Sequencing Facility.

All cDNA constructs of α\textsubscript{1C} and mutants were inserted into the same vector, pGEM-HE-GSB (Shistik et al., 1998), which is a derivative of pGEM-HE (Liman et al., 1992). This vector provides the necessary 5’ and 3’ untranslated regions (UTR) from Xenopus α-globin. Therefore, only the coding sequences of all α\textsubscript{1C} deri- vatives, without any residual original UTRs, were inserted into the vector. To minimize any variability that may be caused by variations in quality and quantity of RNAs, in each series of experiments all tested RNAs (all mutants of α\textsubscript{1C} under study and the wt α\textsubscript{1C}) were synthesized anew on the same day.

Oocyte Culture and Electrophysiology

All the experiments were performed in accordance with the Tel Aviv University Institutional Animal Care and Use Committee (permits no. 11-09-47 and 11-05-064). Xenopus laevis frogs were maintained and operated, and oocytes were collected, defollicu- lated, and injected with RNA as previously described (Dascal and Lotan, 1992). In brief, female frogs, maintained at 20 ± 2°C on an 11-h light/13-h dark cycle, were anesthetized in 0.15% solution of procaine methanesulfonate (MS222), and portions of ovary were removed through a small incision on the abdomen. The incision was sutured, and the animal was returned to a separate tank until it had fully recovered from the anesthesia, and afterwards was returned to a large tank where, together with the other post- operational animals, it was allowed to recover for at least 4 wk until the next surgery. The animals did not show any signs of postoperative distress. The oocytes were injected with the mRNAs of α\textsubscript{1C} or its mutants, α\textsubscript{1C}, and β\textsubscript{2a}, according to the design of experiment (0.3–5 ng for electrophysiology and 2.5–5 ng for imaging). Unless indicated otherwise, equal amounts (by weight) of different RNAs were injected. The oocytes were incubated at 3–5 d at 20–22°C in ND96 solution (96 mM NaCl, 2 mM KC\textsubscript{1}, 1 mM Mg\textsubscript{2+}, 1 mM Ca\textsubscript{2+}, 5 mM HEPES, pH 7.5) supplemented with 2.5 mM Na-pyruvate and 50 μg/ml gentamycin. In some batches with high endogenous chloride currents, oocytes were injected with 25–30 nl/oocyte of the Ca\textsuperscript{2+} chelator EGTA (50–100 mM), giving a final concentration of 2–5 mM within the oocyte (assuming oocyte’s free water volume of 0.5 μl).

Whole cell currents were recorded using the Gene Clamp 500 amplifier (Axon Instruments) using the two-electrode voltage clamp technique, in a solution containing 40 mM Ba(OH)\textsubscript{2}, 50 mM NaOH, 2 mM KOH, and 5 mM HEPES, titrated to pH 7.5 with methanesulfonic acid (Shistik et al., 1995). Stimulation, data acquisition, and analysis were performed using pCLAMP software (Axon Instruments). Current–voltage (I-V) relation of Ba\textsuperscript{2+} currents was measured by 60-ms, 10-mV steps given every 10 s from a holding potential of ~80 mV. In each cell, the net I\textsubscript{Ba} was obtained by subtraction of the residual currents recorded with the same protocols after applying 200 μM Cd\textsuperscript{2+}.

**Immunocytochemistry and Confocal Imaging**

Immunocytochemistry in giant PM patches was done essentially as previously described (Singer-Lahat et al., 2000; Peleg et al., 2002), as illustrated in Fig. 3. Oocytes were devitellinized by peeling off the vitelline membrane in ND96, and placed on plastic coverslips (Thermanox plastic coverslip; Nunc). After sticking to the coverslip, the oocyte was removed mechanically and/or by washing with a strong jet of solution. Pieces of membranes strongly attached to the coverslip were continuously washed until the membrane patch became transparent without any visible cytosolic content and pigment granules. After fixation for 10 min in 1% formaldehyde, the membranes were washed three times with 1% BSA dissolved in TBS solution (135 mM NaCl, 10 mM Tris-HCl, pH 7.4). Blocking of nonspecific binding sites was done with donkey immunoglobulin G (IgG, whole molecule, 1/200, Jackson ImmunoResearch Laboratories) for 30 min. Each coverslip was incubated for 1 h with CT4 antibody against α\textsubscript{1C} COOH terminus (1:500; provided by M. Hosey, Northwestern University, Chicago, IL; see Gao et al., 2001) or against L2 (1:500; Alomone Labs.). Residual antibody was washed out with 1% BSA three times, 5 min each. This was followed by a 30-min incubation with secondary antibody (Cy3 donkey anti-rabbit IgG, 1:400; Jackson ImmunoResearch Laboratories). Free secondary antibody was then washed out with 1% BSA three times, 5 min each in darkness and the coverslips were mounted on a glass slide. The fluorescent labeling was examined by a confocal laser scanning microscope (LSM 410 or LSM 510, Zeiss, Germany). 40X NA/1.2 C-apochromat water-immersion lens (Axiovert 135 M, Zeiss) was used for imaging. The Cy3-conjugated secondary antibody was excited at 488 nm and the emitted light at >568 nm was collected. The fluorescent signals were analyzed by measuring total luminosity (optical density) of the whole image using the Tino 2.1 (Raytest Isotopenmelgerilte GmbH) or Carl Zeiss MicroImaging, Inc. LSM5 software. In all confocal imaging procedures, care was taken to completely avoid saturation of the signal. The gain of the photomultiplier was kept <75% of maximum. In each experiment, all oocytes from the

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different groups were studied using a constant set of imaging parameters. The normalized intensities were always calculated relative to the control group of the same experiment. Net fluorescence intensity per unit area was obtained by subtracting an averaged background signal measured in the same way in membranes of native (uninjected) oocytes from the same batch.

Immunocytochemistry and imaging of whole oocytes has been done as follows. 3–4 h after the injection of RNA, the oocytes were fixed in 4% formaldehyde (37%) in Ca-free ND96 solution for 15 min. Blocking of nonspecific binding sites was done by 5% skim milk for 1 h. Then the oocytes were incubated for 1 h with the mouse monoclonal IgG2a antibody against HA (Santa Cruz Biotechnology), diluted 1:400 in 2.5% skim milk. Residual antibody was washed out with 2.5% skim milk three times, 5 min each. This was followed by 1 h incubation with the secondary antibody (Alexa-conjugated anti-mouse IgG, 1:400; Jackson ImmunoResearch Laboratories) in dark. Free secondary antibody was then washed out with Ca-free ND96. Oocytes were placed in a chamber with a transparent bottom, and fluorescence imaging of optical slices was performed with LSM 510 (×20 objective, zoom = 1, pinhole 3 Airy units). Alexa was excited at 594 nm and the emitted light was collected using long-pass (LP) 615-nm filter. The fluorescent signals were usually analyzed as described in Fig. 4 D. Alternatively, the intensity of fluorescence in the PM was measured by averaging the signal obtained from six standard circular regions of interest. Net fluorescence intensity per unit area was obtained by subtracting the background signal measured in native oocytes.

**Data Analysis**

Current–voltage (I–V) curve was fitted to the Boltzmann equation in the form

$$I_{Ih} = G_{max}(V_m - V_{rev})/(1 + \exp(-(V_m - V_{1/2})/K_v)),$$

where $K_v$ is the slope factor, $V_{1/2}$ is the voltage that causes half maximal activation, $G_{max}$ is the maximal macroscopic conductance, $V_m$ is membrane voltage, $I_{Ih}$ is the current measured at the same voltage, and $V_{rev}$ is the reversal potential of $I_{Ih}$. The obtained parameters of $G_{max}$ and $V_{rev}$ were then used to calculate fractional conductance at each $V_m$, $G/G_{max}$ using the equation

$$G/G_{max} = I_{Ih}/(G_{max}(V_m - V_{rev})).$$

where $G$ is the total macroscopic conductance at $V_m$. The conductance–voltage (G–V) curves were plotted with the values of $V_{1/2}$ and $K_v$ obtained from the fit of the I–V curves, using the following form of the Boltzmann equation:

$$G/G_{max} = 1/(1 + \exp(-(V_m - V_{1/2})/K_v)).$$

The results were summarized from many groups of oocytes from different donors (“batches”). To avoid series resistance artifacts associated with very large currents (Schreibmayer et al., 1994), or inaccuracies resulting from a contribution from the small endogenous oocyte’s Ca”+,” Cl”, or K+ channel currents when the macroscopic $I_{Ih}$ was low (mainly in oocytes expressing αIC+δ without β3C; Dascal, 1987; Dascal et al., 1992), these quantitative analyses were performed only in experiments in which, at the peak of the I–V curve, 0.1 μA ≤ $I_{Ih}$ ≤ 6 μA.

The calculation of changes in $P_{o,max}$ was based on the following considerations. The total macroscopic conductance, estimated by measuring peak currents at different voltages, is a linear function of $P_o$:

$$G = \gamma \times N \times P_o,$$

where $\gamma$ is the single channel conductance, and $N$ is the total number of functional channels in the PM (Hille, 2002). In Ca$\text{\textsubscript{2}}$ channels, $P_o$ is voltage dependent whereas $\gamma$ and $N$ are not. $P_o$ reaches a maximal value, $P_{o,max}$ at positive voltages where a maximal macroscopic conductance, $G_{max}$ is attained. Both $G_{max}$ and $P_{o,max}$ are empirical parameters that are considered voltage independent and are interrelated as follows:

$$P_{o,max} = G_{max}/(\gamma \times N).$$

The fold change in $N$ caused by a treatment, $R_N$, is defined as $N_{treatment}/N_{control}$. From here, if $\gamma$ is constant, the change in $P_{o,max}$ caused by a treatment is given by

$$Fold change in P_{o,max} = P_{o,max(treatment)}/P_{o,max(control)} = (G_{max(treatment)}/G_{max(control)})/R_N.$$
almost all of the cytosolic part of the NT (Kanevsky and Dascal, 1991; Dascal et al., 1992; Singer-Lahat et al., 1994). Therefore, endogenous Cav channels, which are non–L type (Singer et al., 2000), but most of the actions of β2b are similar in the presence or absence of αδ (see Table IV). In oocytes expressing α1C+αδ or α1C+αδ+β2b, the contribution of endogenous channels to I_Ba was negligible; I_Ba was reduced by >95% by 10 μM of the dihydropyridine L-type Ca^{2+} channel blockers nitrendipine and nifedipine (Singer-Lahat et al., 1994; unpublished data).

Fig. 2 A shows the routine experimental protocol used to analyze the I-V characteristic of the expressed channels. Ca^{2+} channel currents were elicited by 60-ms depolarizing pulses from a holding potential of −80 mV, in 10-mV steps, in a solution containing 40 mM Ba^{2+} using the two-electrode voltage clamp technique. Net I_Ba was obtained by subtracting currents elicited by the same voltage protocol in the presence of 200 μM CdCl_2 (Fig. 2A, left). The absolute amplitude of I_Ba depended on the amount of RNA injected, period of incubation, and also varied among oocyte batches. The amount of the injected RNA (of both α1C-wt and α1Cδ) varied in our experiments between 1 and 5 ng/oocyte, depending on the experimental design. In this range, greater amounts of RNA always resulted in larger I_Ba. The right panel of Fig. 2A shows a typical I-V curve of net I_Ba, averaged from six oocytes of the same batch (donor frog). Without Caδ, I_Ba was maximal at ~30 mV. In the following, to compare I_Ba across different groups and treatments,
we chose to use the value of $I_{Ba}$ measured at $+40 \text{ mV}$ ($I_{40}$) rather than at the peak of the I-V curve, because the latter varied between different treatments. Also, at $+40 \text{ mV}$, the whole-cell $Ba^{2+}$ conductance is close to maximum under most conditions (Figs. 2 and 4), therefore changes in $I_{40}$ should approximately reflect changes in $G_{\text{max}}$.

As reported previously (Shistik et al., 1999), in the $\alpha_{1C}\Delta5$ mutant, $I_{40}$ increased about twofold compared with $\alpha_{1C}\delta$wt, without any significant change in the voltage dependence of activation (Fig. 2B). The calculated increase in $G_{\text{max}}$ in the $\alpha_{1C}\Delta5$ mutant was 66% (Table II). In comparison, the deletion of 20 or more aa of the long-NT initial segment caused a robust seven to ninefold increase in $I_{40}$ and in the calculated $G_{\text{max}}$ (Fig. 2, Ba and Ca, and Table II). For a summary of the absolute values of $G_{\text{max}}$ in the different groups, injected with the same RNA amount of 1 ng/oocyte, see Table S1 (available at http://www.jgp.org/cgi/content/full/jgp.200609485/DC1).

As reported earlier (Wei et al., 1996; Shistik et al., 1998), the I-V curves in $\alpha_{1C}\Delta46$ and $\alpha_{1C}\Delta139$ appeared similar to $\alpha_{1C}\delta$wt. The I-V curve shift was observed in all cases. Fitting I-V curves to Boltzmann equation (solid lines in Fig. 2Cb) indicated a statistically significant 5–7 mV shift in the half activation voltage, $\Delta V_{1/2}$, in all NT deletion mutants studied except $\alpha_{1C}\Delta5$ (Table I). The leftward shift in the G-V curves is more clearly seen in conductance–voltage (G-V) curves that were drawn through the data points using the Boltzmann equation parameters obtained in I-V curve fits (Fig. 2Cc). In addition, the slope appears to be increased by the NT deletions. Indeed, the slope factor $K_a$ was slightly but statistically significantly reduced, from 9.6 mV in $\alpha_{1C}\delta$wt to ~7.9 mV in most mutants (Table I). None of the mutations caused any changes in the reversal potential of $I_{Ba}$ (see Table S1).

The robust increase in $I_{Ba}$ and the shift in G-V curve were also observed in the $\alpha_{1C}\Delta6-20$ mutant lacking the 16 aa of the partial homology region. Mutation of the conserved TxxYxP motif produced similar but milder changes (Fig. 2 and Table I). The changes in $G_{\text{max}}$ (calculated from the Boltzmann fits) were similar to changes in the measured $I_{40}$ (Table II). Taken together, these data imply that amino acids 6–20 of the long-NT isoform constitute a crucial part of the NTI module. The increase in $I_{Ba}$ and in $G_{\text{max}}$ is accompanied by a moderate hyperpolarizing shift in the G-V curve; this effect is also fully dependent on the presence of the same 16 aa. The $T_{10\delta}/Y_{13}\delta/P_{15}\delta$ mutation interferes with the function of the NTI module by a mechanism yet to be determined.
Previously, controversial results have been reported regarding the surface expression of α1C with NT deletions of 40 aa or more from a long-NT α1C, expressed in *Xenopus* oocytes. Wei et al. (1996) reported an approximately sixfold increase in surface expression on the basis of measurements of Q\textsubscript{max} in cut-open oocytes. In contrast, we did not observe any changes in surface expression in α1C\textsubscript{Δ5} either by counting channels in cell-attached patches, or by immunoprecipitating α1C from manually separated plasma membranes (Shistik et al., 1998). Here we confirm these results by an independent imaging method (Singer-Lahat et al., 2000; Peleg et al., 2002). Proteins expressed in the PM were visualized by immunostaining in giant membrane patches in which the cytosolic surface of the PM is exposed to external solution (Fig. 3 A). The membrane protein in the patch is labeled with an antibody against a cytosolic segment, then with a fluorescently labeled secondary antibody, and visualized using a confocal microscope (see Materials and Methods for details). The image captures a randomly selected 105 × 105 μm area within a (larger) patch. The large size of the imaged area ensures a fair averaging of channel density even if the channels are clustered. Patches from many tens of oocytes can be screened during a 1-d experiment, providing statistically reliable data. This is a big advantage over the previously employed method of immunoprecipitation of α1C from manually separated PM of metabolically labeled oocytes (Shistik et al., 1995, 1998), which produced one experimental point for 20–30 whole-oocyte membranes.

Fig. 3 B shows images of representative membrane patches from oocytes of one batch expressing α1C\textsubscript{wt} or various NT mutants of α1C without the β subunit. The antibody labeling was clearly detected in all channel constructs, and no significant labeling was observed in un.injected oocytes at these imaging parameters. The data from two oocyte batches are summarized in Fig. 3 Ca, showing that there were no significant differences in the expression of any of the channel variants tested. This result was reproduced later in a separate series of experiments (see Fig. 4, A and B) and also using an alternative imaging method (see Fig. 7 C). At the same time, I\textsubscript{Ba} measured in oocytes of one of these two batches was enhanced by the NT mutations, as usual (Fig. 3 Cb). We used the nonpalmitoylated np-β\textsubscript{2b} (Hullin et al., 1992), which is the rabbit orthologue of human β\textsubscript{2b} (96% identity), the most abundant cardiac β subunit in

### TABLE I

| Construct | No β\textsubscript{2b} | With β\textsubscript{2b} | No β\textsubscript{2b} | K\textsubscript{m} mV | With β\textsubscript{2b} |
|-----------|--------------------------|--------------------------|--------------------------|-------------------------|--------------------------|
| α\textsubscript{1C-wt} | 15.66 ± 1.23 (56) | 1.49 ± 0.79 (56) | 9.62 ± 0.31 (56) | 7.2 ± 1.13 (56) |
| α\textsubscript{1C-5} | 15.83 ± 3.17 (6) | 0.91 ± 0.37 (9) | 9.48 ± 0.92 (6) | 5.59 ± 0.08 (9) |
| α\textsubscript{1C-20} | 9.28 ± 0.98 (19)b | −1.14 ± 1.14 (19)d | 7.87 ± 0.18 (19)b | 6.41 ± 0.23 (19)b |
| α\textsubscript{1C-46} | 9.49 ± 0.99 (30)b | −1.40 ± 0.80 (20)d | 7.88 ± 0.24 (30)b | 5.87 ± 0.18 (20)b |
| α\textsubscript{1C-139} | 11.02 ± 1.00 (16)a | −1.85 ± 3.58 (13)d | 7.93 ± 0.15 (16)b | 5.38 ± 0.35 (13)b |
| α\textsubscript{1C-6-20} | 8.61 ± 1.56 (8) | −1.28 ± 2.21 (14)c | 7.83 ± 0.23 (8) | 6.29 ± 0.23 (14)b |
| α\textsubscript{1C-1C-HA} | 10.28 ± 0.94 (23)b | −2.36 ± 0.57 (12)d | 8.88 ± 0.24 (23) | 6.13 ± 0.27 (12)d |
| α\textsubscript{1C-1C-wt} | 9.83 ± 1.28 (22)b | −2.19 ± 1.20 (26)d | 7.15 ± 0.28 (22)b | 6.06 ± 0.19 (26)b |
| α\textsubscript{1C-1C-D46} | 20.75 ± 1.73 (9) | 5.68 ± 1.93 (12)d | 9.15 ± 0.33 (9) | 6.94 ± 0.11 (12)d |
| NT\textsubscript{1C} | 9.77 ± 1.40 (13)a | −1.89 ± 2.59 (18)d | 8.03 ± 0.17 (13)b | 5.95 ± 0.3 (18)b |
| NT\textsubscript{1C} | 13.86 ± 2.18 (13) | −3.59 ± 2.9 (9)d | 6.58 ± 0.97 (13)b | 4.45 ± 0.62 (9)b |

The values of V\textsubscript{1/2} and K\textsubscript{m} were obtained in each cell by fitting the IV curve to the Boltzmann equation (Eq. 1 in Materials and Methods). Mean ± SEM are shown, with numbers of cells in parentheses.

*Statistically significant difference in V\textsubscript{1/2} or K\textsubscript{m} as compared with α1C\textsubscript{wt} (or α1C-HA) by one-way ANOVA followed by the Dunnett’s test. P < 0.05.

*Statistically significant difference in V\textsubscript{1/2} or K\textsubscript{m} as compared with α1C\textsubscript{wt} (or α1C-HA) by one-way ANOVA followed by the Dunnett’s test. P < 0.01.

*Statistically significant difference compared to no β\textsubscript{2b} group by t test. P < 0.05.

*Statistically significant difference compared to no β\textsubscript{2b} group by t test. P < 0.001.
5 ng/oocyte.

of one of these batches (b).

The amount of injected RNA was

1C. (A) A simplifi ed presentation of the method

expression of $\alpha$

mutations do not alter the surface expression of

the typical differences in I Ba (measured at

used to measure the surface expression of

22 Regulation of P o of L-type Channel by

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the traffi cking of

Xenopus oocytes, 

et al., 1994; Chien et al., 1998). When expressed in

1C (Olcese et al., 1994; Chien et al., 1998). When expressed in

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$\alpha$

$\alpha$

(Altier et al., 2002). Ba 2+

currents via channels formed by $\alpha$C-HA, coexpressed with $\alpha$/$\beta$ with or without $\beta$2b, did not differ in amplitude or voltage dependency from

$\alpha$C-wt (unpublished data; Table I). Surface expression

of the HA label was measured using a confocal micro-

scope in whole intact oocytes fi xated with formaldehyde

and treated with an anti-HA antibody and a secondary

antibody conjugated to a fl uorescent dye (Fig. 4 D). As

shown in Fig. 4 Da, the expression of $\beta$2b caused a

clear increase in surface expression of $\alpha$C-HA. The

results were quantifi ed as explained in Fig. 4 legend, and

showed a 2.09 ± 0.34-fold increase in the intensity of fl uorescence caused by $\beta$2b (Fig. 4 Dc). This estimate, though somewhat higher than by measuring the effect of $\beta$2b in giant PM patches, was not signifi cantly different when compared in the same oocyte batch (not depicted). These results unequivocally demonstrate that, despite the theoretical possibility that some of the fl uorescent signal in the giant membrane patches arises from $\alpha$C found in submembrane ER, the latter method provides a realistic estimate of $\alpha$C levels in the PM. We conclude that, despite the presence of an endogenous Ca,$\beta$ in the oocytes, the expressed $\beta$2b further increases the surface expression of Ca1,2 channels about twofold (in the presence of $\alpha$,$\delta$). The ability of $\beta$2b to do so is not affected by the elimination of the NTI module.

Coexpression of $\beta$2b also increased the macroscopic currents in wt and all NT mutants. Examples are shown in Fig. 5 A for $\alpha$C-wt and $\alpha$C$\Delta$46. Note that $\beta$2b accelerated the inactivation kinetics in both cases, despite the fact that without Ca,$\beta$, they were already faster in $\alpha$C$\Delta$46 than in $\alpha$C-wt. This was a recurrent result in most mu-

nants (unpublished data). Thus, although VDI is out of the scope of this paper, and although it is clear that the NH2 terminus itself plays a role in VDI (see also Shistik et al., 1998; Kobrinsky et al., 2004), we construe that the elimination of the NTI module does not impair the ability of $\beta$2b to speed up the VDI.

rat and humans (Colecraft et al., 2002; Hullin et al.,

2003). To avoid confusion between palmitoylated and nonpalmitoylated forms of $\beta$2b, we refer to np-$\beta$2b as $\beta$2b.

Unlike the palmitoylated splice variant isoform ($\beta$2a) of the same gene, $\beta$2b does not slow down the VDI and does not reside in PM in the absence of $\alpha$C (Olcese et al., 1994; Chien et al., 1998). When expressed in

Xenopus oocytes, $\beta$2b accelerates the VDI of Ca1,2, and in addition causes a robust increase in the whole-cell current and affects all gating parameters (Hullin et al., 1992; Shistik et al., 1995). However, it is not clear whether coexpression of either $\beta$2a or $\beta$2b improves the traffi cking of $\alpha$C to the PM in

Xenopus oocytes. No change (Neely et al., 1993, 2004) or a mild ~50% increase (Shistik et al., 1995) have been reported (see Table IV and Discussion).

Using the imaging method shown in Fig. 3, we have examined the effect of coexpression of $\beta$2b on the amount of $\alpha$C-wt, $\alpha$C$\Delta$20, $\alpha$C$\Delta$46, and $\alpha$C-TYP in two to four batches of oocytes; $\alpha$2$\delta$ was always coexpressed (Fig. 4). As before, in the absence of Ca,$\beta$, the surface expression of NT mutants was similar to that of $\alpha$C-wt (Fig. 4, A and B). In contrast, $\beta$2b induced a mild but reproducible and statistically significant ~70% increase (P < 0.001) in the surface expression in all $\alpha$C constructs (Fig. 4, A and C).

The effect of $\beta$2b on surface expression of $\alpha$C was additionally verified using an independent method, using a modified $\alpha$C (termed $\alpha$C-HA) with an extracellular HA tag inserted in the extracellular loop following the S5 segment of domain I. A similar construct was previously used to study the traffi cking of $\alpha$C in mammalian cells (Altier et al., 2002). Ba2+ currents via channels formed by $\alpha$C-HA, coexpressed with $\alpha$2/3 with or without $\beta$2b, did not differ in amplitude or voltage dependency from $\alpha$C-wt (unpublished data; Table I). Surface expression

of the S5 segment of domain I. A similar construct was previously used to study the traffi cking of $\alpha$C in mammalian cells (Altier et al., 2002). Ba2+ currents via channels formed by $\alpha$C-HA, coexpressed with $\alpha$2/3 with or without $\beta$2b, did not differ in amplitude or voltage dependency from $\alpha$C-wt (unpublished data; Table I). Surface expression of the HA label was measured using a confocal micro-

scope in whole intact oocytes fi xated with formaldehyde

and treated with an anti-HA antibody and a secondary

antibody conjugated to a fl uorescent dye (Fig. 4 D). As

shown in Fig. 4 Da, the expression of $\beta$2b caused a

clear increase in surface expression of $\alpha$C-HA. The

results were quantifi ed as explained in Fig. 4 legend, and

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Coexpression of $\beta$2b also increased the macroscopic currents in wt and all NT mutants. Examples are shown in Fig. 5 A for $\alpha$C-wt and $\alpha$C$\Delta$46. Note that $\beta$2b accelerated the inactivation kinetics in both cases, despite the fact that without Ca,$\beta$, they were already faster in $\alpha$C$\Delta$46 than in $\alpha$C-wt. This was a recurrent result in most mu-

nants (unpublished data). Thus, although VDI is out of the scope of this paper, and although it is clear that the NH2 terminus itself plays a role in VDI (see also Shistik et al., 1998; Kobrinsky et al., 2004), we construe that the elimination of the NTI module does not impair the ability of $\beta$2b to speed up the VDI.

![Figure 3](image_url)

**Figure 3.** NT mutations and deletions do not alter the surface expression of $\alpha$C. (A) A simplifi ed presentation of the method used to measure the surface expression of $\alpha$C in giant membrane patches (see Materials and Methods for details). A devitellinized intact oocyte is placed for 10–20 min on coverslip with its animal (dark) hemisphere facing the coverslip (a). After attachment, the oocyte is swept away and patches of membrane (usually >100 μm in diameter) remain stuck to the coverslip (b). The cytosolic sur-

face of the PM, facing the external solution, is thoroughly washed until the membrane appears transparent (c) and then stained with an antibody directed against a cytosolic part of the channel. (B) Examples of confocal images of $\alpha$C-wt, $\alpha$C$\Delta$46, $\alpha$C$\Delta$20, and $\alpha$C-TYP in giant patches of oocytes of the same batch. (C) NT mutations do not alter the surface expression of $\alpha$C (a), despite the typical differences in I Ba (measured at +20 mV) in oocytes of one of these batches (b). The amount of injected RNA was 5 ng/oocyte.
TABLE II

| Construct                  | Fold increase in PM α1C labeling | Fold increase in G\text{max} | Fold increase in I\text{40} | Fold increase in P\text{o,max} from G\text{max} | Fold increase in P\text{o,max} from I\text{40} |
|----------------------------|---------------------------------|-----------------------------|-----------------------------|-----------------------------------------------|-----------------------------------------------|
| α\text{IC-wt}             | 1 ± 0.05 (40; 7)                | 1 ± 0.04 (56; 11)           | 1 ± 0.07 (56; 11)           |                                               |                                               |
| α\text{IC-Δ5}             | 1.21 ± 0.18 (6; 2)              | 1.66 ± 0.088 (9; 2)         | 1.76 ± 0.24 (9; 2)          | 1.37                                          | 1.45                                          |
| α\text{IC-Δ20}            | 1.22 ± 0.12 (24; 3)             | 7.64 ± 0.98 (8; 30; 6)      | 7.68 ± 0.34 (8; 30; 6)      | 6.26                                          | 6.3                                           |
| α\text{IC-Δ6}             | 0.99 ± 0.12 (16; 3)             | 7.63 ± 0.81 (4; 30; 6)      | 7.75 ± 0.95 (4; 30; 6)      | 7.7                                           | 7.82                                          |
| α\text{IC-Δ139}           | Not examined                    |                             |                             |                                               |                                               |
| α\text{IC-TYP}            | 0.86 ± 0.1 (17; 3)              | 5.71 ± 0.93 (4; 23; 3)      | 5.95 ± 0.95 (4; 23; 3)      | 6.63                                          | 6.91                                          |
| α\text{IC-Δ2-20}          | Not examined                    | 8.22 ± 1.01 (4; 14; 3)      | 8.6 ± 1.23 (4; 14; 3)       |                                               |                                               |
| α\text{IC-short}          | 1.16 ± 0.14 (9; 2)              | 8.56 ± 0.98 (4; 12; 2)      | 8.71 ± 1.71 (4; 12; 2)      | 7.38                                          | 7.5                                           |
| NT\text{SL-HA}            | 1.13 ± 0.19 (11; 2)             | 5.77 ± 0.56 (4; 16; 3)      | 5.98 ± 0.74 (4; 16; 3)      | 5.1                                           | 5.3                                           |
| H\text{A}-labeled constructs |                                |                             |                             |                                               |                                               |
| α\text{IC-HA}             | 1 ± 0.12 (10; 1)                | 1 ± 0.11 (9; 3)             | 1 ± 0.31 (9; 3)             |                                               |                                               |
| NT\text{IC-β2b HA}        | 1.15 ± 0.09 (11; 1)             | 13.6 ± 1.85 (4; 15; 3)      | 14.08 ± 2.04 (4; 15; 3)     | 11.8                                          | 12.2                                          |
| α\text{IC-Δ21-46-HA}      | 0.88 ± 0.09 (11; 1)             | 1.41 ± 0.29 (9; 3)          | 1.47 ± 0.29 (9; 3)          | 1.6                                           | 1.67                                          |

Surface expression was measured by confocal imaging of immunostained α1C in giant membrane patches or, for HA-labeled constructs, in intact oocytes. Numbers in parentheses indicate n: N (the total number of oocytes and the number of oocyte batches tested). To calculate fold change in a parameter, normalization procedure was performed as described in Materials and Methods. Statistical comparisons were performed using one way ANOVA (multiple comparisons versus control group, α\text{IC-wt} or α\text{IC-HA}, in oocytes injected with the same amount of RNA, followed by Tukey’s test).

*In two batches where α\text{IC-wt} and α\text{IC-Δ5} were compared, the statistical analysis has been done separately from other batches, since the amount of injected RNA was 2.5 ng per oocyte rather than 1 ng. Averaged normalized fold increase in G\text{max} and I\text{40} for α\text{IC-wt} in these two batches was 1 ± 0.16 (n = 9, N = 2) and 1 ± 0.2 (n = 9, N = 2), respectively. The statistical analysis was done using t test.

\*P < 0.05.
\*P < 0.01.
\*P < 0.001.

The I-V curves of all α\text{IC} mutants, and of the α\text{IC-wt}, were shifted to the left by coexpression of β\text{2b}. Examples of averaged I-V curves of oocytes from representative batches are shown in Fig. 5 B, and a full summary of normalized I-V and G-V curves averaged from all oocytes is presented in Fig. 5 (C and D). The hyperpolarizing shift produced by β\text{2b} is observed both in α\text{IC-wt} and in all mutants lacking the NTI module. A closer examination of the results of Boltzmann fits in Table I reveals that the net change in V_{1/2} and K_0 parameters caused by β\text{2b} in α\text{IC-wt} (14 and 2.4 mV, respectively) was somewhat greater than in some of the NT mutants (11–13 and 1.5–2 mV). However, these differences are small and may be within the experimental or fitting error. In all, we conclude that elimination of NT inhibitory module does not impair the ability of β\text{2b} to cause a hyperpolarizing shift in the voltage dependency of channel activation.

In contrast to parameters considered so far, the extent of increase in I_{Ba} and in G_{\text{max}} caused by coexpression of β\text{2b} was altered dramatically by NT mutations (Figs. 5 B, Fig. 6, and Table III). In agreement with previous reports regarding α\text{IC-Δ20}, α\text{IC-Δ46}, and α\text{IC-Δ139} (Shistik et al., 1998, 1999), all mutations that impaired the function of the NTI module also greatly reduced the ability of β\text{2b} to increase I_{Ba} (Fig. 6, A and B). The differences were more pronounced at less positive potentials, because at least part of the increase in I_{Ba} at these potentials is due to the leftward shift in the I-V curve, as illustrated in Fig. 6 A for some of the mutants. However, even at +40 mV, the difference between α\text{IC-wt} and the various mutants is still very substantial (Fig. 6 B). The β2b-induced increase in the calculated G_{\text{max}} which in α\text{IC-wt} was 4.65 ± 0.39-fold, was diminished to only 1.92 ± 0.23-fold in α\text{IC-Δ46} (P < 0.01; Table III). A similar, though slightly milder, reduction was observed in α\text{IC-Δ20} and α\text{IC-TYP} (Table III). The only outlier is α\text{IC-Δ5}. The β2b-induced change in I_{40} was even greater in this mutant than in α\text{IC-wt}, as measured in two batches of oocytes (Fig. 6 C). This is at odds with a previous observation (Fig. 2 F in Shistik et al., 1999), but a comprehensive investigation into the reasons of controversy is not possible, because in the experiment reported in 1999, the effect of β\text{2b}, was studied only marginally (Shistik et al., 1999); I_{Ba} was measured only at +10 mV, and no analysis of voltage dependency has been performed. Thus, dose-dependent or batch-dependent effects of β\text{2b} on α\text{IC-Δ5} cannot be excluded at present.

To summarize, the main conclusion from these experiments is that the ability of β\text{2b} to increase G_{\text{max}} is greatly reduced by the removal of a crucial part of the NT inhibitory module, aa 6–20. The unique aa 2–5 of the long-NT channel are a part of the NTI module, since their removal already causes a mild increase in P_{o,max}, however, it also potentiates the enhancing effect.
of $\beta_{2b}$ on $P_{o,max}$ instead of reducing it. This peculiar effect deserves further attention in the future.

The $\beta_{2b}$-induced increase in $G_{max}$ still remaining after the elimination of the NTI module is comparable to the increase in the surface expression caused by $\beta_{2b}$. This indicates that in these mutants, the increase of the macroscopic $\mathrm{Ca}^{2+}$ channel conductance caused by $\beta_{2b}$ may be the result of an increase in the amount of the functional channels, $N$, whereas $P_{o,max}$ is not substantially changed. This is illustrated by comparing the values of $\beta$-induced change in $P_{o,max}$ calculated using Eq. 6 (Table III).

**The Initial Segment of the Short-NT Isoform of $\alpha_{1C}$ Is Not an NTI Module**

Are $aa$ 6–20 of long-NT sufficient to act as an NTI module? Does the initial segment of the short-NT isoform ($aa$ 2–16) act as an inhibitory module, similarly to the partially homologous $aa$ 6–20 segment in the long-NT? To answer these questions, we created a cDNA encoding for a short-NT isoform, $\alpha_{1C}$-short, in which exon 1a of the long-NT was replaced by the exon 1 of short-NT $\alpha_{1C}$ (Fig. 7 A). The rest of the molecule was left as in the cardiac, long-NT $\alpha_{1C}$. This design was intended to single out the effects of NT and to avoid additional changes in channel’s properties that can arise if one is using one of the several cloned short-NT isoforms, with additional variations in the other parts of the protein (Abernethy and Soldatov, 2002). We also created cDNAs encoding two chimeric proteins in which parts of short- and long-NT initial segments were switched (Fig. 7 A): (1) NTSL, which is a short-NT $\alpha_{1C}$ in which $aa$ 6–20 are replaced by aa 6–20 of the long-NT $\alpha_{1C}$; (2) NTLS, which is a long-NT $\alpha_{1C}$ in which $aa$ 2–16 of the short-NT $\alpha_{1C}$ are replaced by aa 6–20 of the long-NT $\alpha_{1C}$; (3) NTLS, which is a long-NT $\alpha_{1C}$ in which $aa$ 2–16 are replaced by aa 2–16 of the short-NT. Two additional groups tested in these experiments included the standard $\alpha_{1C}$Δ46 mutant (which misses the entire initial segment), and a new internal deletion of long-NT $\alpha_{1C}$, $\alpha_{1C}$Δ21-46. External HA-tagged constructs were also made for all new mutants except short-NT $\alpha_{1C}$; they exhibited G-V curves and macroscopic $I_{Ba}$ very similar to the untagged counterparts (Table I; unpublished data).

Immunohistochemical measurements in giant membrane patches or in intact oocytes (for HA-tagged expression of $\alpha_{1C}$-wt and of NT mutants. (A) Examples of confocal images of $\alpha_{1C}$-wt, $\alpha_{1C}$Δ46, $\alpha_{1C}$Δ20, and $\alpha_{1C}$TYP in giant PM patches, in absence and presence of $\beta_{2b}$. (B) Surface expression of the mutant proteins in $\alpha_{1C}$ subunit composition, without $\beta_{2b}$, normalized to $\alpha_{1C}$-wt. This series of experiments included two oocyte batches and was separate from that shown in Fig. 3. Injected RNA was 5 ng/oocyte. (C) Summary of the effects of $\beta_{2b}$ on surface expression of $\alpha_{1C}$ mutants. For each construct, the surface expression in the presence of $\beta_{2b}$ was normalized to the average expression measured without $\beta_{2b}$. (D) The effect of $\beta_{2b}$ on surface expression of $\alpha_{1C}$-HA: panel a shows representative confocal images of whole oocytes, either native (uninjected) or expressing $\alpha_{1C}$-HA; panel b presents optical density profiles. The intensity was defined as the integral of the shaded area. The width of this area was defined as constant for all images taken in an experiment. The net intensity in each $\alpha_{1C}$-HA-expressing oocyte was calculated by subtracting the average intensity measured in un.injected oocytes of the same experiment, and then normalized to the average net intensity of $[\alpha_{1C}$-HA+$\alpha_{1C}$-δ]-expressing oocytes of this experiment. The normalized values, shown in c, were summarized across all experiments ($N=3$).
constructs) showed that all new constructs expressed similarly to $\alpha_{1C}$-wt in the PM, and coexpression of $\beta_{2b}$ increased the amount of all constructs in the PM similarly to $\alpha_{1C}$-wt (Fig. 7, B and C; Tables II and III).

Fig. 8 (A–C) reports on the results of two series of experiments (a and b) in which the macroscopic properties of IBa in the new constructs were tested, in different combinations and at two RNA doses. Without the $\beta$ subunit, IBa in $\alpha_{1C}$-short, NTSL, and NTLS was substantially greater than in $\alpha_{1C}$-wt, and a small but significant leftward shift in $V_{1/2}$ and a decrease in $K_a$ were also observed. All these parameters were comparable to those of $\alpha_{1C}$-$\Delta_{46}$ (Fig. 8; Tables I and II). $I_{10}$ tended to be somewhat smaller in NTSL than in $\alpha_{1C}$-short or $\alpha_{1C}$-$\Delta_{46}$, but the difference did not reach statistical significance. In contrast, both macroscopic IBa and all G-V curve parameters of $\alpha_{1C}$-$\Delta_{21-46}$ were very similar to $\alpha_{1C}$-wt.

Coexpression of $\beta_{2b}$ shifted the G-V curve to the left by 12–17 mV in all constructs (Fig. 8; Tables I and II). $I_{10}$ increased much more than in $\alpha_{1C}$-$\Delta_{46}$ but less than in $\alpha_{1C}$-wt, whereas $\alpha_{1C}$-$\Delta_{21-46}$ again behaved almost exactly as $\alpha_{1C}$-wt (Fig. 8 C). The increase in $P_{o,max}$ caused by $\beta_{2b}$ almost disappeared in $\alpha_{1C}$-$\Delta_{46}$ and NTLS mutants; in the $\alpha_{1C}$-short, the calculation even showed a small decrease in $P_{o,max}$ by $\beta_{2b}$ (Table III). In contrast, in NTLS, the calculated $P_{o,max}$ was increased by $\beta_{2b}$ almost like in the long-NT $\alpha_{1C}$-wt. $\alpha_{1C}$-$\Delta_{21-46}$ behaved exactly like $\alpha_{1C}$-wt (Fig. 8 Cb). Taken together, these results strongly suggest that aa 1–20 of long-NT fully restore the function of the NTI module, whereas aa 6–20 of long-NT alone are insufficient. aa 2–16 of short-NT cannot replace the homologous 15 aa of the long-NT as a crucial component of NTI module.

The difference in effects of $\beta_{2b}$ on $\alpha_{1C}$-wt and the various NT deletion mutants is further explored in Fig. 8 D, where the $\beta_{2b}$-induced increase in IBa at each voltage was normalized to that observed in $\alpha_{1C}$-wt. The rationale for this analysis is as follows. If the NTI module regulates the effect of $\beta_{2b}$ on $P_{o,max}$ (a nominally voltage-independent parameter) and not on the voltage dependence of activation, then the removal of the NTI module should alter the effect of $\beta_{2b}$ to a similar extent at all voltages. In general, this was indeed the case (Fig. 8 D), although the relative effect of $\beta_{2b}$ showed a slight voltage sensitivity; the difference between $\alpha_{1C}$-wt and NT mutants was somewhat greater at more negative voltages. This, however, is a predictable consequence of the mild hyperpolarizing shift in the G-V curve caused by the removal of the NTI module (see Fig. 2 C and Fig. 8 B). The latter leads to a slightly stronger voltage dependency.
relative $I_{Ba}$ in the various constructs; numbers of assayed cells are indicated above the bars. $N = 2–7$ experiments. (C) The increase in $I_{Ba}$ caused by coexpression of $\beta_{2b}$ is potentiated in the $\alpha_{1C}\Delta\delta$ mutant. $N = 2$.

of $\beta_{2b}$-induced increase in $I_{Ba}$ in $\alpha_{1C}\text{-}\text{wt}$ than in the mutants. Fig. 8 D also illustrates the fact that, of all constructs tested, the increase in $I_{Ba}$ by $\beta_{2b}$ was the greatest in $\alpha_{1C}\text{-}\text{wt}$ and the smallest in $\alpha_{1C}\text{-\text{short}}$ (even compared with $\alpha_{1C}\Delta\alpha\Delta\gamma\Delta\delta$). The substantial recovery of the effect of $\beta_{2b}$ in NTSL and the full recovery in $\alpha_{1C}\Delta\alpha\Delta\gamma\Delta\delta$ are also clearly visualized.

**DISCUSSION**

**Overview of Findings and Conclusions**

Despite the importance of regulation of Ca$^{2+}$ channels by $\beta$ subunits and the wide interest in structure, function, and interactions of Ca $\alpha$ and Ca $\beta$, controversies still abound regarding the mechanisms and even the phenomenology of the effects of Ca $\beta$. In this study we attempt to sort out some of the discrepancies, and to better understand the mechanism by which Ca $\beta$ increases Ca$^{2+}$ channel currents, focusing on a single type of Ca $\alpha_{\text{1C}}$ and Ca $\beta$.

(a) We show that Ca $\beta$-induced increase in maximal open probability ($P_{\text{o, max}}$), at least in Ca1.2, is separable from the other mechanisms (shift in G-V curve and change in PM expression) that underlie the increase in macroscopic Ca$^{2+}$ currents by Ca $\beta$. This effect is crucially dependent on the presence of an NTI module. This separability, and the separability of trafficking from all gating effects, implies that Ca $\beta$ acts on $\alpha_{1C}$ to increase $P_{\text{o, max}}$ via molecular determinants (in $\beta$ and/or in $\alpha_{\text{1C}}$) different from those used to change trafficking or to shift the G-V curve.

(b) Using deletion mutagenesis and chimeric constructs, we map the necessary and sufficient component of the NTI module to aa 1–20 of the long-NT and demonstrate that the NTI reduces $P_{\text{o, max}}$, thus restraining the activation of the channel at all voltages. Loss of NTI module enhances $I_{Ba}$ by increasing $P_{\text{o, max}}$ ~6–10-fold and, in parallel, specifically eliminates the effect of Ca $\beta$ on $P_{\text{o, max}}$; addition of the module restores the small amplitude of $I_{Ba}$ and the enhancement of $P_{\text{o, max}}$ by Ca $\beta$. This correlation supports the hypothesis (Shistik et al., 1998) that part of the effect of $\beta$ on $\alpha_{1C}$ is due to a suppression of the inhibitory action of the NTI module. In the absence of the NTI module, Ca $\beta$ cannot further increase $P_{\text{o, max}}$, which is already high.

**TABLE III**

| Group | Fold increase in PM $\alpha_{1C}$ labeling | Fold increase in $G_{\text{max}}$ | Fold increase in $I_{Ba}$ | Fold increase in $P_{\text{o, max}}$ |
|-------|------------------------------------------|---------------------------------|--------------------------|-------------------------------|
| $\alpha_{1C}\text{-\text{wt}}$ | 1.60 ± 0.19 (20; 4) | 4.65 ± 0.39 (45; 7) | 4.9 ± 0.37 (45; 7) | 2.75 | 2.9 |
| $\alpha_{1C}\Delta\alpha\Delta\gamma\Delta\delta$ | 1.65 ± 0.11 (11; 2) | 2.48 ± 0.58 (19; 4) | 2.36 ± 0.26 (19; 4) | 1.5 | 1.43 |
| $\alpha_{1C}\Delta\alpha\Delta\gamma\Delta\delta$ | 1.63 ± 0.08 (20; 3) | 2.01 ± 0.22 (28; 5) | 1.92 ± 0.23 (28; 5) | 1.25 | 1.18 |
| $\alpha_{1C}\text{-\text{TYP}}$ | 1.79 ± 0.04 (9; 2) | 2.49 ± 0.21 (20; 3) | 2.24 ± 0.23 (20; 3) | 1.39 | 1.25 |
| $\alpha_{1C}\text{-\text{short}}$ | 1.64 ± 0.13 (12; 3) | 1.25 ± 0.14 (11; 2) | 1.31 ± 0.15 (11; 2) | 0.76 | 0.8 |
| NTSL | 1.58 ± 0.17 (9; 2) | 4.12 ± 0.41 (16; 3) | 4.07 ± 0.49 (16; 3) | 2.6 | 2.57 |
| HA-labeled constructs | | | | |
| $\alpha_{1C}\text{-\text{HA}}$ | 2.22 ± 0.21 (13; 1) | 8.04 ± 0.93 (13; 3) | 8.49 ± 1 (15; 3) | 3.6 | 3.82 |
| NTSL-HA | 1.99 ± 0.22 (7; 1) | 2.36 ± 0.31 (9; 3) | 2.29 ± 0.28 (9; 3) | 1.18 | 1.15 |
| $\alpha_{1C}\text{-\text{421-46-HA}}$ | 2.86 ± 0.38 (11; 1) | 10.9 ± 1.38 (12; 3) | 11.03 ± 1.54 (12; 3) | 3.8 | 3.85 |

All comparisons and normalization procedures are as in Table II.

*P < 0.01, compared with $\alpha_{1C}\text{-\text{wt}}$. 
We establish that the NTI module is completely missing from a short-NT Cav1.2, which represents a class of Ca\textsubscript{v}1.2\textsubscript{\alpha} isoforms abundant in the smooth muscle and brain. This is a striking example of a physiologically meaningful disparate regulation of two very similar isoforms of an ion channel by an auxiliary subunit.

Using two independent methods, we unequivocally demonstrate that coexpression of Ca\textsubscript{v}\textsubscript{\beta} increases the surface expression of Ca\textsubscript{v}\textsubscript{\alpha}1C in Xenopus oocytes approximately twofold, independently of the presence of the NTI module. By a critical examination of literature and separation of studies that used different NT isoforms, we resolve apparent controversies and single out remaining problems, regarding the effects of Ca\textsubscript{v}\textsubscript{\beta} on Ca\textsubscript{v}\textsubscript{\alpha}1C.

**A Summary of the Effects of Ca\textsubscript{v}\textsubscript{\beta} on Ca\textsubscript{v}\textsubscript{\alpha}1C**

It is known that the effects of \( \beta \) subunits differ among the various \( \alpha \) subunits (Dolphin, 2003). However, even for a single species of Ca\textsubscript{v}\textsubscript{\alpha}, the details and magnitude of effects of coexpressed Ca\textsubscript{v}\textsubscript{\beta} often remain controversial. For instance, estimates of the increase in macroscopic maximal \( I_{Ba} \) of Ca\textsubscript{v}1.2 (\( \alpha_{1C} \)) in mammalian cells range between 15 and 20-fold (Lory et al., 1993; Takahashi et al., 2004) to almost no effect (Yasuda et al., 2004). The comparison between the different reports is thwarted by the unavoidable difference in recording conditions and often by an absence of quantitative estimates of some of the electrophysiological parameters.

To understand the origins of the controversies, we summarized the data on Ca\textsubscript{v}\textsubscript{\beta} effects on Ca\textsubscript{v}1.2 from reports that contain an explicit quantitative estimation of parameters that affect the macroscopic Ca\textsubscript{2+} channel currents (Table IV). Data collected from short-NT (or mutants of \( \alpha_{1C} \) missing the initial NT segment) and the long-NT isoforms of \( \alpha_{1C} \) are separated. Macroscopic inactivation is unimportant in determining the whole-cell current when Ba\textsubscript{2+} is used as the charge carrier (see below), and is left out of scope. Reports in which effects of different Ca\textsubscript{v}\textsubscript{\beta} have been compared but there are no data on channels expressed without Ca\textsubscript{v}\textsubscript{\beta} are not included. If several \( \beta \) subunits have been compared in the same study, we usually show \( \beta_{2a} \) or np-\( \beta_{2a} \) (\( \beta_{2b} \)), which were the most widely used. While the summary in Table IV may not be exhaustive, it is quite revealing.
28 Regulation of \( P_o \) of L-type Channel by \( \beta \) Subunit

A Distinction between \( P_o \) and \( P_{o,max} \)

Whole-cell \( \text{Ca}^{2+} \) channel currents and/or conductance (G) depend both on the amount of functional channels (N) and on the open probability, \( P_o \), such that \( G = \gamma \times N \times P_o \), and \( G_{\text{max}} = \gamma \times N \times P_{o,max} \) (see Materials and Methods, Eqs. 4 and 5). Treatments used in our experiments (NT deletions or coexpression of \( \beta_{2b} \)) do not affect \( \gamma \) (Shistik et al., 1995, 1998; Hullin et al., 2003), thus only N and \( P_o \) were changing. N is voltage independent, and assessing the impact of changes in N (once monitored) on G is straightforward.

In comparison, the analysis of \( P_o \) and the assessment of its effect on G are not trivial. First, in voltage-dependent channels, \( P_o \) is voltage dependent, being described by the same Boltzmann equation as \( G/G_{\text{max}} \) (Hille, 2002) (Eq. 3). \( P_o \) ranges from zero at negative potentials to \( P_{o,max} \) at “saturating” membrane voltages where the G-V curve reaches a plateau. Note that in this classical treatment \( G_{\text{max}} \) and \( P_{o,max} \) are voltage independent. An agent that improves the coupling between gating charge movement and pore opening, such as \( \text{Ca}_2^+ \beta \) (Neely et al., 1993), and shifts the G-V curve to the left will increase \( P_o \) (and macroscopic currents) in a range of “nonsaturating” voltages, whether \( P_{o,max} \) is changed or not. In contrast, an increase in \( P_{o,max} \) leads to an increase in \( \text{Ca}^{2+} \) conductance at all voltages except those at which the channel is fully shut. Therefore, to understand the molecular mechanism of \( \beta \)’s actions, it is important to distinguish between changes in \( P_o \) caused by a G-V curve shift vs. an increase in \( P_{o,max} \).

In single-channel recordings it is possible to directly determine \( P_o \), which is the fraction of time that a channel spends in open state(s), out of total observation time (Colquhoun and Sigworth, 1995). Although estimation of \( P_o \) can be distorted by the presence of inactivated states (Colquhoun and Sigworth, 1995), in \( \text{Ca}_{1.2} \) with \( \text{Ca}^{2+} \) as charge carrier, the inactivation is slow, and such distortion can be avoided by using short analyzed time segments (a few tens of milliseconds). Unfortunately, the estimation of changes in \( P_{o,max} \) caused by \( \text{Ca}_2^+ \beta \) is almost impossible in single-channel recording of \( \text{Ca}_2 \) channels. Most publications report single channel currents recorded between -10 and +10 mV in high-\( \text{Ba}^{2+} \) solutions, to ensure high signal-to-noise ratio; a full I-V curve is usually not constructed. (An exception is a study in cardiomyocytes [Colecraft et al., 2002], where a full I-V relation was explored; see Table IV.) Under these conditions, G (and thus \( P_o \)) is very far from reaching a maximum, as evident from typical G-V curves shown in Figs. 2, 5, and 8, and the measured \( P_o \) does not provide any estimate of \( P_{o,max} \).

In comparison, measurements of changes in macroscopic \( G_{\text{max}} \) in conjunction with a reliable estimation of N should provide a good measure of relative changes in \( P_{o,max} \) (Wei et al., 1994; Takahashi et al., 2004). Here, \( G/G_{\text{max}} \) is calculated using the values of peak macroscopic current that are independent of the duration of voltage step, therefore the latter does not affect the calculated relative change in \( P_{o,max} \). Our estimates of \( P_{o,max} \) were not affected by changes in the steady-state
inactivation curve in the NT deletion mutants, because the availability of the channels for opening at the holding potential of $-80$ mV remained 100% (Shistik et al., 1998). There are two possible sources of inaccuracy. First, acceleration of the inactivation process by $\beta_{2b}$ may decrease peak $I_{Ba}$ if the rate constants of activation and inactivation are comparable (Aldrich et al., 1983; Hille, 2002). However, this artifact is probably negligible in $\alpha_{1C}$. Here, the time constant of the faster of the two components of macroscopic VDI of $I_{Ba}$ is $\sim 75$ ms for a short-NT isoform (Shi and Soldatov, 2002) and $>150$ ms with the long-NT $\alpha_{1C}$ (unpublished data). This is at least twofold slower than first latency time constant observed in single channel recordings ($\sim 25$–$35$ ms, see Hulín et al., 2003). Another artifact may arise when $G_{\text{max}}$ is estimated by fitting experimental $I$-$V$ curves to Boltzmann equation. In this case, $G$ usually appears to reach its maximum at $+40$ to $+50$ mV. $G$-$V$ curves based on the measurement of tail currents, which are devoid of certain inaccuracies inherent to $I$-$V$ curves (such as very small $I_{Ba}$ at $V_m$ close to the reversal potential), often imply that the $G$-$V$ curve has two components and does not reach a plateau at $+40$ to $+50$ mV, but close to $100$ mV (e.g., Takahashi et al., 2004). Assuming that this is the case and using the data from Takahashi et al. (2004), we calculated that we might have overestimated the changes in $G_{\text{max}}$ by no more than 30% (unpublished data). Since in $\alpha_{1C}$-wt the changes in $G_{\text{max}}$ caused by NT deletions or by $\beta_{2b}$ are between 260 and 770% (Tables II and III), such an error would not affect our main conclusions.

$\text{Ca}_\beta$-induced Changes in Voltage Dependency (G-$V$ curve) and in $P_{o,\text{max}}$ Are Separable

(a) Only the $\text{Ca}_\beta$-induced increase in $P_{o,\text{max}}$, but not the shift in $G$-$V$ curve, depends on the presence of the NTI module. In all mutants lacking the crucial part of the NTI module (aa 6–20 of long NT), $G$-$V$ curve shifts are like in $\alpha_{1C}$-wt, but $P_{o,\text{max}}$ is not changed by $\beta_{2b}$ (Table III). The mild approximately twofold increase in $G_{\text{max}}$ (or $I_{\text{d10}}$) caused by $\beta_{2b}$ in these mutants can be almost fully accounted for by the increase in $N$, which is 1.6–2.2-fold. Strikingly, in the short-NT isoform, the coexpression of $\beta_{2b}$ even causes an $\sim 20\%$ decrease in calculated $P_o$, despite a well-pronounced threefold increase in $I_{Ba}$ at 0 mV (a $V_m$ relevant for comparison with single-channel records). This increase is undoubtedly governed by the increase in $N$ (see below) and the leftward shift in the $G$-$V$ curve.

Published results (Table IV) fully support our data; separation of long-NT and short-NT isoforms eliminates controversies and allows solid conclusions. Coexpression of $\beta$ subunits increases the $P_o$ measured in single-channel patches at $-10$ to $+10$ mV in high-$\text{Ba}^{2+}$, in oocytes and in mammalian cells. However, the increase is consistently greater in the long-NT isoform (range: six to eightfold) than in the short-NT $\alpha_{1C}$ forms (range: two to fivefold). Data necessary for the calculation of the relative change in $P_{o,\text{max}}$ are available from fewer works, but also fit our results; coexpression of $\text{Ca}_\beta$ increases $P_{o,\text{max}}$ of the long-NT $\alpha_{1C}$ two to fourfold, whereas a mild $\sim 20\%$ decrease is observed in a short-NT $\alpha_{1C}$ isoform.

(b) Changes in $P_{o,\text{max}}$ caused by coexpression of $\text{Ca}_\beta$ are similar in Xenopus oocytes and mammalian cells (Table IV). In contrast, there is a consistent and striking difference in the reported shifts in the $G$-$V$ curve: changes in $\Delta V_{1/2}$ range from 12 to 22 mV in oocytes but are always $<10$ mV in mammalian cells. This intriguing difference calls for study and may provide new clues to understanding VDCC gating; but it also supports the disparity of mechanisms of the effects of $\text{Ca}_\beta$ on $P_{o,\text{max}}$ and on voltage dependence of activation.

On the basis of these arguments, we conclude that, most intriguingly, the two gating actions of $\text{Ca}_\beta$ that are most important in determining the amplitude of whole-cell $\text{Ca}^{2+}$ currents are separable, and therefore may rely on distinct molecular mechanisms. It is remarkable that one of the most prominent actions of $\text{Ca}_\beta$ can be selectively eliminated by removing a short segment of $\alpha_{1C}$ that does not even bind $\beta$, whereas other functions of $\text{Ca}_\beta$ remain unchanged. This finding provides a hint at how a single $\text{Ca}_\beta$ may regulate several different functions of $\alpha_{1C}$, without the need to assume the involvement of multiple $\beta$ subunits.

Changes in Surface Expression Caused by $\beta_{2b}$

Surface expression of voltage-dependent ion channels can be gauged by quantitative immunohistochemistry of various kinds, or by measuring the maximal nonlinear gating charge movement, $Q_{\text{max}}$. The immunohistochemical methods that use monitoring of external surface-exposed parts of channel with or without genetically introduced epitopes reliably monitor the total number of channels in PM (e.g., Zerangue et al., 1999; Altier et al., 2002; Canti et al., 2005; Leroy et al., 2005) but do not guarantee that all detected channels are conducting (functional). Similarly, $Q_{\text{max}}$ reflects the displacement of the voltage sensors in all channels in the PM, whether conducting or not. Relative changes in number of channel ($N$) caused by a regulatory factor (e.g., $\text{Ca}_\beta$) are faithfully reflected in changes of either surface labeling or $Q_{\text{max}}$; however, the latter is also altered by factors that alter the charge or movement of the voltage sensors (Aggarwal and MacKinnon, 1996; Barrett et al., 2005).

The prevailing view is that the $\text{Ca}_\beta$-induced increase in the amount of $\alpha_{1C}$ in the PM, reproducibly observed in mammalian cells including even cardiomyocytes (Table IV), does not occur in Xenopus oocytes. This notion is based on the reported absence of a significant $\text{Ca}_\beta$-induced increase in $Q_{\text{max}}$ measured in the oocytes using...
Regulation of $P_o$ of L-type Channel by $\beta$ Subunit

**A. Short-NT isoforms of $\alpha_{1C}$**

| Surface expression (fold increase) | $\Delta V_{1/2}$ (mV) | $P_{o,max}$ (fold increase) | $P_o$ at $-10$ to $0$ mV (fold increase) | Type of $\beta$ | Type of $\alpha_{1C}$ | Reference |
|-----------------------------------|-----------------------|-----------------------------|----------------------------------------|----------------|---------------------|-----------|
| Oocyte Oocyte Mamm cells Oocyte Mamm cells Oocyte Mamm cells Oocyte Mamm cells | | | | | | |
| 1.85\* | 16 | 0 | $0.83\*$ | | | |
| $\beta_{1a}$ | | Schultz | | | | Yamaguchi et al., 1998 |
| 3.4a | 16 | 0 | $0.83\*$ | | | |
| $\beta_{1a}$ | | Schultz | | | | Josephson and Yaradi, 1996 |
| 1.25\* | 19.5 | | | | | |
| $\beta_{1a}$ | | Schultz | | | | Yamaguchi et al., 2000 |
| 1.6 | 18.4 | | | | | |
| $\beta_{1a}$ | | Schultz | | | | Yamaguchi et al., 2000 |
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the cut-open voltage clamp, in which gating and ionic currents are measured from a large part of an internally perfused cell (Neely et al., 1993, 2004). In contrast, Yamaguchi et al. (1998, 2000) reported a threefold increase in α1C caused in Xenopus oocytes by either injecting a purified β3 subunit or by coexpressing β2a. Although imaging of an intracellular HA tag in permeabilized oocytes, used in these works to monitor surface expression of α1C, does not exclude the labeling of α1C located in submembrane ER, the approximately threefold increase is also supported by observing the enhancement of I_Ba at different times after the injection of the β3 protein (Yamaguchi et al., 1998; see Table IV legend for details). We have previously detected a β2a-induced, 20–55% increase in N measured by counting channels in cell-attached patches and by immuno-precipitating α1C from manually separated PM (Shistik et al., 1995), but it did not reach statistical significance. Interestingly, in the absence of αδ, even this small increase was not observed (Table IV).

To address the controversy and to measure changes in surface expression of α1C in oocytes as accurately as possible, in this work we employed two additional, independent methods: immunolabeling of α1C, tagged by an extracellular HA epitope, in whole oocytes; and imaging of immunolabeled α1C in giant membrane patches of oocytes. Both methods clearly demonstrated a reproducible, highly statistically significant, isoform-independent increase in PM expression of α1C induced by coexpression of β2a. The estimates obtained by the widely accepted external tag measurement were slightly but usually insignificantly greater than by imaging in giant patches, when measured with the same construct of the channel (2.2-fold vs. 1.7-fold increase in α1C wt, respectively). We conclude that β2a causes an approximately twofold increase in the PM content of α1C, in the presence of αδ. The reason for the discrepancy with Neely et al. (1993, 2004) remains unclear; it would be interesting to see whether a change in Q_max occurs when αδ is present. The increase in surface expression caused by coexpression Caβ is absolutely independent of NTI module, in sharp contrast with Po_max. These results corroborate the previously demonstrated separability of the effects of Caβ on surface expression and gating (Canti et al., 1999, 2001; Gerster et al., 1999).

The NTI Module and the Effect of Caβ

In this report we demonstrated that α1C variants lacking the NTI module (the various mutants and the short NT isoform) express in the PM at the same level as the long-NT isoform, though they give much larger macroscopic currents. These and our previous results (Shistik et al., 1998) firmly establish the role of the initial segment of long-NT as a regulator of gating, rather than expression, of α1C. In support, Agler et al. (2005) recently found that the NT also constitutes an inhibitory module in the N-type (Ca_{2.2}) channel; in contrast to Ca_{1.2}, in Ca_{2.2} the inhibitory effect of the NT is G protein gated.

Since the main effect of NTI is to regulate the voltage-independent parameter Po_max, it is possible that the removal of the NTI module causes a modal shift in gating, like Ca_{1β} (Costantin et al., 1998; Colecraft et al., 2002). If NTI module “tonically” restrains the opening of the channel’s gate at all voltages (Shistik et al., 1998), its removal would cause a voltage-independent transition from a low-Po mode to a high-Po mode, in which the voltage independent transition from the last closed to the open state is more favorable than in the low-Po mode. The slight change in the voltage dependence of activation in NT mutants lacking the NTI module may be due to a reequilibration among closed states, the transitions between which are voltage dependent. Further studies including single channel recordings will be required to test this conjecture.

We find that aa 6–20 constitute a crucial and necessary part of the inhibitory module; its deletion causes a maximal enhancement of Po_max and an almost complete disappearance of the enhancing effect of β2a, on Po_max. Nevertheless, aa 6–20 alone only partially recover the function of the NTI when added at the NH2 terminus of a α1CΔ46 mutant, which lacks all 46 initial amino acid residues (NTSL chimera). Full recovery of both functions of NTI (reduction in Po_max and enhancement of β2a-induced increase in Po_max) is achieved by the addition of aa 1–20 of the long-NT, which is thus established as a necessary and sufficient structural determinant of the NTI module. The function of aa 21–46, which link between the NTI module and the beginning of the highly conserved part of NT encoded by exon 2, is unknown at present. Our data conclusively demonstrate that the initial 16-aa NT segment of the short α1C isoform is not an inhibitory module. These 16 aa were unable to restore the NTI functions even in the context of a full 46-aa initial segment, i.e., in the presence of aa 2–5 and 21–46 of the long NT (NT1.5 chimera).

The interacting partners of NT in Ca_{1.2} are unknown. The regulation of inactivation kinetics of α1C (Ca_{1.2α}) by β2a involves voltage-dependent rearrangements of NT regions of α1C and β2a, as deduced from fluorescent resonance energy transfer (FRET) experiments (Kobrinsky et al., 2004). However, no physical interaction between NT of α1C and Caβ could be detected by direct biochemical measurements (Pragnell et al., 1994; Shistik et al., 1998), and the exact molecular interaction underlying the proximity between the NT of α1C and β2a, indicated by FRET is yet to be determined. No direct interaction of NT with L1 (which would explain the intensive interplay of Caβ function) could be detected in α1C (Agler et al., 2005). Another possible interactor is the CT of α1C. Similarly to NT, CT acts to prevent channel opening (Wei et al., 1994; Klockner et al., 1995; Gao et al., 2001; Mikala et al., 2003). Joint removal of NT
and distal CT causes a more-than-additive increase in $I_{Na}$ (Ivanina et al., 2000), suggesting that the relaxed state of the channel is a high-$P_o$ one, and the termini are vital to prevent uncontrolled entry of Ca$^{2+}$.

**Using Xenopus Oocytes vs. Mammalian Cells to Study the Effects of Ca$\beta$ on Ca$^{2+}$ Channels**

The validity of conclusions regarding Ca$\beta$ effects, especially changes in surface expression, obtained in *Xenopus* oocytes has often been met with reservations, because of the presence of an endogenous $\beta$ subunit. However, HEK cells also contain endogenous $\beta$ (Leroy et al., 2005). In this work we demonstrate that all major effects of Ca$\beta$ can be reproducibly observed and reliably studied in *Xenopus* oocytes. The Ca$\beta$-induced increase in surface $\alpha_{IC}$ in oocytes is in qualitative agreement with that in mammalian systems, although quantitatively the increase reported in mammalian cells is usually greater, more than fivefold (Table IV; but see Yasuda et al., 2004). The variability among different cell types may reflect variable levels of endogenous Ca$\beta$. The changes in gating parameters are also at least qualitatively (shift in G-V curve; see above) and quantitatively (change in $P_{o,max}$) similar.

**Physiological Significance**

Ca$\alpha_{1.2}$ channels are crucial for contraction of cardiac and smooth muscles, hormone secretion, and regulation of gene expression in the brain (Reuter, 1983; Finkbeiner and Greenberg, 1998; Catterall, 2000; West et al., 2001; Lipscombe et al., 2004). In humans, the Ca$\alpha_{1.2}$ gene, CACNA1C (Soldatov, 1994) contains 55 exons, of which 19 are subject to alternative splicing, giving rise to a very large number of isoforms (Tang et al., 2004). The exact exon combinations underlying the isoforms of $\alpha_{IC}$ in different tissues are yet to be catalogued (Abernethy and Soldatov, 2002; Jurkat-Rott and Lehmann-Horn, 2004; Tang et al., 2004). Long-NT isoform (isoforms?) is predominant in the heart but is also present in the brain, short-NT isoforms prevail in smooth muscle and brain (Slish et al., 1989; Koch et al., 1990; Snutch et al., 1991; Shistik et al., 1999; Blumenstein et al., 2002; Dai et al., 2002; Saada et al., 2003). At present we cannot exclude the possibility that in some of the short-NT isoforms of $\alpha_{IC}$ the function of the NT initial segment is different than in the isoform that we used here, due to an interaction with some alternative distant parts of $\alpha_{IC}$; this remains to be investigated.

The isoforms may substantially differ in their physiological and pharmacological properties. For instance, splice variants in the COOH-terminal region regulate the inactivation of the channel and its sensitivity to certain pharmacological agents (Abernethy and Soldatov, 2002). The present work reveals a strikingly disparate regulation of two very similar isoforms of a Ca$\alpha$ channel by a Ca$\beta$ subunit. It is notable that, in addition to the two isoforms of $\alpha_{IC}$ corresponding to splice variants with the alternative first exons 1 and 1a, another short-NT isoform lacking either of these exons and starting from exon 2 may exist in human tissues (Schultz et al., 1993; Saada et al., 2003). This would correspond to the truncation mutant $\alpha_{IC}\Delta46$ or, if the protein starts with the first methionine of exon 2, to $\alpha_{IC}\Delta59$ (see Fig. 1). The differences in function of NH$_2$ termini of these isoforms, discovered in this paper, are expected to have significant physiological implications. They will result in substantial differences in amplitudes of macroscopic Ca$^{2+}$ currents and in the effects of Ca$\beta$ (whose association with $\alpha_1$ may change in development and growth; see Spafford et al., 2004) or regulators that act in a Ca$\beta$-dependent manner, such as protein kinase A (Bunemann et al., 1999).

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