Voltage-Gated Rearrangements Associated with Differential β-Subunit Modulation of the L-Type Ca\(^{2+}\) Channel Inactivation

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ABSTRACT Auxiliary β-subunits bound to the cytoplasmic α\(_1\)-interaction domain of the pore-forming α\(_{1C}\)-subunit are important modulators of voltage-gated Ca\(^{2+}\) channels. The underlying mechanisms are not yet well understood. We investigated correlations between differential modulation of inactivation by β\(_{1a}\) and β\(_{2}\) subunits and structural responses of the channel to transition into distinct functional states. The NH\(_2\)-termini of the α\(_{1C}\) and β-subunits were fused with cyan or yellow fluorescent proteins, and functionally coexpressed in COS1 cells. Fluorescence resonance energy transfer (FRET) between them or with membrane-trapped probes was measured in live cells under voltage clamp. It was found that in the resting state, the tagged NH\(_2\)-termini of the α\(_{1C}\) and β-subunit fluorophores are separated. Voltage-dependent inactivation generates strong FRET between α\(_{1C}\) and β\(_{1a}\) suggesting mutual reorientation of the NH\(_2\)-termini, but their distance vis-à-vis the plasma membrane is not appreciably changed. These voltage-gated rearrangements were substantially reduced when the β\(_{1a}\)-subunit was replaced by β\(_{2}\). Differential β-subunit modulation of inactivation and of FRET between α\(_{1C}\) and β were eliminated by inhibition of the slow inactivation. Thus, differential β-subunit modulation of inactivation correlates with the voltage-gated motion between the NH\(_2\)-termini of α\(_{1C}\) and β-subunits and targets the mechanism of slow voltage-dependent inactivation.

INTRODUCTION

Calcium channels respond to membrane depolarization with an increase in membrane permeability that develops within a few milliseconds and then spontaneously decreases (inactivates) with characteristic kinetics. In the case of the L-type class C voltage-sensitive Ca\(^{2+}\) channel (Ca\(_{1.2}\)), the replacement of Ca\(^{2+}\) with Ba\(^{2+}\) ions as the charge carrier eliminates Ca\(^{2+}\)-induced inactivation, and the Ba\(^{2+}\) current inactivates essentially in a voltage-dependent manner. Given the critical role of Ca\(_{1.2}\) channels in cell signaling (Soldatov, 2003), investigation of molecular correlates of inactivation has been an objective of a number of laboratories, including those cited in this article.

The Ca\(_{1.2}\) channel is composed of the pore-forming α\(_{1C}\)-subunit and the auxiliary β- and α\(_{2}\)δ-subunits. All three subunits can be colocalized along the plasma membrane in transfected cells expressing the functional recombinant channel (Gao et al., 1999). The subunits remain associated with each other after solubilization of the channel protein complex in mild nonionic detergents (Chang and Hosey, 1988) suggesting close association between the subunits in vivo. In fact, binding motifs involved in interactions between the α\(_{1C}\) and β-subunits (Pragnell et al., 1994) as well as between the α\(_{1C}\)- and α\(_{2}\)δ-subunits (Gurnett and Campbell, 1996) have been identified.

The β-subunits are important modulators of the Ca\(^{2+}\) channel activity (Birnbaumer et al., 1998; Yamaguchi et al., 1998; Colecraft et al., 2002; Hullin et al., 2003; Takahashi et al., 2003). They are located on the cytoplasmic side of plasma membrane and generate the molecular signal necessary for correct plasma membrane targeting of the functional Ca\(_{1.2}\) complexes. Each of the four identified different types of β-subunit (β\(_{1}\), β\(_{2}\), β\(_{3}\), and β\(_{4}\)) binds, via the NH\(_2\)-terminal part of the second common conserved region (amino acids 262–290 in β\(_{1}\) and 215–243 in β\(_{2}\)), to the α\(_{1}\)-interaction domain (AID), a conserved motif in the cytoplasmic linker between repeats I and II of the α\(_{1}\)-subunit (Pragnell et al., 1994; De Waard et al., 1994). This binding was shown to be a molecular correlate for the well-established effect of β-subunits on the kinetics of Ca\(_{1.2}\) channel gating (Singer et al., 1991). Inactivation of the Ca\(_{1.2}\) channel is accelerated by the replacement of β\(_{2}\) with the β\(_{1a}\)-subunit. The modulatory effect of the β-subunit was observed in other types of Ca\(^{2+}\) channels as well (Hummer et al., 2003). For example, in Ca\(_{2.1}\) (α\(_{1A}\)) P/Q-type channel, De Waard and Campbell (1995) identified β\(_{2a}\) as the least potent among four other types of β-subunit in acceleration of inactivation. Thus, β-subunits modulate both the functional expression and the gating of ion conductance.

Recently we have developed a new method for measuring dynamic structural responses to changes in the cell membrane potential (Kobrinsky et al., 2003). Using measurements of fluorescence resonance energy transfer (FRET) between enhanced cyan (ECFP) and yellow (EYFP) fluorescent proteins under voltage-clamp conditions in live cells, we provided evidence that ECFP/EYFP-tagged...
cytoplasmic tails of the Ca,1.2 channel are moving parts, responsive to voltage gating. The voltage-dependent mobility of the COOH-terminal tail was found to be essential for Ca\(^{2+}\)-dependent inactivation and Ca\(^{2+}\) signal transduction.

Here we have applied FRET imaging in voltage-clamped living COS1 cells to directly compare the interaction between \(\alpha_{1C}\) and different \(\beta\)-subunits in different functional states of the channel, and to determine molecular motions of the \(\beta\)-subunits vis-à-vis \(\alpha_{1C}\) and the plasma membrane. Two pore-forming \(\alpha_{1C}\)-subunits showing different inactivation properties were used. The \(\alpha_{1C,77}\)-subunit has the structure of the most common \(\alpha_{1C}\) splice variant and thus can be referred to as the “wild-type” isoform. The \(\alpha_{1C,1S,IV}\)-subunit gives rise to a channel that does not show the slow voltage-dependent inactivation (Shi and Soldatov, 2002). This isoform was generated by the mutation of the following four amino acids in the transmembrane segments S6 of the \(\alpha_{1C,77}\)-subunit: S405I in IS6, A752T in IIS6, V1165T in IIIS6, and I1475T in IVS6. These mutations are located in positions −1 (IS6) and −2 (IIS6–IVS6) and thus form a ring of altered amino acid residues at the inner mouth of the pore. The corresponding structure in the \(\alpha_{1C,77}\) channel was named the “annular determinant of slow inactivation” (ADSI) reflecting the specific arrangement of crucial amino acids with regards to the ion-conducting pore.

The \(\beta\)-subunit variants \(\beta_{1a}\), cloned from rabbit skeletal muscle (Mori et al., 1991), and \(\beta_{2}\), originally cloned from rabbit heart (Hullin et al., 1992), have been studied in this work as modulators of Ca\(^{2+}\) channel inactivation. The rabbit \(\beta_{2}\)-subunit has 92.6% overall amino acid identity to the rat \(\beta_{2}\) protein (Perez-Reyes et al., 1992) with some variations (17 amino acids) at the NH\(_2\)-terminus, and lacks the NH\(_2\)-terminal cysteine sites of palmitoylation. The differential effect of these \(\beta_{1a}\)– and \(\beta_{2}\)-subunits on voltage-dependent inactivation of the \(\alpha_{1C,77}\) channel expressed in Xenopus oocytes has been described (Soldatov et al., 1997). These results confirmed other reports on the acceleration of the Ba\(^{2+}\) current decay by the \(\beta_{1a}\)-subunit as compared with \(\beta_{2}\) (Birnbaumer et al., 1998; Colecraft et al., 2002). This \(\beta\)-subunit dependence of inactivation, defined here as differential \(\beta\)-subunit modulation of inactivation, may be associated with the different ability of \(\beta\)-subunits, permanently bound to AID, to freely move with the cytoplasmic linker between repeats I and II of the \(\alpha_{1C}\)-subunit, thus serving as a voltage-dependent inactivation particle (Restituito et al., 2000).

The aim of this work was to investigate the correlation between the effects of \(\beta_{1a}\)- and \(\beta_{2}\)-subunits on inactivation of the \(\alpha_{1C,77}\) and \(\alpha_{1C,1S,IV}\) channels and the voltage-dependent rearrangements between the \(\alpha_{1C}\) and \(\beta\)-subunits using FRET microscopy combined with voltage clamp in live cells (Kobrinsky et al., 2003). This method of measuring the dynamic structural responses to changes in cell membrane potential was used to determine the differences in relative proximity and/or angular orientation between the NH\(_{2}\)-terminal regions of different \(\alpha_{1C}\)- and \(\beta\)-subunits, as well as relative to the plasma membrane when channels are in the resting, inactivated, or conducting states.

**MATERIALS AND METHODS**

**Molecular biology**

Preparation of plasmids encoding \(\alpha_{1C,77}\) (Soldatov et al., 1995) and \(\alpha_{1C,1S,IV}\) (Shi and Soldatov, 2002) in pBluescript vector for expression in Xenopus oocytes and plasmids encoding \(\alpha_{1C,77}\), \(\beta_{1a}\) (Mori et al., 1991), and \(\alpha_{1C,77}\)– and \(\beta_{1a}\)-subunits using T7/SP6 mMessage mMachine (Ambion, Austin, TX).

**Cell cultures**

COS1 cells used in experiments did not show appreciable endogenous expression of Ca,1.2 channels (Meir et al., 2000). HEK293 cells were selected for immunoprecipitation and single-channel analyses (Kepplinger et al., 2000), because of the fast time course and high level of transient expression of recombinant Ca,1.2 channels. COS1 cells are particularly useful for single-cell electrophysiology, because they divide slower than HEK293 cells and allow for tighter control over the efficiency of expression of proteins of different size. COS1 cells were grown on poly-d-lysine coated coverslips (MatTek, Ashland, MA) in DMEM supplemented with 10% fetal calf serum. HEK293 cells used for immunoprecipitation and COS1 cells were transfected with cDNAs coding for the indicated \(\alpha_{1C}\), \(\beta\), and \(\alpha_{1C}\)-subunits of the Ca\(^{2+}\) channel (1:1:1, w/w) using the Lipofectamine (Invitrogen, Carlsbad, CA) or Effectene kit (Qiagen, Venlo, The Netherlands), respectively. In some experiments with COS1 cells, (ECFP)\(_{N}\)-labeled PH domains (van der Wal et al., 2001) were coexpressed. HEK293 cells used for single-channel experiments were transfected 24–72 h before recordings with cDNAs coding for \(\alpha_{1C,77}\) or (EYFP)\(_{N}\)-labeled \(\alpha_{1C}\)– and \(\beta\)-subunits using SuperFect transfection reagent (Qiagen). Unlike the \(\alpha_{1C,77}\) channel, inactivation of which was somewhat (≈15%) accelerated by the N-terminal EYFP labeling, the decay of the Ca\(^{2+}\) current through the \(\alpha_{1C,1S,IV}\) was not significantly affected by the double ECFP/EYFP labeling (Kobrinsky et al., 2003). The rationale for using the cDNA coding for (EYFP)\(_{N}\)-labeled \(\alpha_{1C}\)- and \(\alpha_{1C,1S,IV}\) channels was to facilitate identification of the expressing cells. No functional expression of the \(\alpha_{1C,77}\) or \(\alpha_{1C,1S,IV}\) channels was observed in COS1 cells in the absence of \(\beta\)-subunits.

**Immunoprecipitation analysis**

HEK293 cells were transfected in 75-cm\(^2\) flasks with the mixture of \(\alpha_{1C}\), \(\alpha_{1C}\)- and (EYFP)\(_{N}\)-labeled \(\beta_{1a}\) or (ECFP)\(_{N}\)-labeled \(\beta_{2}\)-subunits as described.
above. After 48–72 h, the cells were collected in 0.5 ml of Tris-buffered saline (150 mM NaCl, 50 mM Tris-HCl, pH 8.0) containing a mixture of protease inhibitors composed of 1 mM PMSF, 1 mM DTT, and 5 μl of protease inhibitor cocktail (P8340, Sigma, St. Louis, MO). To remove ‘orphan’ β-subunits, not associated with the membrane-bound α_{iC} channels, cells were quickly freeze-thawed three times and then permeabilized with digitonin (20 μg/ml) for 5 min at 4°C. Lysed cells were pelleted for 30 min at 12,000 x g, 4°C and solubilized with 0.5% Nonidet P-40. The cell extract was centrifuged for 30 min at 12,000 x g, 4°C. Equal amounts of protein extracts, determined by BCA protein assay (Pierce, Rockford, IL), were diluted twofold with 0.3 M KCl containing 50 mM Tris-HCl, pH 8.0, the mixture of protease inhibitors, and 10% glycerol. Extracts were incubated overnight at 4°C on a rotary shaker with 10 μl of Living Color Full-Length A.V. polyclonal antibody (BD Biosciences Clontech) prebound to 60 μl of Protein A Sepharose Fast Flow (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Protein A-sepharose beads were washed at 4°C for 15 min with the wash buffer (0.2% Nonidet P40, 50 mM Tris-HCl, pH 8.0, and protease inhibitors) containing 0.5 M KCl, then quickly washed twice with 0.3 M KCl in wash buffer, and then 30 min with 0.15 M KCl in wash buffer. The resin was finally rinsed twice briefly with 0.5 M KCl in wash buffer, and the retained proteins were eluted by incubation for 5 min at 95°C with 40 μl of SDS-sample buffer containing 2% SDS, 10 mM Tris-HCl (pH 6.8), 7.5% sucrose, and 0.5 M 2-mercaptoethanol. Proteins were separated by SDS-gel electrophoresis on a precasted 4–12% gradient polyacrylamide gel (Invitrogen) and transferred to a 0.45 μm PVDF membrane (BioRad, Hercules, CA). The (ECFP/EYFP) N-labeled α_{iC} subunits were identified on Western blots with Living Color monoclonal antibody JL-8 (1:4000 dilution, BD Biosciences Clontech), whereas the coprecipitated α_{iC}–subunits were detected with the affinity purified rabbit anti-α_{iC} calcium channel polyclonal antibody (1:10,000; Chemicon International, Temecula, CA) using an ECL Plus Western blotting detection system (Amersham Pharmacia Biotech). Expression of labeled β-subunits was routinely monitored by the conventional epifluorescent microscopy.

**FRET imaging**

Images were recorded in live transfected COS1 cells with a Hamamatsu digital camera C4742-95 (Hamamatsu City, Japan) mounted on the Nikon (Melville, NY) epifluorescent microscope TE2000 (60 x 1.2 n.a. objective) equipped with multiple filter sets (Chroma Technology, Rockingham, VT). Excitation light was delivered by a 75-W Xenon lamp (Woburn, MA). C-Imaging (Compix, Tuatulian, OR) and MetaMorph (Universal Imaging, Downingtown, PA) software were used to obtain and analyze FRET images. FRET was quantified with three filter sets: for EYFP cube, excitation filter 500/20 nm, dichroic beam splitter 515 nm, emission filter 535/30 nm; for FRET (ECFP/EYFP) cube, excitation 436/20 nm, dichroic beam splitter 455 nm, emission filter 540/30 nm; and for ECFP cube, excitation filter 436/20 nm, dichroic beam splitter 455 nm, emission filter 480/40 nm. Regions of interest (ROI) were selected from plasma membrane sites where there was obvious signal of fluorescence and little or no interference from fluorescence from the intracellular compartment. Selection of ROIs was carried out using the C-Imaging software where intensity (I) from three filter sets was determined after background subtraction. Fluorescence values were averaged across the ROI (with one ROI per cell). Inside the ROIs some areas did not show substantial FRET. These areas were also included in the analysis. The full scale of digitized resolution ranged from 0 to 255. Because fluorescence in ROIs, containing typically ~1000 pixels, was averaged, the signal/noise ratio was, respectively, increased by two to three orders and thus resolution of FRET detection was more refined. Corrected intensity of FRET \( I_{\text{FRET}} \) was calculated as \( I_{\text{FRET}} = I_{\text{ECFP}} - I_{\text{EYFP}} \), where bleached-through coefficients were experimentally determined by calibration according to Xia and Liu (2001). This calibration is based on the measurements of fluorescence of all ECFP- and EYFP-labeled constructs expressed in COS1 cells in all three filters set to determine bleed-through in CFP, YFP, and FRET filters (for additional details, see Kobrinsky et al., 2003). The typical values of corrected FRET varied from 10 to 40. To quantify FRET, we used a generally accepted correction method (Xia and Liu, 2001). Corrected FRET values were normalized against donor and acceptor levels: \( N_{\text{FRET}} = (I_{\text{FRET}})/(I_{\text{EYFP}} \times I_{\text{ECFP}})^{1/2} \). Under these conditions, FRET was directly demonstrated with the positive control of the coexpressed mixture of EYFP- and ECFP-labeled pleckstrin homology (PH) domains of phospholipase C61 (Kobrinsky et al., 2003). Acceptor photobleaching (on average >90% for 15 min with the 100-W mercury lamp) gave a FRET efficiency of \( 0.209 \pm 0.054 \) (\( n = 17 \)), calculated as \( (I_{\text{ECFP}} - I_{\text{EYFP}})/(I_{\text{EYFP}} + I_{\text{ECFP}}) \), where \( I_{\text{EYFP}} \) and \( I_{\text{ECFP}} \) are the intensities of ECFP fluorescence before and after acceptor (EYFP) photobleaching. The relative changes in FRET signal were determined from the pairs of consecutive FRET acquisitions recorded at two different transmembrane voltages (–90 and +20 mV), stabilizing the channel in different functional states. Acquisitions of fluorescence were obtained during the application of a conditioning pulse with simultaneous recordings of the current at indicated voltages and within the time window, ranging from 50 to 300 ms. In a recent comprehensive study comparing different FRET measurement methods (Berney and Danuser, 2003), it has been shown that use of corrected FRET values to present relative changes in FRET signal is adequate if the ratio of donor (ECFP) to acceptor (EYFP) is \( \geq 1 \). This requirement was fulfilled throughout our study. In some cases, the entire consecutive image was shifted by one or two pixels and required pixel-by-pixel adjustment using the first (reference) image with designated ROI. Such a shift could easily be detected by comparison with the borders of ROI designated for the reference image.

Acceptor (EYFP) photobleaching was performed with custom YFP photobleaching cube (Chroma Technology), consisting of a D553/50 x excitation filter and 100% mirror (instead of a dichroic one); this bleaching cube spared the CFP chromophore in control experiments. The 175-W Xenon lamp was used for illumination.

For illustration purposes, results of the ratio of FRET measurements, not limited just with ROI, are presented as fluorescence images. The first step in the processing of such an illustrating image is subtraction of the background, which is typically 10–12 units on the full fluorescence scale of 255 units. Background was measured in the part of the image that was not affected by cell fluorescence or in a separate dish containing nonfluorescent cells. EYFP and ECFP images with subtracted background were fractionally subtracted from raw FRET images based on measurements of bleed-through coefficients. The fractional subtraction generated corrected FRET images, showing sensitized FRET in COS1 cells. Because C-Imaging software converted possible negative values of fluorescence to zero, after this procedure, an average ‘corrected background’ was typically 0 or 1. Although subtraction of images recorded at –90 and +20 mV gives absolute changes of FRET, the ratio of images allows for comparison between different experiments and thus was used in our research. To obtain illustrating images of the FRET ratio at ±20 mV/–90 mV, an empirical constant value, typically equal to 5 on the fluorescence scale, was added to each pixel of both images corrected by the background subtraction. This was required to avoid division by zero background fluorescence of a corrected image. With noise ranging from 0 to 12 and the full scale of the measurement at 255, this operation did not substantially affect the relationship between the processed image and the actual calculation of corrected FRET in ROI, which did not include such processing.

**Electrophysiology**

Three different types of electrophysiological measurements were used. Two-electrode voltage clamp recording of Ba^{2+} currents through the wild-type and mutated Ca^{2+} channels expressed in Xenopus oocytes was carried out as described previously (Shi and Soldatow, 2002). To inhibit Ba^{2+}-activated Cl− current, the oocytes were injected with 50 nl of 100 mM 1,2-bis(o-aminophenoxy)ethane-N,N',N'-tetraacetate (BAPTA)-Cs (pH 7.4) 30 min before recordings.
All FRET measurements were combined with whole-cell patch clamp. Patch-clamp recording of the Ba$^{2+}$ current in COS1 cells was carried out using the Axopatch 200B amplifier (Axon Instruments, Union City, CA) 48–72 h after transfection. The extracellular bath solution contained (in mM): NaCl 100, BaCl$_2$ 20, MgCl$_2$ 1, glucose 10, HEPES 10, pH 7.4. The pipettes had resistance 3–6 MΩ and were filled with pipette solution containing (in mM): CsCl 110, MgATP 5, BAPTA 10, tetraethylammonium 20, cAMP 0.2, HEPES 20, pH 7.4 (Soldatov et al., 2000). Currents were sampled at 2.5–5 kHz and filtered at 1 kHz. Voltage protocols were generated and data were digitized, recorded, and analyzed using pClamp 8.1 software (Axon). Calculated parameters are means ± SEM. The unpaired t-test was used to compare the $\alpha_{1C,77}/\beta$ and $\alpha_{1C,IS-IV}/\beta$ channels.

In single-channel recordings, the bath solution contained (in mM): t-aspartic acid 110, KCl 20, MgCl$_2$ 2, EGTA 2, HEPES 20, pH 7.4 (KOH). The pipette solution contained (in mM): BaCl$_2$ 96, HEPES 5, pH 7.4 (NaOH). The pipettes were made of borosilicate glass, coated with SigmaCote (Sigma) to reduce pipette capacitance, heat polished, and showed resistances of 3–4.5 MΩ. Voltage protocols were applied and single-channel current traces were recorded using pClamp 6 software (Axon). Interpulse potential was held at −80 mV for 5 s, after depolarization pulse to 0 mV (maximum of activity) for 1 s. Currents were sampled at 10 kHz and filtered at 1 kHz. Analysis of single-channel data (average open probability, P_o) was performed with Matlab software (MathWorks, Natick, MA) using algorithms according to Schmid et al. (1995) and Baumgartner et al. (1997). In experiments where more than one single channel was visible, the number of channels was estimated by the maximum number of overlapping openings seen within the 80–140 traces as described by Baumgartner et al. (1997). All measurements were carried out at 20–22°C.

RESULTS

Lack of differential β-subunit modulation of the $\alpha_{1C,IS-IV}$ channel inactivation

For comparison with our previous results (Shi and Soldatov, 2002), the electrophysiological study of inactivation of the $\alpha_{1C,77}$ and $\alpha_{1C,IS-IV}$ channels were initially carried out in the *Xenopus* oocyte expression system. Expression in oocytes provided control over subunit composition of the Ca$^{2+}$-channels and yielded results fully compatible with those acquired by the whole-cell patch clamp recording in COS1 cells used for the FRET microscopy (see below). Differential β-subunit modulation of the Ba$^{2+}$ current through the wild-type ($\alpha_{1C,77}$) channel is illustrated in Fig. 1 A as a difference in kinetics of the current inactivation conveyed to the channel by coexpression of $\beta_{1a}$ and $\beta_{2}$-subunits. Replacement of the $\beta_{2}$-subunit with $\beta_{1a}$ significantly accelerated decay of the Ba$^{2+}$ current, which supports results of other studies reviewed by Birnbaumer et al. (1998).

Fig. 1 B shows results obtained with the $\alpha_{1C,IS-IV}$ channel coexpressed with the $\beta_{1a}$- and $\alpha_{8}$-subunits in *Xenopus* oocytes. In complete agreement with our previous report (Shi and Soldatov, 2002), ≈50% of the maximum Ba$^{2+}$ (or Ca$^{2+}$) current through the $\alpha_{1C,IS-IV}/\beta_{1a}$ channel was inactivated with a single-exponential decay ($\tau_i = 11.7 ± 0.3$ ms; $n = 34$), did not show Ca$^{2+}$-dependent inactivation, and completely recovered from voltage-dependent inactivation within < 100 ms. The sustained fraction of the current (45.2 ± 2.1%, $n = 18$) lasted for up to 30 s without decay (Fig. 1B, top trace). Coexpression of the $\beta_{2}$-subunit instead of $\beta_{1a}$ did not change the electrophysiological properties of the $\alpha_{1C,IS-IV}$ channel except for some increase (≈10%, $p < 0.05$) in the amplitude of the sustained Ba$^{2+}$ current to 56.6 ± 4.4% ($n = 5$) of the peak current (Fig. 1 B, bottom trace). The time constant for the fast component of inactivation was not significantly changed ($\tau_i = 12.4 ± 0.7$ ms; $n = 5$).

Data summarized in Fig. 2 show that none of the tested kinetics or voltage-dependent characteristics of the Ba$^{2+}$ current through the $\alpha_{1C,IS-IV}$ channel were notably affected by the coexpression of the $\beta_{2}$-subunit instead of $\beta_{1a}$. The sustained current was observed over a wide range of membrane potentials (Fig. 2 A) and, on average, accounted for 50–55% of the peak current. No significant differences in the current-voltage relationships for the peak and sustained Ba$^{2+}$ current or the time course of fast inactivation (Fig. 2 B) were found between the $\alpha_{1C,IS-IV}$ channels coexpressed with $\beta_{1a}$ and $\beta_{2}$-subunits. The values for half-maximal activation $V_{0.5} = 2.1 ± 1.3$ mV (slope factor $k_{1V} = −9.1 ± 1.3$ mV; $n = 10$) with $\beta_{1a}$ and 3.9 ± 2.0 (k$_{1V} = −8.9 ± 0.7$; $n = 3$) with $\beta_{2}$ were not significantly different ($p < 0.002$; unpaired t-test). The sustained Ba$^{2+}$ current did not show decay even with prolonged depolarization (Fig. 2 C). Similar to the $\alpha_{1C,IS-IV}/\beta_{1a}$ channel, the $\alpha_{1C,IS-IV}/\beta_{2}$ channel was characterized by high steady-state availability at positive voltages (Fig. 2 D) and rapid recovery from inactivation within ≈100 ms (data not shown). Thus, it seems that inhibition of slow voltage-dependent inactivation interferes with differential β-subunit modulation of the kinetics of inactivation. Based on this observation, we hypothesize that
β-subunits modulate the kinetics of the Ba\(^{2+}\) current decay by targeting the slow inactivation mechanism of the channel.

Comparison of the effects of the β\(_{1a}\) and β\(_{2}\)-subunits on Ca\(_{1.2}\) channels with different inactivation properties

To investigate whether the inhibition of slow inactivation of the Ca\(_{1.2}\) channel affects the interaction between the α\(_1c\) and β-subunits, commounprecipitation of the β\(_{1a}\) or β\(_{2}\)-subunits with the wild-type α\(_{1c,77}\) and mutated α\(_{1c,IS-IV}\)-subunits was analyzed by Western blot assay. The (EYFP/ECFP)-tagged β\(_{1a}\) or β\(_{2}\)-subunits were functionally coexpressed in HEK293 cells with the α\(_{2}\)- and α\(_{1c,77}\)-subunits. Putative “orphan” cytosolic β-subunits not associated with membrane-bound α\(_1c\) were removed by cell lysis upon three cycles of freeze-thaw followed with brief permeabilization by low concentration of digitonin.

The crude membrane particulate fraction of HEK293 cells contained “orphan” α\(_{1c}\)-subunits and those associated with the labeled β-subunits. The latter were immunoprecipitated from solubilized membranes as α\(_{1c}/(ECFP)\_N-β\) complexes by antibody against GFP variants, thus eliminating contamination with the “orphan” α\(_{1c}\)-subunits. The immunoprecipitated complexes of the α\(_{1c}/(ECFP)\_N-β\) complexes were analyzed with SDS-polyacrylamide gel electrophoresis and immunoblotting (Fig. 3 A). Western blots showed that both types of the β-subunit pulled down the α\(_{1c,77}\) and α\(_{1c,IS-IV}\) proteins and thus confirmed physical association of the β\(_{1a}\) or β\(_{2}\)-subunits with either channel. Supporting experiment with commounprecipitation of the unlabeled β\(_{1a}\)- or β\(_{2}\)-subunits with the (EYFP)/(ECFP)-tagged α\(_{1c,77}\) and α\(_{1c,IS-IV}\) subunits corroborated this result (data not shown).

Recordings of the single-channel activity (Fig. 3 B) of α\(_{1c,77}\) and α\(_{1c,IS-IV}\) coexpressed with the α\(_{2}\)- and β\(_{1a}\) or β\(_{2}\)-subunits in HEK293 cells provided insight into their gating behavior, particularly at the end of the 1-s depolarizing pulse where the differences in β-subunit modulation of inactivation become especially clear and where FRET images were taken (see below). It is evident (Fig. 3 B, left) that single-channel activity with the α\(_{1c,77}/β_2\) channel is sustained at the end of a 1-s depolarization in contrast to the rather low activity of the α\(_{1c,77}/β_1a\) channel. Confirming data obtained with macroscopic currents, the α\(_{1c,IS-IV}\)-mutant with β\(_{1a}\) or β\(_{2}\) showed a similar low activity that was independent of the type of the coexpressed β-subunit and was sustained during depolarization (Fig. 3 B, right). Consistent with this observation was the average open probability (N.p) of the Ba\(^{2+}\) current calculated for the time window of 800–950 ms at the end of each 1-s depolarization (Fig. 3 C). The α\(_{1c,77}/β_2\) channel demonstrated the highest N.p\(_{800-950\text{ms}}\), whereas the α\(_{1c,77}/β_1a\) channel and α\(_{1c,IS-IV}\) coexpressed with either β\(_{1a}\) or β\(_{2}\)-subunit show no significant difference in low N.p\(_{800-950\text{ms}}\) values. These various patterns of activity (N.p\(_{800-950\text{ms}}\)) are not due to different number of channels N, but occurred rather due to different p\(_{800-950\text{ms}}\) as evident from Fig. 3 D. Furthermore, the ratio of average open probabilities determined between the 0–50-ms and 800–950-ms time windows of each recording (Fig. 3 B, boxed) during a 1-s depolarization was significantly higher for the α\(_{1c,77}/β_1a\) channel compared to the other α\(_{1c}/β\)-subunit combinations (Fig. 3 E). This result corresponds to the most extensive inactivation of the α\(_{1c,77}/β_1a\) channel among the channels tested in the whole-cell experiments and is consistent with the similarly low extent of inactivation for the α\(_{1c,77}/β_2\) channel and those of α\(_{1c,IS-IV}\) coexpressed with either β\(_{1a}\) or β\(_{2}\)-subunit. Taken together, these data indicate that mutation of ADSI does not abolish the stable association between β- and α\(_1c\)-subunits, but leads to the loss of differential β-subunit modulation of inactivation.
Voltage-dependent rearrangement between the αIC,77- and β1a-subunits

FRET is a result of the molecular interaction between the labeled partners (Lakowicz, 1999). FRET measurement with simultaneous voltage clamp gives an opportunity to eliminate possible artifacts associated with “orphan” subunits in live cells and focus exclusively on tagged αIC- and β-subunits of fully functional channels. One of the most commonly used approaches is to measure FRET efficiency by acceptor photobleaching. Specially designed Chroma D535/50 × excitation filter (Erickson et al., 2003) provided ≈90% photobleaching of EYFP in 2 min, a time sufficient to determine FRET efficiency under conditions when stability of a patch is not compromised. Recently Tour et al. (2003) reported on the high photosensitivity of labeled Cav1.2 channel to rundown. We found that EYFP photobleaching greatly accelerated rundown of the (EYFP)N-αIC,77 or (EYFP)N-αIC,IS-IV channel expressed with αIC- and β1a- or β2-subunits in HEK293 cells. Currents were elicited by 1-s depolarizations to 0 mV from the holding potential of −80 mV. Boxed are 0–50 ms and 800–950 ms episodes used for the subsequent analysis. Average open probabilities determined as N.p (C) or p (D) at the 800–950 ms time window of a 1-s depolarization (boxed in B). For each independent experiment, between 80 and 140 traces were analyzed (number of experiments is shown in parentheses). Only the average open probability for αIC,77β1a channel was significantly (p < 0.05) different to the other αIC/β channels. (E) Ratio of average open probabilities (determined as N.p) between the 0–50-ms and 800–950-ms time windows (boxed in B) during a 1-s depolarization. Only the ratio calculated for the αIC,77/β1a channel was significantly (p < 0.05) different from the other αIC/β channels.
Additionally normalized to the direct donor and acceptor intensity according to Xia and Liu (2001). Fluorescence images b and c (Fig. 4A) indicate that coexpressed (ECPF)N-Ca1c,77 and (EYFP)N-Ca1c,77 subunits are similarly distributed in the cell. We focused our analysis on the plasma membrane region, where the voltage-gated functional channel molecules reside. A FRET measurement of the same cell recorded at −90 mV (panel d) shows that N\textsubscript{FRET} between these subunits was rather small. Depolarization to +20 mV (Fig. 4A, panel e) resulted in a substantial increase in N\textsubscript{FRET} between the (ECPF)N-Ca1a and (EYFP)N-Ca1c,77 subunits (Table 1), which was confined to the rim of the cell, consistent with plasma membrane localization (see digitally magnified regions at the bottom of the whole-cell images).

FRET experiments were designed to estimate energy transfer between the fluorophores of the interacting subunits that was exclusively the result of structural rearrangements of the channel induced by the controlled changes of transmembrane voltage. When a cell is maintained at the holding potential of −90 mV, the (ECPF)N-Ca1a/(EYFP)N-Ca1c,77 channels are in the resting closed state characterized by low FRET (Table 1). Depolarization to +20 mV, corresponding to the maximum of the current-voltage relationship, induced opening of the (ECPF)N-Ca1a/(EYFP)N-Ca1c,77 channels followed by their voltage-dependent inactivation recorded as a rapid decay of the Ba\textsuperscript{2+} current. Therefore, the +20 mV image (Fig. 4A, panel e) corresponding mainly to the inactivated state of the channels was recorded only after inactivation was essentially complete (i.e., the whole-cell current decayed to the dotted line, which marks the initial zero level of the current). The respective time window for the acquisition of the +20 mV image is labeled by the red bar above the current trace on Fig. 4A. The ratio of the two consecutive images recorded at −90 and +20 mV (Fig. 4A, panel f) represents only those changes in FRET that were due to the voltage-gated rearrangements in the channel from the resting to the predominantly inactivated state. Measured FRET correlates with the state of the channel, because reversible changes of FRET between (EYFP)N-Ca1c,77 and (ECPF)N-Ca1a were observed every time inactivation had developed in response to depolarization from −90 mV to +20 mV.

When coexpressed with the unlabeled Ca1c,77- and Ca5δ-subunits, the (ECPF)N-labeled Ca1a- and Ca1b-subunits did not show voltage-dependent changes in ECPF fluorescence. The ratio of ECPF fluorescence intensity measured at +20 mV to those at −90 mV did not significantly differ from 1.0 and was found to be 0.991 ± 0.006 (n = 44) with (ECPF)N-Ca1a/(ECPF)N-Ca1c,77; 1.018 ± 0.016 (n = 40) with (ECPF)N-Ca1b/(ECPF)N-Ca1c,77; 1.006 ± 0.004 (n = 22) with (ECPF)N-Ca1a/(ECPF)N-Ca1c/IS-IV, and 0.997 ± 0.008 (n = 22) with (ECPF)N-Ca1b/(ECPF)N-Ca1c/IS-IV. Conversely, acceptor (EYFP) fluorescence measured at +20 mV was not significantly different from those at −90 mV when the (EYFP)N-labeled Ca1c,77- and Ca1c/IS-IV-subunits were coexpressed with the unlabeled Ca1a- and Ca1b-subunits. Because a voltage-induced change of the acceptor fluorescence in EYFP cube will be subtracted from the FRET cube measurement, we did not expect a decline of the EYFP fluorescence ratio at +20 and −90 mV from 1 with our three-cube filter system even if acceptor hypothetically shows voltage-sensitive change of fluorescence. Indeed, the ratio of EYFP fluorescence intensity measured at +20 mV to those at −90 mV was on average not different from 1.0: 1.003 ± 0.007 (n = 38) with (EYFP)N-Ca1c,77/Ca1a; 1.002 ± 0.007 (n = 10) with (EYFP)N-Ca1c/IS-IV/Ca1a; 0.995 ± 0.010 (n = 26) with (EYFP)N-Ca1c,77/Ca1b, and 1.011 ± 0.011 (n = 16) with (EYFP)N-Ca1c/IS-IV/Ca1b. This control indicates that voltage-dependent changes of fluorescence of the individual ECPF or EYFP fluorophores fused to the NH2-termini of the Ca1c- and Ca1b-subunits do not contribute to the voltage-dependent changes in FRET between them recorded in this study.

Efficiency of FRET depends on the distance between the donor and acceptor fluorophores and their mutual angular orientation. Although (ECPF)N-Ca1a and (EYFP)N-Ca1c subunits were expressed at 1:1 molar ratio, the uncertainties in their relative concentration can be minimized by the ratiometric analysis of FRET (Xia and Liu, 2001). Relative changes in normalized FRET values (N\textsubscript{FRET}) were detected for all combinations of the Ca1c- and Ca1a-subunits, with the exception of the Ca1c,77/Ca1b channel (Table 1; Figs. 4, B and C). In the resting state (−90 mV), all tested channels were characterized by low FRET between the tagged NH2-termini of the Ca1c- and Ca1b-subunits. Depolarization to +20 mV caused almost complete inactivation of the (ECPF)N-Ca1c,77/(ECPF)N-Ca1a channels within ≈1 s (see current trace in Fig. 4A) and produced a fully reversible significant increase in N\textsubscript{FRET} (Table 1; Fig. 4A, panel e) measured in the predominantly inactivated state (marked by red bar above the current trace). This result suggests substantial rearrangement between the Ca1c- and Ca1b-subunits in the inactivated state accompanied by a reduction of the apparent distance between the donor and acceptor fluorophores and/or more parallel orientation of the respective dipoles.

**Voltage-dependent rearrangements between the Ca1c,77- and Ca1b-subunits**

Replacement of the Ca1a-subunit for Ca1b produced an increase in the Ba\textsuperscript{2+} current amplitude corresponding to higher efficiency of expression of the Ca1c,77 channel. Complete inactivation of the Ba\textsuperscript{2+} current through the Ca1c,77/Ca1b channel was delayed, even with a 45-s depolarization at +20 mV (Fig. 4B, bottom trace in the left panel), but not inhibited like in the case of the Ca1c/IS-IV channel. No measurable current was observed when Ca1c,77 was expressed in COS1 cells in the absence of Ca1b-subunits (data not shown), thus precluding comparison of the current decay in the absence of the Ca1b-subunit modulation. Fluorescence measurements
corroborate the results of the immunoprecipitation/Western blot analysis and additionally confirm the expression of the β2-subunit and its association with α1C,77. In the case of the (EYFP)N-α1C,77/(ECFP)N-β1a channel, equally strong ECFP and EYFP fluorescence was observed (Fig. 4 A, panels b and c), similar to that found for the (EYFP)N-α1C,77/(ECFP)N-β1a channel (compare with Fig. 4 A, panels b and c). Moreover, FRET images (Fig. 4 B, panels d and e, and the respective digitally magnified membrane regions) clearly point to colocalization of the labeled α1C,77- and β2-subunits (notice different scale in FRET images when comparing Fig. 4 B with Fig. 4 A, panels d-f). Taken together, the large amplitude of the Ba2+ current, the immunoprecipitation analysis, the equally strong expression of the fluorescent α1C,77- and β2-subunits, and their colocalization by FRET suggest that the low state-dependent change in FRET de-

FIGURE 4 Voltage-gated rearrangements between the (ECFP)N-β and (EYFP)N-α1C subunits of the functional Ca2+ channels determined by FRET combined with patch clamp in COS1 cells. (A and B) Voltage-dependent corrected FRET between the (EYFP)N-α1C,77 and (A) (ECFP)N-β1a, or (B) (ECFP)N-β2 subunits. Schematic diagram (boxed) depicts the arrangement of fluorophores. Panel a, phase-contrast cell image with a shadow of patch pipette. Panels b and c, images of the same cell with YFP and CFP filters, respectively. Arrows point to the plasma membrane distribution of the labeled proteins. Panels d and e, representative corrected images of FRET between the (ECFP)N-β and (EYFP)N-α1C,77 subunits recorded, respectively, at the resting (~90 mV) and inactivated ((ECFP)N-β1a) or predominantly conducting ((ECFP)N-β2) states of the channel (+20 mV). The time window of the +20-mV acquisition is marked on the left panel above the 1-s Ba2+ current trace by a red horizontal bar. Panel f, ratio of images e/d showing the voltage-dependent change of corrected FRET. Image areas indicated by arrows were digitally magnified to demonstrate the confinement of FRET to the plasma membrane. Three traces of the Ba2+ current on the left panel in B, recorded in response to the 1-, 5- and 45-s depolarizations, demonstrate that inactivation was not complete, and FRET imaging on panel c represents channels almost equally distributed between the inactivated and open states (ratio of the Ba2+ current after 1 s to the peak current, r1s ≈ 0.56). (C) Absence of marked FRET between the (ECFP)N-β2a and (EYFP)N-α1C,77 subunits at the end of a 5-s depolarization to +20 mV that caused an additional decrease in the fraction of open channels (r5s ≈ 0.33). Panels a and b, representative corrected images of FRET obtained at ~90 mV and +20 mV at the end of a 5-s pulse at the time window marked by the red bar above the current trace (panel e). Panel c, the ratio of corrected FRET images b/a sequentially recorded at +20 and ~90 mV; right panels, digitally magnified areas of the image pointed by arrows. Panel d, phase-contrast image of the expressing cell with a shadow of patch pipette. (D and E) Voltage-dependent corrected FRET between the (EYFP)N-α1C,IS-IV and (ECFP)N-β1a (D) or (ECFP)N-β2 (E) subunits shown as a ratio of the sequentially recorded +20 mV/~90 mV images (panel a). Panel b, phase-contrast images of the expressing cell. Note that in every case the Ba2+ current recordings (shown below the respective images) provide evidence that the sustained conducting state was achieved before the +20-mV FRET image acquisitions (at time windows marked by red bars). Scaling bars, 5 μm.
TABLE 1 Voltage-dependent changes of normalized FRET between the (ECFP)\textsubscript{N}β subunits and (EYFP)\textsubscript{N}\textalpha subunits determined at –90 mV and +20 mV under whole-cell voltage clamp in COS1 cells

<table>
<thead>
<tr>
<th>Ca channel subunits</th>
<th>β\textsubscript{1a}\textalpha\textsubscript{C,77}</th>
<th>β\textsubscript{1a}\textalpha\textsubscript{C,IS-IV}</th>
<th>β\textsubscript{1a}\textalpha\textsubscript{C,77}</th>
<th>β\textsubscript{1a}\textalpha\textsubscript{C,IS-IV}</th>
</tr>
</thead>
<tbody>
<tr>
<td>–90 mV</td>
<td>0.01 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.00 ± 0.005</td>
<td>–0.0 ± 0.03</td>
</tr>
<tr>
<td>+20 mV</td>
<td>0.09 ± 0.02</td>
<td>–0.01 ± 0.01</td>
<td>0.17 ± 0.04</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Paired t-test</td>
<td>p &lt; 0.05 (n = 4)</td>
<td>ns (n = 39)</td>
<td>p &lt; 0.05 (n = 6)</td>
<td>p &lt; 0.01 (n = 10)</td>
</tr>
</tbody>
</table>

Averages were compiled from different cells, where \( n \) stands for the number of cells. \( p \), paired t-test; \( ns \), nonsignificant difference.

terminated for the ratio of images recorded at +20/–90 mV (Fig. 4 B, panel f) was not due to the lack of functional expression of the (EYFP)\textsubscript{N}\textalpha\textsubscript{C,77}/(ECFP)\textsubscript{N}β channel.

Insignificant change in relative \( N_{FRET} \) (Table 1) recorded at the end of the 1-s depolarization pulse to +20 mV from the holding potential of –90 mV (marked by the red bar above the top current trace in Fig. 4 B) corresponded to a transition of the (EYFP)\textsubscript{N}\textalpha\textsubscript{C,77}/(ECFP)\textsubscript{N}β channel from the resting to a predominantly noninactivated state, as seen from the slow and incomplete decay of the whole-cell Ba\textsuperscript{2+} current and single-channel-gating behavior in Fig. 3, B and C. An average fraction of inactivated channels in 1-s recordings accounted for \( \approx 44\% \). Prolongation of +20 mV depolarization pulse to 5 s (Fig. 4 C) increased the fraction of the inactivated channels to \( \approx 67\% \). In the case of the (EYFP)\textsubscript{N}\textalpha\textsubscript{C,77}/(ECFP)\textsubscript{N}β\textsubscript{1a} channel, a similar fractional inactivation would correspond to almost fivefold increase in relative \( N_{FRET} \). However, differential FRET between the (EYFP)\textsubscript{N}\textalpha\textsubscript{C,77} and (ECFP)\textsubscript{N}β\textsubscript{1a} subunits recorded at –90 mV before and at the end of the 5-s pulse to +20 mV (Fig. 4 C, panel c) was not substantially increased. Thus, in sharp contrast with the \( \beta_1 \)-subunit, no significant voltage-dependent changes of FRET between the NH\textsubscript{2}-terminal fluorescent tags of the \( \alpha_1 \)-subunit and \( \beta_2 \)-subunits were found. This result suggests that the voltage-gated rearrangements of the labeled parts in the (EYFP)\textsubscript{N}\textalpha\textsubscript{C,77}/(ECFP)\textsubscript{N}β channel complex are either insignificantly small or occur outside the detection limits of the method (i.e., when linear distance between the fluorophores becomes >10 nm, or the dipoles approach perpendicular orientation, or both). Overall, the difference in voltage-dependent FRET between (EYFP)\textsubscript{N}\textalpha\textsubscript{C,77} and (ECFP)\textsubscript{N}β\textsubscript{1a} or \( \beta_2 \)-subunits (Table 1) reflects the differential \( \beta \)-subunit modulation of inactivation of the \( \alpha_1 \)-subunit (‘‘wild-type’’) calcium channel.

Voltage-dependent rearrangement between \( \alpha_1 \)- and different \( \beta \)-subunits

In full agreement with the data obtained in oocyte expression system, the replacement of the \( \beta_1 \)-subunit for \( \beta_2 \) did not affect the kinetics of inactivation of the Ba\textsuperscript{2+} current through the \( \alpha_1 \)-channel expressed in COS1 cells (compare current traces in panels c in Fig. 4, D and E). On average, the fast time constant of inactivation was 13.0 ± 1.2 ms (\( n = 13 \)) with the \( \beta_1 \)-subunit and 12.9 ± 0.8 ms (\( n = 12 \)) with the \( \beta_2 \)-subunit. The sustained fraction of the current (52 ± 8% with \( \beta_1 \) and 50 ± 9% with \( \beta_2 \)) lasted for the duration of depolarization (up to 30 s) without decay. The relatively small current amplitudes obtained with the \( \alpha_1 \)-channel is characteristic for other Ca\textsubscript{1.2} channel mutants with impaired Ca\textsuperscript{2+}-induced inactivation property (Soldatov et al., 1998). This was found to be due to a lower open probability and ~15% reduction in the single-channel conductance (Kepplinger et al., 2000), and had no significant impact on the measurement of intramolecular FRET between the termini of the \( \alpha_1 \)-channel (Kobrinsky et al., 2003).

The lack of differential \( \beta \)-subunit modulation of the \( \alpha_1 \)-channel inactivation correlated with the absence of the \( \beta \)-subunit specificity of voltage-dependent FRET between the (ECFP)\textsubscript{N}β and (EYFP)\textsubscript{N}\textalpha\textsubscript{C,IS-IV} subunits. \( N_{FRET} \) between the NH\textsubscript{2}-terminal tags of the \( \alpha_1 \)-subunit and either \( \beta \)-subunits was found to increase in the sustained depolarizing state (Fig. 4, D and E; Table 1). However, this increase in FRET essentially did not depend on the type of the coexpressed \( \beta \)-subunit. The transition of the (EYFP)\textsubscript{N}\textalpha\textsubscript{C,IS-IV} channel into the conducting noninactivated state, stabilized by depolarization to +20 mV (Fig. 4, D and E, panels c), showed an equally large (by unpaired \( t \)-test) increase in \( N_{FRET} \) with either (EYFP)\textsubscript{N}β\textsubscript{1a} (to 0.17 ± 0.04) or (ECFP)\textsubscript{N}β\textsubscript{2} subunit (to 0.19 ± 0.03). This result may reflect the loss of the \( \beta \)-subunit specificity of voltage-dependent rearrangements between the NH\textsubscript{2}-terminal regions of the \( \alpha_1 \)- and \( \beta \)-subunits and correlates with the lack of differential \( \beta \)-subunit modulation of the Ba\textsuperscript{2+} current through the \( \alpha_1 \)-channel.

Voltage-dependent rearrangements of the \( \alpha_1 \)- and \( \beta \)-subunits vis-à-vis the plasma membrane

FRET microscopy combined with whole-cell patch clamp was used to estimate whether the relative proximity of the \( \alpha_1 \)- and \( \beta \)-subunits to the plasma membrane is changed in different functional states of the channel (Fig. 5). As membrane probes we used the ECFP/EYFP-labeled pleckstrin homology (PH) domains of phospholipase C61 (van der Wal et al., 2001) that localize via PI\textsubscript{3}P to the inner leaflet of the plasma membrane. Unlike the (EYFP)\textsubscript{C}-channel labeled at the C-terminal tail (Kobrinsky et al., 2003), which did not show FRET with the labeled PH domains (data not shown), all NH\textsubscript{2}-terminally labeled \( \alpha_1 \) and \( \beta \)-subunits showed substantial FRET (Fig. 5 A). This finding suggests...
that the NH$_2$-termini of the $\alpha_{1C}$- and $\beta$-subunits are situated near the plasma membrane within the linear detection limits of the method ($\approx$100 Å), i.e., close enough to determine their state-dependent rearrangements with regards to the lipid bilayer.

The results of the measurements of the voltage-dependent rearrangements of $\beta$-subunits vis-à-vis the plasma membrane are shown in Fig. 5, A and B. FRET was measured between the donor fluorophore of (ECFP)$_N$-$\beta$ and the acceptor fluorophore of (EYFP)$_N$-PH. The latter was expressed at $\approx$1:1 molar ratio to an (ECFP)$_N$-$\beta$ subunit, and distributed predominantly over the cytoplasmic surface of the plasma membrane, most likely in a random and homogeneous manner. Control experiments showed that there was no FRET between (EYFP)$_N$-PH and (ECFP)$_N$-$\beta$ unless the latter was coexpressed with the untagged $\alpha_{1C}$- and $\alpha_2\delta$-subunits as a part of the channel complex. In the absence of membrane-anchoring $\alpha_{1C}$- and $\alpha_2\delta$-subunits, the labeled $\beta_{1a}$- and $\beta_2$-subunits exhibited similarly diffuse distribution in the cytoplasm without preferential targeting of the plasma membrane (data not shown). However, as parts of the functional Ca$^{2+}$ channel, (ECFP)$_N$-labeled $\beta$-subunits displayed strong FRET with (EYFP)$_N$-PH domain (Fig. 5 A). This FRET was probably due to not only the close proximity and the effective alignment of the fluorophores, but also the global or local deviation from the 1:1 donor/acceptor ratio (Lakowicz, 1999; Xia and Liu, 2001). Therefore it would be incorrect to compare individual FRET measurements obtained at a single potential, e.g., +20 mV (Fig. 5 B, panels b). To compensate for possible artifacts, ratios were
calculated from FRET images that were sequentially recorded at \(-90\) and \(+20\) mV (Fig. 5 B, panel c). Using this approach, it was found that none of the \(\beta\)-subunits showed a state-dependent change of FRET, with the possible exception of \((\text{ECFP})_N\beta_{1a}\) in the \(\alpha_{1C,IS-IV}\) channel (Fig. 5 A). Assuming randomized orientation of the independent fluorophores (see scheme in Fig. 5 A), one may conclude that the NH2-termini of either the \(\beta_{1a}\) or \(\beta_2\)-subunit of the functional \(\alpha_{1C,77}\) channel do not significantly change their proximity with respect to the plasma membrane in response to depolarization. Inhibition of slow inactivation in the \(\alpha_{1C,IS-IV}\) channel may facilitate the voltage-gated mobility of the \((\text{ECFP})_N\beta_{1a}\) subunit, which is not associated with inactivation of the channel.

Fig. 5, C and D, show that FRET between the \((\text{ECFP})_N\)-PH domain and the \((\text{EYFP})_N\)-tagged \(\alpha_{1C,77}\) or \(\alpha_{1C,IS-IV}\) channels containing the \(\beta_{1a}\)-subunit is essentially voltage independent. No significant changes in FRET between the NH2-terminal fluorophores of the \(\alpha_{1C}\)-subunits and the plasma membrane PH domain probes were observed for the channels in the resting and the inactivated \((\alpha_{1C,77})\) or conducting \((\alpha_{1C,IS-IV})\) states.

**DISCUSSION**

Results of our work show that voltage-gated rearrangements between the NH2-termini of the \(\alpha_{1C}\)- and \(\beta\)-subunits, associated with inactivation of the \(\alpha_{1a,1.2}\) channel, depend on ADSI, the type of the \(\beta\)-subunit, and differential \(\beta\)-subunit modulation of inactivation.

**Differential \(\beta\)-subunit modulation and slow inactivation**

The effect of \(\beta\)-subunits on the kinetics of the \(\text{Ba}^{2+}\) current is mediated by their interactions with the ion-conducting \(\alpha_{1C}\)-subunits. However, is not clear whether inactivation of the \(\alpha_{1a,1.2}\) channel is inherent to association between the \(\alpha_{1C}\)- and \(\beta\)-subunits. Earlier, Ferreira et al. (1997) observed in tsA201 cells an expression of the functional cardiac rabbit \(\alpha_{1C}\) channel in the absence of the rat brain \(\beta_{2}\)-subunit. In their report, a coexpression of the \(\beta_{2}\)-subunit predominantly affected the amplitude of the \(\text{Ba}^{2+}\) current but did not accelerate the kinetics of inactivation. In contrast, our studies in COS1 cells showed no functional expression of the \(\alpha_{1C,77}\) and \(\alpha_{1C,IS-IV}\) channels in the absence of \(\beta\)-subunits. Thus, we investigated the \(\beta\)-subunit modulation of inactivation as a differential effect of two different \(\beta\)-subunits, \(\beta_{1a}\) and \(\beta_2\), on two variants of the \(\alpha_{1a,1.2}\) channels showing completely different inactivation properties.

Inactivation of the \(\text{Ba}^{2+}\) current through the “wild-type” \(\alpha_{1C,77}\) channel is characterized by fast and slow components and is subject to differential \(\beta\)-subunit modulation (Fig. 1 A). Inhibition of the voltage-dependent slow inactivation \((\alpha_{1C,IS-IV})\) abolished differential \(\beta\)-subunit modulation of the \(\text{Ba}^{2+}\) current (Fig. 1 B). The replacement of the \(\beta_{1a}\)-subunit with \(\beta_2\) did not cause appreciable changes in the inactivation kinetics of the \(\alpha_{1C,IS-IV}\) channel over a wide range of membrane potentials (Figs. 2 and 3 B) and produced only marginal reduction of the fast component of the current (Fig. 1 B). This result is not due to a disruption of physical association between the \(\alpha_{1C}\) and \(\beta\)-subunits. The immunoprecipitation assay (Fig. 3 A) and direct measurements of FRET between the \(\beta\)- and \(\alpha_{1C}\)-subunits (Fig. 4, A and B, panels d and e) independently confirmed their colocalization. Given the strong structural binding of \(\beta\)-subunits to the AID locus of the I-II linker of the \(\alpha_{1C}\)-subunit (Pragnell et al., 1994; De Waard et al., 1994), this result is not surprising, because the I-II linker remains unchanged in the tested \(\alpha_{1C}\)-subunits. Taken together, these findings suggest that association of \(\beta\)-subunits with AID is not sufficient for differential \(\beta\)-subunit modulation of inactivation that seems to selectively target the slow inactivation of the \(\alpha_{1C,77}\) channel, a mechanism that is inhibited in the \(\alpha_{1C,IS-IV}\) channel.

**Structural rearrangements between the \(\alpha_{1C}\) and \(\beta\)-subunits**

The second essential finding of this study shows that the differential \(\beta\)-subunit modulation of the \(\alpha_{1a,1.2}\) channel inactivation is associated with specific differences in voltage-gated molecular rearrangements between the NH2-terminal regions of the \(\alpha_{1C}\) and \(\beta\)-subunits. This relationship was highlighted with reversible voltage-dependent differential corrected FRET measurements between the donor \((\text{ECFP})_N\beta\) and acceptor \((\text{EYFP})_N\alpha_{1C}\) fluorophores of the functional \(\text{Ca}^{2+}\) channel complexes expressed in voltage-clamped living COS1 cells (Fig. 4). This method of patch clamp combined with FRET measurement was designed to determine the differences in relative proximity and/or angular orientation of the fluorescent FRET partners when the channel conformation is changed from the resting state (stabilized at \(-90\) mV) to the inactivated \((\alpha_{1C,77})\) or sustained conducting \((\alpha_{1C,IS-IV})\) states, induced by depolarization to \(+20\) mV (Table 1). The transition of the channels into each of these states before FRET imaging was controlled by voltage clamp and verified, in real time, by monitoring of the \(\text{Ba}^{2+}\) current during FRET measurements.

The reporter fluorophores were genetically fused to the NH2-termini of the \(\alpha_{1C,77}\) and \(\alpha_{1C,IS-IV}\)-subunits without considerable interference with the properties of the channels (Kepplinger et al., 2000; Kobrinsky et al., 2003). We find that the NH2-terminal region is a convenient site for the attachment of GFP variants to the \(\beta\)-subunit. Earlier Wei et al. (2000) have described acceleration of the \(\text{Ba}^{2+}\) current inactivation imparted by the NH2-terminal fusion of GFP to the \(\beta_{2a}\)-subunit cloned from the rat heart (Perez-Reyes et al., 1992). However, the rabbit \(\beta_2\)-subunit (Hullin et al., 1992) was (ECFP)_N-labeled in our work without substantial change.
in the inactivation properties (see Fig. 4 B, left traces). Most important, the characteristic difference in modulation of the Ca\(^{2+}\) channel by the rabbit \(\beta_{1\alpha}\) and \(\beta_{2}\)-subunits was retained after the (ECFP)\(_N\)-labeling, as one can see, for example, from the comparison of the representative 1-s current traces in Fig. 4, A and B. The nature of this difference in terms of molecular dynamics of the \(\alpha_{1\alpha}\) and \(\beta\)-subunits in a functional channel has been investigated here by differential state-dependent FRET microscopy.

Differential corrected FRET microscopy eliminates artifacts associated with the tagged orphan subunits and, by focusing on the relative changes in corrected FRET between the resting (−90 mV) and different inactivation states (+20 mV) of the channels, provides an estimate of the extent of voltage-gated intermolecular motion. We assume that the environment of the fluorophores may be structured by physical association of the subunits, and their segmental motions may not independently randomize the orientations (orientation factor \(\kappa^2 \leq 2/3\)) (Lakowicz, 1999). These assumptions generally complicate interpretation of the data in terms of translational distances between donor and acceptor, but appear to be physiologically relevant.

Differential corrected FRET microscopy did not reveal significant voltage-dependent changes in proximity to the plasma membrane of the (ECFP)\(_N\)-tags of \(\beta\)-subunits in the functional \(\alpha_{1\alpha\text{CIS-IV}}\) channel (Fig. 5). However, large voltage-dependent differences were observed in \(N_{\text{FRET}}\) between the N-terminal fluorophores of the \(\beta\)- and \(\alpha_{1\alpha\text{CIS-IV}}\)-subunits (Table 1).

In the resting state of the channel, corrected FRET between the (ECFP)\(_N\)-labeled \(\beta_{1\alpha}\) or \(\beta_{2}\)-subunit and (EYFP)\(_N\)-\(\alpha_{1\alpha\text{CIS-IV}}\) was small (Table 1) and close to the detection limit of the method thus not permitting comparison between the \(\alpha_{1\alpha\text{CIS-IV}}/\beta_{1\alpha}\) and \(\alpha_{1\alpha\text{CIS-IV}}/\beta_{2}\) channels. Inactivation of the \(\alpha_{1\alpha\text{CIS-IV}}/\beta_{1\alpha}\) channel was accompanied by a substantial rearrangement between the NH2-terminal regions of the \(\beta_{1\alpha}\) and \(\alpha_{1\alpha\text{CIS-IV}}\)-subunits. The respective increase in corrected FRET between the donor (ECFP)\(_N\)-\(\beta_{1\alpha}\) and acceptor (EYFP)\(_N\)-\(\alpha_{1\alpha\text{CIS-IV}}\) fluorophores (Fig. 4 A, panel f) may reflect differential \(\beta\)-subunit modulation, because it was not seen with the \(\beta_{2}\)-subunit (Table 1).

Lack of state-dependent FRET between the NH2-terminal \(\beta_{2}\) and \(\alpha_{1\alpha\text{CIS-IV}}\) fluorophores contrasts with strong state-dependent FRET in the \(\alpha_{1\alpha\text{CIS-IV}}/\beta_{1\alpha}\) channel. There are essentially two explanations to this difference in terms of molecular rearrangements. One possibility suggests that state-dependent rearrangements between the NH2-terminal fluorophores in the \(\alpha_{1\alpha\text{CIS-IV}}/\beta_{2}\) channel occur in a direction opposite to those observed in the \(\alpha_{1\alpha\text{CIS-IV}}/\beta_{1\alpha}\) channel complex, e.g., further apart from the low-FRET resting arrangement and, thus, outside of the detection limits of the method. This might correspond, for example, to an increase in a linear distance between the NH2-terminal fluorophores of the \(\alpha_{1\alpha\text{CIS-IV}}\) and \(\beta_{2}\)-subunits over 10 nm, and/or to perpendicular orientation approached by the ECFP/EYFP dipoles. The other possibility suggests that bulky \(\beta_{2}\) might impede the voltage-gated mobility between the subunits as compared to \(\beta_{1\alpha}\). The FRET data (Table 1) don’t allow one to discriminate between these two scenarios with sufficient confidence because the \(N_{\text{FRET}}\) values for the \(\alpha_{1\alpha\text{CIS-IV}}/\beta_{2}\) channel are small at both −90 and +20 mV. However, they indicate that the voltage-dependent rearrangement between the \(\beta_{2}\) and \(\alpha_{1\alpha\text{CIS-IV}}\) fluorophores is fundamentally different from those between the \(\beta_{1\alpha}\) and \(\alpha_{1\alpha\text{CIS-IV}}\)-subunits. This difference may be related to differential \(\beta\)-subunit modulation of inactivation, because both were eliminated by inhibition of the slow inactivation mechanism of the \(\alpha_{1\alpha\text{CIS-IV}}\) channel (Fig. 4, D and E). It was proposed that ADSI is crucial for a state-dependent folding of the pore-underlying polypeptide bundle associated with specific arrangement of the C-terminal Ca\(^{2+}\) sensors of inactivation (Soldatov, 2003). The \(\beta\)-subunit constitutes another component of that arrangement involved in AID binding and other interactions essential for slow inactivation. Robust state-dependent FRET between the \(\alpha_{1\alpha\text{CIS-IV}}\) and \(\beta\)-subunits NH2-tails lacks \(\beta\)-subunit specificity probably because the mutation of ADSI causes desintegration of the bundle and eliminates physical constrains for the \(\beta\)-subunit movement (Fig. 6).

Molecular determinants of differential \(\beta\)-subunit modulation of inactivation

We found that voltage-gated rearrangements between the NH2-terminals of the \(\beta\)- and \(\alpha_{1\alpha\text{CIS-IV}}\)-subunits occur in a plane parallel to the plasma membrane (Fig. 6). Shistik et al. (1998) have proposed that \(\beta\)-subunits modulate the Cav1.2 channel by relieving an inhibitory control of the NH2-terminus of \(\alpha_{1\alpha\text{CIS-IV}}\) over the activity of the channel. Lack of state-dependent FRET between the \(\alpha_{1\alpha}\) cytoplasmic NH2-tail and the plasma membrane may be due to an impediment of the NH2-tail voltage-gated conformational mobility by \(\beta\)-subunits that might generate a molecular signal for gating facilitation in a \(\beta\)-subunit specific manner.

The rabbit \(\beta_{2}\)-subunit studied in our work does not have NH2-terminal cysteine sites of palmitoylation that may limit its voltage-gated mobility by anchoring to the plasma membrane (Chien et al., 1998; Qin et al., 1998; Gao et al., 1999; Restituito et al., 2000). As cytosolic proteins, \(\beta\)-subunits can’t be subject to voltage-dependent rearrangements, which are apparently transduced to them via the AID locus of the linker between transmembrane segments IS6 and IIS1 of \(\alpha_{1\alpha}\). Previously, Stotz and Zamponi (2001) proposed a role for the \(\beta\)-subunit-binding I-II linker of the \(\alpha_{1\alpha}\)-subunit as a plug docking to segments IS6 and IIS6 in a “hinged-lid” inactivation mechanism. Among other structural factors, we find ADSI to be a crucial determinant. The role of \(\beta\)-subunits as modulators of slow voltage-dependent inactivation shown in this, and other reports (Sokolov et al., 2000) suggests a rationale for the model assuming that \(\beta\)-subunits differentially interfere with the slow inactivation mechanism. Experiments support this
assumption and show that ADSI mutation uncouples the interaction of either β-subunit with the slow inactivation mechanism, equates, and probably facilitates the voltage-gated rearrangements between the NH2-terminals of the β1a- or β2β1, and α1C,IS-IV-subunits.

In conclusion, we observed voltage-dependent rearrangements of NH2-terminal regions of the α1C,77 and β-subunits. A β-subunit is shown bound via AID to the I-II loop of α1C,77. Four amino acids (Ser, Ala, Val, and Ile) of ADSI at the cytoplasmic ends of transmembrane segments S6 are indicated in a single-letter code in circles. The C-terminal tail (where CAM stands for tethered calmodulin) and other polypeptide bundle components, connected to the pore via ADSI, but not investigated in this work, are shown by shaded lines. We suggest that ADSI is crucial in communicating the voltage-dependent rearrangements in the pore to the AID locus. Shown are two types of rearrangements (double-headed arrows), associated with voltage-gated inactivation of the channel, that might affect relative proximity between the NH2-terminals of the α1C,77 and β1,2-subunits without changing their proximity to the plasma membrane (dashed arrows). Such rearrangements are hindered in the case of the α1C,77 and β2β1-subunits.

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