Ca\textsuperscript{2+} Channel Modulation by Recombinant Auxiliary \(\beta\) Subunits Expressed in Young Adult Heart Cells

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Abstract—L-type Ca\textsuperscript{2+} channels contribute importantly to the normal excitation-contraction coupling of physiological hearts, and to the functional derangement seen in heart failure. Although Ca\textsuperscript{2+} channel auxiliary \(\beta_{1-4}\) subunits are among the strongest modulators of channel properties, little is known about their role in regulating channel behavior in actual heart cells. Current understanding draws almost exclusively from heterologous expression of recombinant subunits in model systems, which may differ from cardiocytes. To study \(\beta\)-subunit effects in the cardiac setting, we here used an adenoviral-component gene-delivery strategy to express recombinant \(\beta\) subunits in young adult ventricular myocytes cultured from 4- to 6-week-old rats. The main results were the following. (1) A component system of replication-deficient adenovirus, poly-L-lysine, and expression plasmids encoding \(\beta\) subunits could be optimized to transfect young adult myocytes with 1% to 10% efficiency. (2) A reporter gene strategy based on green fluorescent protein (GFP) could be used to identify successfully transfected cells. Because fusion of GFP to \(\beta\) subunits altered intrinsic \(\beta\)-subunit properties, we favored the use of a bicistronic expression plasmid encoding both GFP and a \(\beta\) subunit. (3) Despite the heteromultimeric composition of L-type channels (composed of \(\alpha_{1C}, \beta, \) and \(\alpha_{2}\)), expression of recombinant \(\beta\) subunits alone enhanced Ca\textsuperscript{2+} channel current density up to 3- to 4-fold, which argues that \(\beta\) subunits are “rate limiting” for expression of current in heart. (4) Overexpression of the putative “cardiac” \(\beta_{2}\) subunit more than halved the rate of voltage-dependent inactivation at +10 mV. This result demonstrates that \(\beta\) subunits can tune inactivation in the myocardium and suggests that other \(\beta\) subunits may be functionally dominant in the heart. Overall, this study points to the possible therapeutic potential of \(\beta\) subunits to ameliorate contractile dysfunction and excitability in heart failure.

Key Words: Ca\textsuperscript{2+} channel ■ \(\beta\) subunit ■ adenovirus ■ gene delivery

Voltage-gated L-type Ca\textsuperscript{2+} channels provide the essential trigger Ca\textsuperscript{2+} that initiates excitation-contraction coupling in heart.\textsuperscript{1} Not only do these channels figure prominently in the normal functioning of the heart, there are tantalizing hints that L-type channels contribute importantly to the functional derangement of heart failure\textsuperscript{2-4} that includes prolonged action-potential durations (APDs)\textsuperscript{4-6} and depressed contractility.\textsuperscript{5-7} These dysfunctional properties can contribute to arrhythmogenesis and pump failure.\textsuperscript{8} For example, in pacing-induced canine heart failure, a major causative factor in APD prolongation appears to be blunted L-type channel inactivation, perhaps secondary to altered intracellular Ca\textsuperscript{2+} dynamics playing through Ca\textsuperscript{2+}-dependent inactivation.\textsuperscript{9,10} In human heart failure, single-channel work raises the possibility of spatial heterogeneity in channel open probability (\(P_o\)), with readily opened channels located superficially, and low-\(P_o\) channels situated deep in t-tubules.\textsuperscript{11} Such spatial heterogeneity could explain the decline of excitation-contraction coupling gain\textsuperscript{12,13} that may underlie the contractile depression in heart failure.

However, fundamental deficits in our basic understanding of L-type channels hinder definitive evaluation of how these channels contribute to the (patho-)physiology of the heart. Among the more salient deficiencies is the lack of information about the role of Ca\textsuperscript{2+} channel auxiliary \(\beta\) subunits in actual heart cells. Although L-type channels are heteromers, the auxiliary \(\beta\) subunits are arguably the most powerful modulators of expression, open probability, activation, and inactivation.\textsuperscript{14-16} So far, 4 genes encoding \(\beta_{1-4}\) subunits have been identified, along with multiple splice variants.\textsuperscript{17} Differ-
ent β subunits impart distinctive patterns of channel expression and function, and therefore possess enormous potential for in vivo tuning of channel behavior. A major shortcoming in understanding the biological role of β subunits is that current knowledge draws almost exclusively from heterologous expression of recombinant subunits in model systems. Such systems may differ from cardiocytes, given the possible existence of as-yet-unknown regulatory molecules, anchoring proteins, and post-translational modification in native heart. For example, in presynaptic termini, Ca2+ channel inactivation may be different from that in heterologous systems, as interactions with SNARE complex proteins (present at terminals) alters inactivation.

Here, we therefore investigated the effects of expressing recombinant β subunits in ventricular myocytes cultured from young adult rats. To our knowledge, these experiments are the first to transfect foreign genes in young adult myocytes using an adenoviral-component gene-delivery method and to characterize β-subunit modulation in native heart cells. Although gene delivery proved more difficult in young adult rather than neonatal heart cells, we emphasized the older myocyte platform as a prelude to future experiments concerned with adult heart failure models. Neonatal heart cells have immature variants of L-type Ca2+ channels, excitation-contraction coupling, and sarcomeric structure. After optimization of the adenoviral-component method, we observed that expression of β subunits alone induced a large increase in L-type current density, despite the heteromultimeric composition of Ca2+ channels. β subunits could also strongly modulate inactivation rate in the native setting. These findings raise the possibility that gene delivery of β subunits could counter contractile depression and excitability disorders in diseased hearts.

Materials and Methods

Adult Rat Heart Cell Isolation and Culture

The methods for cell isolation and culture have been described previously. Briefly, hearts were excised from young adult (4 to 6 weeks old) Sprague-Dawley rats, and ventricular heart cells were dispersed enzymatically. After centrifugation through a discontinuous Percoll gradient, ~80% rod-shaped cells were usually obtained. Myocytes were cultured relatively sparsely at a density of 50 000 to 80 000 cells per mL on laminin-coated coverslips, in medium 199 containing, in mmol/L, sodium bicarbonate 26 and HEPES 25 as buffers, glucose 5.6, acetate 0.6, and amino acids as metabolic substrates), which was supplemented with (in mmol/L) carnitine 5, creatine 5, taurine 5, and (in μg/mL) penicillin 100, streptomycin 100, and amphotericin 0.25. Cultures were equilibrated with 5% CO2/95% air at 37°C. As reported previously by others, using similar culture conditions, we observed little change in L-type channels over 4 to 5 days in culture.

Vectors for Transfection

Transfection of green fluorescent protein (GFP) alone was accomplished with an expression plasmid containing a cytomegalovirus (CMV) promoter driving “enhanced GFP,” pEGFP (Clontech). The rat β3-C-GFP construct (Figure 3B) was created by polymerase chain reaction (PCR) amplification of approximately the first one third of the coding region of rat β3 (33) so as to place a BglII site immediately before the second amino acid codon of rat β3. The upstream and downstream primers for PCR amplification were, respectively, AAGATCTCATGGGAGCTGGCA and GACCTCTATACCCCTTCAG. This PCR fragment was ligated into rat β3/pGW13 by BglII and Apol sites. The entire resulting coding region was then transferred into pEGFP-C1 (Clontech) by BglII and EcoRI sites. The rat β2, C-GFP construct (Figure 3C) was created by ligating the PCR-amplified coding region of rat β2 (33) into BglII and PstI sites of pEGFP-N3 (Clontech). The upstream and downstream primers for PCR amplification were, respectively, AAGATCTCATGGGAGCTGGCA and TCTGCAAGTTGCGGATTGTTACAATCCTCC. The rat β2-N-GFP construct (Figure 3D) was created by PCR amplification of approximately the first one half of the coding region of rat β2 (33) so as to place a BglII site immediately before the second amino acid codon of rat β2. The upstream and downstream primers for PCR amplification were, respectively, AAGATCTTATGCGACTCTCAGTG and GCCAGTGAGTGTCTTGCGTCTGT. This PCR fragment was ligated into rat β2/pGW13 by BglII and Nhel sites. The entire resulting coding region was then transferred into pEGFP-C1 (Clontech) by BglII and EcoRI sites. pFu polymerase (Stratagene) was used in PCR reactions to increase fidelity, and the region between cloning sites in final products was verified by thermocycle sequencing.

The mammalian expression vector pCG(EGF-IR) (provided by Drs. David Johns and Eduardo Marín, Johns Hopkins University, Baltimore, Md) was used as the base for the bicistronic vector, rat β2 IR-GFP (Figure 4A). The vector contains a CMV promoter, followed by a gene encoding enhanced GFP (EGFP), then an internal ribosomal entry site (IRES) from encephalomyocarditis virus, a multiple cloning site, and poly A region. The rat β1, IR-GFP construct was created by non directional ligation of cDNA encoding the rat β1 subunit into the EcoRI site of pCG(EGF-IR), which was just downstream from the IRES. The final product was verified by diagnostic restriction enzyme digests.

For transfection with the adenoviral-component system, all cDNA constructs were purified by CsCl centrifugation. Transfection rates were considerably lower for cDNAs purified by anion-exchange resins (Qiagen), possibly because supercoiled plasmid DNA is required for proper complexation of component elements.

Adenovirus Preparation and Storage

High-quality, replication-deficient human adenovirus (type 5 mutant Ad5d5312) was critical to transfection efficiency by the adenoviral-component system. We propagated adenovirus in complementing cells (HEK 293) cells and then purified virus as previously described. Briefly, we infected confluent HEK 293 cells grown in 15-mm plates with ~5 μL adenoviral stock solution (1015 to 1016 viral particles per mL in 10 mmol/L Tris-HCl buffer as described below). About 20 plates were processed per preparation. After ~48 hours, cells from all plates were harvested as a pellet and resuspended in 20 mL of buffer containing (in mmol/L) Tris-HCl 10, MgCl2 1, and CaCl2 1 (pH 8.0). Cells were lysed by 5 freeze-thaw cycles, and then cellular debris was pelleted by centrifugation. The virus-laden supernatant was then subjected to 1-hour density-gradient ultracentrifugation with CsCl step gradients of 1.25, 1.33, and 1.45 g/mL. The now-pure viral bands was collected and dialyzed against the Tris-HCl buffer described above. After dialysis, glycerol was added to a final concentration of 10% (vol/vol), and the viral stock solution was stored at −80°C. Resuming adenoviral stock solutions contained 1012 to 1013 particles per mL with a ~30:1 particle/plaque-forming unit ratio. Stocks were stored for 4 to 6 months at −80°C without degradation of transfection efficiency. With increasing storage time, the transfection efficiency declined appreciably.

Transfection of Heart Cells Using Adenoviral-Component System

Transfections were performed on cells that had been cultured for 1 day. An adenoviral-component mixture was used, which was composed of replication-deficient adenovirus (prepared as above), poly-L-lysine (molecular weight 34 000 to 48 000, Sigma), and expression plasmids. The transfection procedure was similar to that described...
previously, with modifications as noted below. Briefly, ∼2 to 20 μL of Ad5Δ3132 virus stock (10^7 to 10^9 viral particles per mL in 10 mmol/L Tris-HCl stock solution described above) was combined with 14 μL poly-l-lysine stock (33 μg/mL) and diluted in medium 199 to a final volume of ∼267 μL, resulting in final concentrations of ∼8×10^10 viral particles per mL and ∼1.7 μg/mL poly-l-lysine. After a 30-minute incubation at room temperature, 0.7 μL of plasmid DNA stock (1 μg/μL in water) was added to the mixture, yielding a final DNA concentration of ∼2.5 μg/mL. This mixture was then incubated for another 30 minutes. Another aliquot of poly-l-lysine stock solution (∼10 μL) was added, resulting in a final poly-l-lysine concentration of ∼2.5 μg/mL. This mixture was subsequently incubated for another 10 minutes. Medium 199 (275 μL) was then added, resulting in a total volume of 550 μL, with final component concentrations of ∼4×10^10 viral particles per mL, ∼1.25 μg/mL poly-l-lysine, and ∼1.25 μg/mL plasmid DNA. Transfection was then initiated by replacing the culture medium with 250 μL of this transfection mixture per 17-mm-diameter well. After a 3-hour incubation at 37°C, the reaction was slowed by addition of 3 times volume of medium. This dilution prevents the damage sometimes observed with prolonged exposure to full-strength component mixture. Cells were cultured in medium, as described above, for ∼72 hours before further analysis.

**Transfection of HEK 293 Cells**

HEK 293 cells were cultured and transfected by Ca^2+ phosphate precipitation as previously described. CMV expression plasmids encoding α1C, α6, and various β-subunit constructs (10 μg each) were used per 10-cm plate.

**Electrophysiology**

Whole-cell recordings were obtained at 20 to 22°C using standard patch-clamp techniques. The external solution contained (in mmol/L) NaCl 138, KCl 4, CaCl2 2, MgCl2 1, 10 mmol/L Tris-HCl stock solution described above) was combined with 14 μL poly-l-lysine stock (33 μg/mL) and diluted in medium 199 to a final volume of ∼267 μL, resulting in final concentrations of ∼8×10^10 viral particles per mL and ∼1.7 μg/mL poly-l-lysine. After a 30-minute incubation at room temperature, 0.7 μL of plasmid DNA stock (1 μg/μL in water) was added to the mixture, yielding a final DNA concentration of ∼2.5 μg/mL. This mixture was then incubated for another 30 minutes. Another aliquot of poly-l-lysine stock solution (∼10 μL) was added, resulting in a final poly-l-lysine concentration of ∼2.5 μg/mL. This mixture was subsequently incubated for another 10 minutes. Medium 199 (275 μL) was then added, resulting in a total volume of 550 μL, with final component concentrations of ∼4×10^10 viral particles per mL, ∼1.25 μg/mL poly-l-lysine, and ∼1.25 μg/mL plasmid DNA. Transfection was then initiated by replacing the culture medium with 250 μL of this transfection mixture per 17-mm-diameter well. After a 3-hour incubation at 37°C, the reaction was slowed by addition of 3 times volume of medium. This dilution prevents the damage sometimes observed with prolonged exposure to full-strength component mixture. Cells were cultured in medium, as described above, for ∼72 hours before further analysis.

**Results**

**Delivery of Foreign Genes to Cultured Heart Cells by an Adenoviral-Component System**

A prerequisite step in this study was the introduction of genes encoding Ca^2+ channel β subunits into young adult heart cells. Delivery of plasmid DNA to adult myocytes by conventional means, such as Ca^2+ phosphate precipitation and lipofection, has proven challenging, although there are instances of highly successful variations of these techniques in cultured neonatal cells. By contrast, recombinant adenoviral constructs can transfect adult rat and neonatal myocytes with nearly 100% efficiency, but considerable time and effort are required to produce new recombinant constructs, and genes of interest are size-limited (<7 kilobases). Recently, a hybrid strategy has been introduced that combines some of the efficiency of adenovirus-mediated transfection with the convenience of plasmid-based strategies. In this approach, cultured neonatal heart cells could be transfected with ∼70% efficiency, simply by application of a trinary complex containing replication-deficient adenovirus, poly-l-lysine, and expression plasmids encoding a gene of interest. Here, we adapted the adenoviral-component gene-delivery method to ventricular myocytes cultured from young adult rats. Figure 1 diagrams the overall time course of the experiments in this study, along with the proposed mechanisms underlying the transfection strategy.

Figure 2 illustrates the characteristic efficiency of gene transfection by the adenoviral-component method in young adult ventricular myocytes, using GFP as a reporter gene. For baseline comparison, the top row displays brightfield (Figures 2A and 2B) and fluorescence (Figure 2C) micrographs of ventricular myocytes that have not been transfected by the compound system. The cells were isolated from 4-week-old rats and are shown after 4 days in culture.
using previously established methods. The regular striations in the brightfield view, seen more clearly at higher magnification (Figure 2A), are characteristic of the adult phenotype. Under fluorescence illumination by blue light, the same field of view as in Figure 2B is now dark (Figure 2C), indicating that GFP could serve as a selective reporter gene. The bottom row of Figures 2D through F displays photomicrographs of transfected myocytes with GFP. A, Brightfield view (×400) of transfected myocytes after 4 days in culture. B, Low-power (×100), brightfield view of young adult ventricular myocytes, after 4 days in culture after isolation from a 4-week-old rat. C, Fluorescence image of the same view as in panel B, using a B2A Nikon fluorescence cube. The lack of appreciable green fluorescence indicates the absence of confounding, endogenous fluorophore. D, Low-power (×100), fluorescence image of a parallel set of cells (from a 4-week-old rat) transfected with GFP. E, Same cells as in panel E, but now under fluorescence illumination.

**Variations in the Use of GFP as Reporter of Successful β-Subunit Transfection**

The results from the previous section indicated that a component adenoviral system could transfert foreign genes into cultured adult heart cells, albeit with a lower efficiency (1% to 10%) than previously found with neonatal cells (~70%). Such a gene-delivery strategy would still prove useful for patch-clamp studies if there were a convenient method for detecting successfully transfected myocytes.

A first approach was to engineer plasmids encoding fusion proteins of GFP to various β subunits, as schematized at the top of Figure 3. To test whether these novel constructs were functionally active as channel β subunits, and to determine whether their properties were unchanged from those of native subunits, we examined the characteristics of recombinant L-type Ca\(^{2+}\) channels expressed in HEK 293 cells from cDNAs encoding the main α\(_{1C}\) subunit, the auxiliary α\(_{1D}\) subunit, and various fusions of GFP to β subunits. Figure 3A shows the characteristic properties of L-type channels containing wild-type rat β\(_{2a}\) subunits, which have been proposed as a dominant “cardiac” isoform. To focus the analysis on voltage-dependent channel gating behavior, Ba\(^{2+}\) serves as the charge carrier, here and throughout, so as to minimize Ca\(^{2+}\)-dependent inactivation that would otherwise be present with Ca\(^{2+}\) as charge carrier. The exemplar current trace (Figure 3A1) illustrates important features imparted to channels containing the rat β\(_{2a}\) subunit. First, the currents are comparatively large, because β subunits help chaperone and/or stabilize the main α\(_{1C}\) subunit at the surface membrane, as well as to enhance open probability. Recombinant Ca\(^{2+}\) channel currents expressed without a β subunit are typically >10-fold smaller than shown here. Second, voltage-dependent inactivation is quite slow during the 1000-ms depolarizing pulse. Of all the known β\(_{1–4}\) subunits, the rat β\(_{2a}\) subunit results in the slowest inactivation. The population data shown below (Figures 3A2 and 3A3, Table) entirely confirm the two exemplar trends. Plots of average peak (●) and residual (○) current as a function of voltage-step potential (Figure 3A2) corroborate the robust expression of functional channels. Moreover, the average fraction of current remaining at the end of 1000-ms depolarizations (Figure 3A3, r\(_{1000}\)) is large, consistent with slow inactivation.

Figures 3B and 3C show the properties of L-type channels containing GFP-rat β\(_{2a}\) fusions, following the identical format of Figure 3A. In both cases, the expression of current is robust, and the overall voltage dependence of activation is undetectably changed compared with native rat β\(_{2a}\). However, exemplar traces clearly suggest that the fusion of GFP to the rat β\(_{2a}\) subunit...
results in enhanced inactivation (compare Figures 3A1, 3B1, and 3C1). Averaged \( r_{1000} \) data confirm the faster inactivation (Figures 3B3 and 3C3, see also Table), which is obvious by comparison with the wild-type rat \( \beta_2a \) data reproduced as a dashed curve. When GFP is fused to the N terminus of rat \( \beta_2a \) (Figure 3B), the acceleration of inactivation is greatest. However, even with GFP fusion to the C terminus of rat \( \beta_2a \), the enhancement of inactivation is still present (Figure 3C).

**Summary of Peak Current Density and Remaining Fraction of Current After 300-ms Depolarization (\( r_{1000} \)) From HEK 293 Cells Transfected With \( \alpha_{1C}, \alpha_{2a}, \) and Various \( \beta \)-Subunit Constructs**

<table>
<thead>
<tr>
<th>( \beta )-Subunit Construct</th>
<th>Cells, n</th>
<th>Peak Current Density, pA/pF</th>
<th>( r_{1000} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat ( \beta_2a )</td>
<td>7</td>
<td>(-37.8 \pm 9.1)</td>
<td>0.82 ( \pm 0.03)</td>
</tr>
<tr>
<td>Rat ( \beta_{2a}-N)-GFP</td>
<td>7</td>
<td>(-109.1 \pm 22.4^*)</td>
<td>0.58 ( \pm 0.05)</td>
</tr>
<tr>
<td>Rat ( \beta_{2a}-C) -GFP</td>
<td>8</td>
<td>(-58.6 \pm 13.6)</td>
<td>0.76 ( \pm 0.04)</td>
</tr>
<tr>
<td>Rat ( \beta_{2a}-IR)-GFP</td>
<td>7</td>
<td>(-52.6 \pm 14.8)</td>
<td>0.84 ( \pm 0.02)</td>
</tr>
<tr>
<td>Rat ( \beta_{2a}-IR)-GFP (one-fifth amount)</td>
<td>11</td>
<td>(-59.1 \pm 15.9)</td>
<td>0.79 ( \pm 0.03)</td>
</tr>
<tr>
<td>Rat ( \beta_3 )</td>
<td>5</td>
<td>(-48.8 \pm 20.5)</td>
<td>0.57 ( \pm 0.04)</td>
</tr>
<tr>
<td>Rat ( \beta_2a-N)-GFP</td>
<td>8</td>
<td>(-19.8 \pm 6.2)</td>
<td>0.68 ( \pm 0.02)</td>
</tr>
</tbody>
</table>

Holding potential was \(-80\) mV. Test potential was \(+10\) mV.

*\( P<0.05 \) vs rat \( \beta_{2a} \).

By contrast, when GFP was fused to the N-terminus of the rat \( \beta_2 \) subunit (Figure 3D, Table), inactivation was similar, or slightly slowed compared with unfused \( \beta_2 \) (Figure 3D3, dashed curve). The faster inactivation observed with \( \beta_1 \) constructs (Figure 3D) compared with the \( \beta_{2a} \) construct (Figure 3A) fits with prior reports that \( \beta_1 \) imparts the fastest inactivation to \( \alpha_1 \) subunits. In short, although direct fusion of GFP and \( \beta \) subunits would provide an efficient strategy for identifying cells transfected with recombinant \( \beta \) subunits, the properties of such \( \beta \) subunits would be somewhat different than those of parental subunits.

To circumvent these complexities, we undertook a second reporter gene approach using a bicistronic expression plasmid encoding both GFP and rat \( \beta_1 \) subunits (Figure 4A). Here, in the rat \( \beta_{2a}-IR\)-GFP construct, the CMV promoter drives transcription of a single mRNA species that encodes both GFP and rat \( \beta_{2a} \) proteins. In principle, such a construct should give rise to an extremely tight correlation between green fluorescence and recombinant \( \beta \) subunit expression, while at the same time preserving native \( \beta \)-subunit properties.
Figure 4B shows the properties of recombinant L-type channels expressed in HEK 293 cells transfected with α1C, αδ, and rat β2a-IR-GFP constructs. For ease of comparison, the format is identical to that in Figure 3. The expression of current is robust (Figure 4B1 and 4B2), and the exemplar trace suggests that inactivation properties are identical to those observed with the conventional rat β2a construct (compare Figures 4B1 and 3A1). Detailed examination of averaged r1000 data (Figure 4B3, Table) confirms that inactivation is no different than that observed with native rat β2a subunits; the dashed curve reproduces the native subunit data from Figure 3A3.

One final check on the rat β2a-IR-GFP construct concerned the possibility that differential expression levels of β subunits could lead to different overall inactivation rates. For example, if more than one β subunit could associate with a single α1C subunit, then channels with variable numbers of associated β subunits could manifest different inactivation properties. In this scenario, the spectrum of inactivation rates observed across different rat β2a constructs in Figure 3A through 3C could simply reflect variable levels of β subunit expression, rather than intrinsically distinct properties of the different constructs. Likewise, the similar inactivation rates observed between rat β2a and rat β2a-IR-GFP might simply reflect matched expression levels. To exclude this possibility, we examined the properties of recombinant L-type channels when only one fifth the usual amount of plasmid encoding β2a-IR-GFP was transfected (Figure 4C). Relative reduction of β2a-IR-GFP did not change channel inactivation properties, as explicitly confirmed by the average r1000 data (Figure 4C3, Table). Here, the averaged data from Figure 4B3 have been reproduced as the dashed curve. These findings agree with earlier biochemical evidence favoring a 1:1 stoichiometry of α1 and β subunits for L-type channels and suggest that different β-subunit constructs impart distinct inactivation properties to L-type channels, in a manner that is insensitive to the level of β-subunit expression. Even in the case of R-type (α1d) and P/Q-type (α1a) channels, where there is relatively clear biochemical evidence for multiple β-subunit binding sites on the α1 subunit, it remains unclear whether >1 β binding site is functionally important. Overall, the use of the bicistronic expression vector encoding GFP and a β subunit appeared to be the reporter gene strategy that would provide the most faithful assessment of the physiological impact of a particular subunit in myocytes.

Ca2+ Channel Modulation by Recombinant β Subunits in Cultured Heart Cells

We next tested whether the various constructs encoding GFP and β subunits could actually direct recombinant β-subunit expression in heart cells. The first indication that these constructs were functionally active came from the detection of green-fluorescent cells after adenoviral compound transfection of the various constructs, observed with an efficiency comparable to that observed with transfection of GFP alone. Figure 5 shows fluorescence views of heart cells transfected with GFP alone as control (A), β2a-N-GFP (B), β-N-GFP (C), and β2a-IR-GFP (D).

Figure 5. High-power (>400) fluorescence micrographs of rat ventricular myocytes expressing GFP alone as control (A), β2a-N-GFP (B), β-N-GFP (C), and β2a-IR-GFP (D). Cells are shown after 4 days in culture.
Figure 7. Tuning of voltage-dependent inactivation by expression of various recombinant \( \beta \) subunits in ventricular heart cells. A, Normalized \( \mathrm{Ca}^{2+} \) current waveforms, averaged from multiple cells after peak currents were rescaled to unity. Means are shown as solid curves, and bracketing SEM confidence ranges. B, Statistical analysis of observed increases in peak current density (at \(+10 \text{ mV}\)) produced by expression of recombinant \( \beta \) subunits. C, Averaged plots of peak current density as a function of step potential, for cells expressing GFP alone (○) or rat \( \beta_{2a} \)-IR-GFP (●). Averages were drawn from the indicated number of cells. These data demonstrate that the \( \beta \)-subunit enhancement of functional \( \mathrm{Ca}^{2+} \) current was present over a broad range of voltages.

More telling was the strong enhancement of L-type channel current density observed in such green-fluorescent cells (Figure 6). Compared with expression of GFP alone as control, recombinant \( \beta \) subunits caused pronounced amplification of current waveforms, averaged from multiple cells (Figure 6A). The enhancement was confirmed statistically by explicit measurements of peak current density (Figure 6B). The effect appeared strongest for the rat \( \beta_{2a} \)-IR-GFP construct but was still present for every \( \beta \)-subunit construct. The increased current density was manifest over a wide range of step potentials, as demonstrated in multiple cells with the rat \( \beta_{2a} \)-IR-GFP construct (Figure 6C). These findings not only demonstrate that recombinant \( \beta \) subunits were functionally active, they also suggest a surprising result that can only be posed in native heart: \( \beta \) subunits appear to be ‘rate limiting’ for expression of functional \( \mathrm{Ca}^{2+} \) channel current in ventricular heart cells, even though channels are composed of at least 3 subunits (\( \alpha_{1C}, \alpha_{1D}, \alpha_\delta \)). Here, we use the term rate limiting in a functional sense, referring to a limitation either in the \( P_o \) of channels or in the number of active channels in the surface membrane (as detailed in the Discussion).

Another important question was whether recombinant \( \beta \) subunits would affect \( \mathrm{Ca}^{2+} \) channel inactivation in a manner similar to that observed in HEK 293 cells (Figures 3 and 4). Figure 7A shows normalized current waveforms elicited by 1-second depolarizations to \(+10 \text{ mV}\), after averaging across multiple heart cells. It is evident that different constructs produced markedly different inactivation characteristics, according to a rank order that accords with the results from HEK 293 cells (Figures 3 and 4). The effects were statistically resolved, as shown in the bar graph summary of \( r_{1000} \) data from multiple cells (Figure 7B). A similar rank order of effects was observed with analysis of the residual fraction of current measured after 300 ms of depolarization (\( r_{300} \)), confirming that various \( \beta \)-subunit constructs impart a generally uniform slowing or acceleration of inactivation over the course of 1-second depolarizations. In comparison with control cells expressing GFP alone, it is remarkable how slowly currents inactivated in cells overexpressing any of the recombinant \( \beta \) subunits: the slowing was detectable even with the rat \( \beta_{2a} \)-N-GFP construct, but was most marked with the putative ‘cardiac’ isoform of the \( \beta \) subunit (rat \( \beta_{2a} \)-IR-GFP) (Figure 7C). Given that rates of voltage-dependent inactivation were insensitive to variable levels of \( \beta \) subunit expression in HEK 293 cells (Figure 4B and 4C), it is reasonable to expect that the functionally dominant \( \beta \) subunit in heart is not the rat \( \beta_{2a} \) isoform. Whatever the molecular identity of the actual cardiac \( \beta \) subunit turns out to be, it is interesting that this subunit imparts an inactivation rate (GFP control cells) that exceeds that observed with overexpression of rat \( \beta_{2a} \)-N-GFP. In HEK 293 cells, the latter construct gives rise to an inactivation rate similar to that of un fused \( \beta \), which...
yields the fastest rate observed with the known $\beta_{1-4}$ subunits.\textsuperscript{36}

**Discussion**

This report describes the first application of an adenoviral-component gene-delivery method to transfected young adult ventricular myocytes. In addition, we established parameters for the use of GFP to detect expression of foreign Ca\textsuperscript{2+} channel $\beta$ subunits. Fusion of GFP to $\beta$ subunits spared overall subunit function, but resulted in quantitative alterations in inactivation properties. Pairing GFP with $\beta$ subunits in a bicistronic gene cassette fully conserved the functional attributes of $\beta$ subunits. Using these approaches, this study provides the first observations of the effects of expressing recombinant Ca\textsuperscript{2+} channel $\beta$ subunits in cardiac ventricular myocytes, culture from 4- to 6-week-old rats. Previous studies have focused on heterologous expression in model systems. These experiments identified $\beta$-subunit modulatory effects with special relevance to the native setting. In particular, the overall density of L-type channel current was markedly enhanced by $\beta$-subunit expression, and voltage-dependent inactivation was highly sensitive to the type of $\beta$ subunit that was transfected. Here we elaborate on the implications of various $\beta$-subunit effects, as observed in the actual cardiac environment.

**$\beta$ Subunits Are Rate Limiting for Expression of Functional Ca\textsuperscript{2+} Channel Current**

Expression of recombinant $\beta$ subunits alone enhanced Ca\textsuperscript{2+} channel current density up to 3-to-4-fold, arguing that $\beta$ subunits are rate limiting for expression of L-type current in heart. This finding is rather surprising, given the well-established heteromultimeric composition of L-type channels. Although the $\alpha_{1c}$ subunit forms the pore of the channel and specifies overall characteristics of the channel, at least 3 subunits ($\alpha_{1c}$, $\beta$, and $\delta$) are believed to comprise native channels,\textsuperscript{14-16} with other as-yet-unknown subunits still possible as additional elements of the channel complex (eg, Reference 19). One might therefore expect that coexpression of multiple Ca\textsuperscript{2+} channel subunits would be required to enhance the overall level of L-type current.

The large increase in Ca\textsuperscript{2+} channel currents arising from expression of recombinant $\beta$ subunits alone raises several possible scenarios for the heart, none of which are mutually exclusive. First, recombinant $\beta$ subunits could associate with $\alpha_{1c}$ subunits that are already in the surface membrane, thereby increasing open probability of the pore via allosteric interactions favoring the open state.\textsuperscript{50} Second, new $\beta$ subunits may help to translocate excess, preexisting $\alpha_{1c}$ subunits waiting in the Golgi complex to the surface membrane or to stabilize such channel complexes in the cell membrane.\textsuperscript{50,55-57} Either or both of these actions would lead to an increase in the number of functional channels in the sarcolemma. Finally, it is possible that gene expression of $\alpha_{1c}$ and other subunits may be under feedback control that senses $\beta$-subunit expression.

Regardless of the mechanism, the functional result that expression of $\beta$ subunits alone can increase L-type current may prove to be an important simplification for potential gene therapy to reverse the decline in overall current density seen in some forms of heart failure.\textsuperscript{7,58-62} In particular, in failing cardiac allografts with diastolic dysfunction, down-regulation of $\beta$ subunits has been correlated with hemodynamic defects.\textsuperscript{63} On the other hand, $\beta$-subunit supplementation would not be relevant to many forms of heart failure in which there is little decline in Ca\textsuperscript{2+} channel current density.\textsuperscript{5,64} Compared with genes encoding $\alpha_{1c}$ subunits ($\approx$7 kilobases), $\beta$-subunit transcripts are relatively small (1 to 3 kilobases), thereby facilitating gene delivery. Furthermore, expression of a single foreign gene considerably simplifies the technical challenge.

**Voltage Inactivation of L-Type Channels Is Highly Sensitive to the Type of $\beta$ Subunit**

Another important finding is that expression of different $\beta$ subunits produces strikingly different rates of voltage-dependent inactivation of L-type channels, as observed over the course of 1-second depolarizations. Using heterologous expression in HEK 293 cells, we have previously demonstrated that different $\beta$ subunits produce remarkably small differences in the steady-state inactivation of L-type channels by voltage.\textsuperscript{18} In apparent agreement, different $\beta$ subunits ($\beta_{1b}$ versus $\beta_{2a}$) produce very similar rates of voltage-dependent inactivation of recombinant L-type channels expressed in Xenopus oocytes during several-hundred–millisecond depolarizations.\textsuperscript{65} Here, we found that $\beta$, and $\beta_{2a}$ subunits produced very different voltage-dependent inactivation rates during 1-second depolarization of recombinant L-type channels expressed in HEK 293 cells. Importantly, these differences in voltage-dependent inactivation were recapitulated in heart cells transfected with different recombinant $\beta$ subunits. The distinct inactivation rates observed over the span of 100 to 1000 ms are particularly relevant to specifying the APD of heart.\textsuperscript{66} In fact, the observed variation of inactivation rates with different $\beta$ subunits could be even more striking in the native setting, if we consider that both voltage-dependent and Ca\textsuperscript{2+}-dependent mechanisms of inactivation are present when Ca\textsuperscript{2+} serves as charge carrier.\textsuperscript{58,60} Previous studies hint that different $\beta$ subunits would produce larger distinctions in the rate of Ca\textsuperscript{2+}-dependent versus voltage-dependent inactivation,\textsuperscript{65,67} although further work may be necessary to fully establish these intriguing observations.

These findings hold particular pathophysiological relevance, because one of the hallmarks of heart failure is prolongation of the action potential.\textsuperscript{4-6} perhaps secondary to depressed intracellular [Ca\textsuperscript{2+}], resulting in decreased Ca\textsuperscript{2+}-dependent inactivation of L-type channels.\textsuperscript{9,10} Alternatively, a relative shift in the prevalence of certain $\beta$-subunit isoforms could also account for altered Ca\textsuperscript{2+}-dependent inactivation in heart failure. In either case, this study raises the possibility that gene expression of an appropriate $\beta$ subunit could rectify the prolongation of APD attendant to some forms of heart failure.

**Identity of Genuine Cardiac-Specific $\beta$ Subunits**

The remarkably slow inactivation rate observed on expression of the putative “cardiac” isoform of the $\beta$ subunit (rat $\beta_{2a}$) suggested that other types of $\beta$ subunits are functionally dominant in native rat heart. Historically, although the rat $\beta_{2a}$
was cloned from a rat brain library. Northern blot analysis suggested that it was also quite prevalent in heart. However, only indicates that something quite homologous to the rat brain β2 subunit is present in heart. Nonetheless, a highly similar rabbit β2 subunit was cloned directly from rabbit heart. Because the main differences between the rat and rabbit β2 subunits were restricted to the extreme amino terminal region, it seemed reasonable that both of these β2 subunits might be found in heart. However, recent PCR analysis, using isoform-specific primers at the amino terminus, confirmed the presence of only the rabbit β2 species in rabbit myocardium but failed to detect a transcript similar to the rat brain β2 in the same tissue.

Overall, there is considerable indeterminacy in regard to legitimate, cardiac–specific isoforms of β subunits. Beyond confirmation of the rabbit β2 species in rabbit heart, Collin et al cloned β1 splice variants in human heart and showed them to be dominant by Northern blot and reverse transcriptase–PCR. No β2 was detected. Expression of novel β subunits in heart cells will prove useful in establishing the veracity of purported cardiac specificity.

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