Cloning of a Functional Splice Variant of L-type Calcium Channel \( \beta_2 \) Subunit from Rat Heart*

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L-type \( \text{Ca}^{2+} \) channels are heteromultimeric and finely tuned by auxiliary subunits in different tissues and regions. Among auxiliary subunits, \( \beta \) subunit has been shown to play important roles in many functional aspects of \( \text{Ca}^{2+} \) channel. Rat heart was reported to specifically express \( \beta_{2a} \) subunit. However, the slow inactivation rates of \( \text{Ca}^{2+} \) currents recorded from recombinant \( \text{Ca}^{2+} \) channels with the \( \beta_{2a} \) subunit, and the reported inability to detect \( \beta_{2a} \) subunit in rabbit heart by reverse transcription-PCR analysis raise the possibility of the existence of other \( \beta \) subunits. We cloned a splice variant of \( \beta_2 \) subunit from rat heart, using rapid amplification of cDNA 5‘ ends. The splice variant is highly similar to human \( \beta_{2c} \) subunit that was cloned from human ventricular septum. When the \( \beta_{2c} \) subunit was expressed along with \( \alpha_1c \) and \( \alpha_2\delta \) subunits in baby hamster kidney cells, the inactivation rates were comparable with those from native cardiac myocytes, although those with the \( \beta_{2a} \) subunit were slow. Taken together, these observations suggest that the \( \beta_{2c} \) subunit is a functional \( \beta_2 \) subunit expressed in heart and that the short N-terminal region plays a major role in modifying inactivation kinetics.

L-type \( \text{Ca}^{2+} \) channel plays an important role in shaping the action potential of cardiac myocytes and is a major pathway for extracellular \( \text{Ca}^{2+} \) entry into cardiomyocytes (1). Four distinct subunits, i.e. \( \alpha_{1c}, \beta, \) and \( \alpha_2\delta \), compose the L-type \( \text{Ca}^{2+} \) channel in tissues other than skeletal muscle (2). Among auxiliary subunits, \( \beta \) subunit has been shown to be a central player in most, if not all, functional aspects of \( \text{Ca}^{2+} \) channel (3). \( \beta \) subunit promotes the transportation of \( \text{Ca}^{2+} \) channel to the membrane and affects the activation and inactivation kinetics (3). To date, four distinct \( \beta \) subunits, i.e. \( \beta_1, \beta_2, \beta_3, \) and \( \beta_4 \), and their splice variants have been cloned and expressed in different tissues and regions (2–8). All four \( \beta \) subunits are expressed in brain (3–5, 7–9). In contrast, the skeletal and cardiac muscle

\( \text{Ca}^{2+} \) channels are thought to be associated, apparently exclusively, with the \( \beta_{1a} \) and cardiac \( \beta_2 \) subunits, respectively (6). Indeed, rat \( \beta_{2a} \) (8), rabbit \( \beta_{2a} \) (7), and \( \beta_{2b} \) (7) subunits, the difference among which resides only in the N-terminal region, were reported to be respectively expressed in these animal hearts. However, in human heart, mRNA expression of \( \beta_{1b} \) (3, 10, 11), \( \beta_{1c} \) (3, 10), and \( \beta_4 \) (11) subunits has been demonstrated in addition to protein expression of the \( \beta_2 \) subunit (12). Moreover, recent PCR analysis could not detect the rat \( \beta_{2b} \) subunit with the rat \( \beta_{2a} \)-specific primers in rabbit heart, but it was present in brain (13). Therefore, it is not yet clear which \( \beta \) subunits exist in heart and whether heart expresses certain \( \beta \) subunits specifically.

Coexpression of the rat \( \beta_{2a} \) subunit with various \( \alpha \) subunits slowed the inactivation of the \( \text{Ca}^{2+} \) current (I\( \text{Ca} \)), although all the other \( \beta \) subunits accelerated it (5–7, 14). Inactivation rates of I\( \text{Ca} \) from native cardiac \( \text{Ca}^{2+} \) channels are apparently fast, compared with those from L-type \( \text{Ca}^{2+} \) channels containing the rat \( \beta_{2a} \) subunit in a heterologous expression system (15). This fact, by itself, implies that other \( \beta \) subunits and/or regulators may function in rat heart. However, no evidence was found to show that the \( \beta_1, \beta_3, \) and/or \( \beta_4 \) subunits existed in rat heart (4, 5, 8). These observations led us to speculate about the possible existence of unidentified \( \beta \) subunits and/or splice variants in heart.

Indeed, rapid amplification of cDNA 5‘ ends (5‘-RACE) disclosed a \( \beta_2 \) transcript in rat heart, which was only different from other \( \beta_2 \) subunits in the N-terminal region. Here, we named it rat \( \beta_{2c} \) subunit, because the sequence of this subunit was very similar to that of human \( \beta_{2c} \) subunit, which was also cloned from human ventricular septum. When the \( \beta_{2c} \) subunit was expressed in BHK cells along with a pore-forming \( \alpha_{1c} \) and other auxiliary \( \alpha_2\delta \) subunits cloned from rat heart, the inactivation rate was comparable with that from native cardiac myocytes.

EXPERIMENTAL PROCEDURES

Preparation of Single Cardiac Myocytes—Single ventricular myocytes were enzymatically isolated from the ventricle of rat hearts as described previously (16). In brief, the hearts were removed from rats, following anesthesia with pentobarbital, and perfused in a Langendorff apparatus with 0.02–0.04% collagenase (Wako Pure Chemical Industries, Osaka, Japan) dissolved in nominally \( \text{Ca}^{2+} \)-free Tyrode solution. After 30 min of digestion, the left ventricle was rinsed with Krafftbrühe (KB) solution (17), cut into small pieces, and shaken to separate cells.
The composition of the nominally Ca\textsuperscript{2+}-free Tyrode solution was (in mM): NaCl 143, KCl 5.4, MgCl\textsubscript{2} 0.5, NaH\textsubscript{2}PO\textsubscript{4} 0.33, glucose 5.5, and HEPES 10 (pH 7.4, titrated by 1 N NaOH). The KB solution was composed of (in mM): KCl 40, KOH 70, KH\textsubscript{2}PO\textsubscript{4} 20, l-glutamic acid 50, taurine 20, MgCl\textsubscript{2} 0.5, EGTA 1, glucose 10, and HEPES 10 (pH 7.4, titrated by 1 N KOH). The cell suspension in the KB solution was stored in a refrigerator at 4 °C for later use.

**RNA Isolation and RT-PCR Analysis**—Rats weighing 250–350 g were anesthetized and killed with an excessive amount of pentobarbital, and the hearts and brains were removed as quickly as possible and frozen in liquid nitrogen. Total RNAs and messenger RNAs (mRNAs) were anesthetized and killed with an excessive amount of pentobarbital. Ten heart beta-\textsubscript{2} pr-1 specific primer, pGEM-tbeta2-p-308f Forward primer for a full-length heart beta-\textsubscript{2} pr-308R Reverse primer for a full-length heart beta-\textsubscript{2} pr.

| Name                  | Description                                                                 | 5’-Sequence-3’                      |
|-----------------------|------------------------------------------------------------------------------|-------------------------------------|
| TotalPrimer1104f      | Forward primer for a full-length \(a_{\text{a}}\)                            | TGGTACATACGGTCTGTTGTC              |
| TotalPrimer1104r      | Reverse primer for a full-length \(a_{\text{a}}\)                           | TAAAAGACTTCGTCC                   |
| a2primer909f         | Forward primer for a full-length \(\alpha_{\text{d}}\)                      | TCGATGCCGGAGATTGCTGT             |
| a2primer909r         | Reverse primer for a full-length \(\alpha_{\text{d}}\)                      | GGGTTTAGAGGTCATCATATG          |
| total-beta-2-primer114f | Reverse primer for a full-length \(\beta_{2a}\)                           | TCAGCTGGCAGCAGTCCCTAT            |
| total-beta-2-primer114r | Reverse primer for a full-length \(\beta_{2a}\)                           | AAGACACGACAGCTGTTG             |
| beta2primer0302f     | Forward primer for \(\beta_{2a}\)                                           | TAATGCGTTGGCTAGTACG               |
| beta2primer0302r     | Reverse primer for \(\beta_{2a}\)                                           | AGATGTCACAAAGCATCCTCA           |
| beta2primer_for_5’-RACE604r1 | Reverse primer for 5’-RACE of \(\beta_{2a}\) | GAACTGGTACTATGCTCACC            |
| beta2primer_for_5’-RACE604r2 | Reverse primer for 5’-RACE of \(\beta_{2a}\) | TCCCTGGTGTCTGCTCTGT            |
| beta2primer_for_5’-RACE604r3 | Reverse primer for 5’-RACE of \(\beta_{2a}\) | CTCTATGTTGAGCCGGTGTC          |
| beta2primer_for_5’-RACE604r4 | Reverse primer for \(\beta_{2a}\)-specific probe | GCCATCTGCTTACATTCTCC          |
| brain-beta-5’-G1208r  | Forward primer for \(\beta_{2a}\)-specific probe                            | GACTTTTCGCGATGTCG               |
| heart-beta-5’-G1208f  | Reverse primer for \(\beta_{2a}\)-specific probe                            | GGACAAACTGTTGGAGGTAGT            |
| heart-beta-5’-G1208r  | Reverse primer for \(\beta_{2a}\)-specific probe                            | CGGATGTCGCTACCGAACGAGT          |
| betta2-p-308f        | Forward primer for a full-length \(\beta_{2a}\)                           | GCCATGAGGCCCCCAAGATG          |
| betta2-p-308r        | Reverse primer for a full-length \(\beta_{2a}\)                           | GAACAGACCGCCAGACAGG          |

Electrophysiological Study—Cover slip fragments with attached cells or isolated cardiac myocytes were continually perfused on the stage of an inverted microscope. Whole-cell patch-clamp recordings were made using pipettes with resistance of 2–4 M\text{mhos}. Perfusion composition was as follows (in mM): choline chloride 140, CaCl\textsubscript{2} 5.4, MgCl\textsubscript{2} 0.5, and glucose 10, titrated by 1 N NaOH.
HEPES 5, and d-glucose 10 (pH 7.4, titrated by Tris-HCl), with Ba\(^{2+}\) 10, 0.5, or Ca\(^{2+}\) 1.8 as charge carrier. Pipette solution composition was as follows (in mM): l-glutamate acid 110, CaCl\(_2\) 20, CsOH 110, MgCl\(_2\) 1, Na\(_2\)ATP 5, creatine phosphate 5, EGTA 10, and HEPES 5 (pH = 7.4, titrated by 1 N CsOH). We chose BHK and COS-7 cells as heterologous expression systems because they were reported to lack any subunits of L-type Ca\(^{2+}\) channel (20–22). All experiments were done at 37°C. Transfected cells were identified by the expression of hrGFP. Cells were clamped at −80 mV, and whole-cell currents were evoked by 400-ms step depolarization to various test potentials (0.1Hz). Currents were filtered at 2 kHz and digitized at 10 kHz. Series resistance compensation was not applied. Analysis and voltage protocols were performed with the use of an Axopatch 1D amplifier/Digidata 1322A interface (Clampex software, pCLAMP 8.1, Axon Instruments Inc.). The data were analyzed after leak subtraction. Inactivation characteristics of Ba\(^{2+}\) current (\(I_{\text{Ba}}\)) were measured using a two-step voltage clamp protocol. A 3-s conditioning pre-pulse was applied from a holding potential of −80 mV (10-mV increments from −100 mV to 10 mV) followed by a 100-ms test pulse to 10 mV. The intervals between each cycle were 10 s. Recorded peak current amplitudes were normalized to the maximum value. Steady-state inactivation curves were fitted by a Boltzmann function: 

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

where \(I_{\text{Ba}}\) is the normalized peak current, \(V\) is the conditioning pre-pulse voltage, \(V_{1/2}\) is the voltage at half-maximum inactivation, and \(h\) is the slope factor. Inactivation time constants were obtained by fitting a current decay with a two-exponential equation: 

\[ I(t) = I_s + A_1 \exp(-t/\tau_{1}) + A_2 \exp(-t/\tau_{2}) \]

where \(I(t)\) is the time-dependent current, \(I_s\) is the steady-state amplitude of the current, \(A_1\) and \(A_2\) are the fractions of currents that are slow and fast inactivated, respectively, and \(\tau_{1}\) and \(\tau_{2}\) are the time constants that are slow and fast, respectively. The r400 value was calculated as the ratio of the current amplitude at the end of 400-ms depolarization pulse divided by the peak amplitude of the trace.

Statistics—All values are presented in terms of mean ± S.E. When I-V relationships and inactivation characteristics were compared, two-way repeated measures of ANOVA were first carried out to test for any differences among the mean values of multiple subgroups. When a significant F value was obtained by two-way ANOVA, intergroup comparisons were performed by contrast test. When the model with the interactions improved significantly, we interpreted it as indicating different voltage dependences of the groups. The voltages at half-maximum inactivation and the slope factors were compared by Student’s t test. Significance was established at \(P\) values < 0.05.

RESULTS

First, we performed RT-PCR to confirm that the \(\beta_{2c}\) subunit exists in rat heart, using total RNA extracted from rat heart as a template. As shown in lane 1 of Fig. 1B, we were able to obtain a partial fragment of \(\beta_{2c}\) subunit. Then, we attempted to isolate the reported \(\beta_{2a}\) subunit including a full-length ORF. However, as shown in lane 2 of Fig. 1B, we could not observe any PCR product using the \(\beta_{2a}\)-specific primers. As the same primers detected the \(\beta_{2a}\)-specific subunit when total RNA from rat brain (Fig. 1B, lane 5) was used as a template, we speculated that another splice variant of \(\beta_{2a}\) was expressed in rat heart. The \(\beta_{2a}\) subunit is known to have splice variants in the N-terminal region. To prove that the forward primer was not suitable to obtain the \(\beta_{2a}\) subunit in rat heart, we performed RT-PCR analysis using a primer pair of primer_1 and primer_4 and another pair, primer_3 and primer_2 (see Fig. 1A). As shown in lanes 3 and 4 of Fig. 1B, only primer_3 and primer_2 detected the \(\beta_{2a}\) subunit, which indicated that the N-terminal region of the \(\beta_{2a}\) subunit expressed in rat heart was different from the reported \(\beta_{2a}\)-sequence cloned from rat brain.

The upper lane of Fig. 2A shows the partial sequence of the \(\beta_{2a}\) subunit in rat heart that was determined by 5’-RACE (GenBank™ accession number AF394942). The sequence was completely different from the reported \(\beta_{2a}\) sequence in the 5’-untranslated region (UTR) and the first 199 nucleotides of ORF. We set the primers based on this result and succeeded in cloning the full-length \(\beta_{2a}\) subunit (GenBank™ accession number AF394941) expressed in rat heart (Fig. 3A, lane 2). This \(\beta_{2a}\) clone contains 656 amino acids, yielding a protein with a calculated molecular mass of 73.2 kDa. We could also amplify the \(\beta_{2c}\) subunit in rat brain, using the same primers (Fig. 3A, lane 1). Therefore, it was demonstrated that the \(\beta_{2c}\) subunits were expressed in heart and brain.

The deduced partial amino acid sequences for the rat \(\beta_{2c}\) and \(\beta_{2a}\) subunits are compared in Fig. 2B, along with that for the human \(\beta_{2a}\), the rabbit \(\beta_{2a}\), and the rabbit \(\beta_{2c}\) subunits (7, 8, 23). The rat \(\beta_{2c}\) subunit differs only in the N-terminal region from the rat \(\beta_{2a}\), the rabbit \(\beta_{2a}\), and \(\beta_{2b}\) subunits except for some amino acids sequence, considered to be species differences. The N-terminal region of the rat \(\beta_{2a}\) subunit has one potential protein kinase C site and two casein kinase II phosphorylation sites. No consensus site for cAMP-dependent protein kinase was found in this region. In contrast to other \(\beta_{2}\) subunits, the sequence for the N-terminal region of the human \(\beta_{2a}\) subunit, which was also cloned from human ventricular septum (23), is almost identical to that for the rat \(\beta_{2c}\) subunit.

To confirm the PCR result further, Northern blot analysis was performed. The \(\beta_{2a}\)-specific probe hybridized with three mRNA species of −6, 4, and 2 kb in rat heart (Fig. 3B, lane 3). However, the \(\beta_{2a}\)-specific probe hybridized only two transcripts of about 6 and 2 kb (Fig. 3B, lane 1). In rat brain, both the \(\beta_{2a}\)-and the \(\beta_{2c}\)-specific probes cross-reacted with three transcripts of −6, 4, and 2 kb (Fig. 3B, lanes 2 and 4). Perez-Reyes et al. (8) detected mRNA species of 6, 4, and 3.5 kb in rat heart, using \(^{32}\)P-labeled randomly primed cDNA of the \(\beta_{2}\) clone. As the sequence of the \(\beta_{2}\) and the \(\beta_{2a}\) subunits are the same except for the N-terminal region, it is highly possible that the probe used by Perez-Reyes et al. (8) contained the common sequences that recognize both the \(\beta_{2}\) and the \(\beta_{2a}\) subunits. Therefore, the 4-kb transcript, which was not recognized by the \(\beta_{2a}\)-specific probe but by the \(\beta_{2c}\)-specific probe, may be the \(\beta_{2c}\) subunit.

These results, along with the PCR analysis, demonstrated that the \(\beta_{2c}\) subunit was expressed abundantly in both heart and brain (see also “Discussion”).

To test the functional effect of the \(\beta_{2c}\) subunit and to compare the recombinant Ca\(^{2+}\) channel containing the \(\beta_{2c}\) subunit with native one, we cloned a pore-forming subunit and other auxil-
A Functional Splice Variant of $\beta_2$ Subunit

The deduced amino acid sequence of rat $\beta_2$-subunit can be retrieved using GenBank accession number AF394942. Alignment of the deduced amino acid sequence of rat $\beta_2$-subunit. Amino acid sequences of rat $\beta_2$-subunit. Amino acid sequences of rat $\beta_2$, rat $\alpha_2$, human $\beta_2$ (GenBank accession number AF137376), rabbit $\beta_2$ (GenBank accession number X64297), and rabbit $\beta_2$ (GenBank accession number X64298) are aligned for comparison. The potential sites for protein kinase C (●) and casein kinase II (○) phosphorylation are indicated above the line. Identical amino acids are indicated by vertical bars. Dashes represent gaps in the sequence.

Secondary subunits of L-type Ca$^{2+}$ channel, i.e. $\alpha_1c$ and $\alpha_2\delta$ subunits from rat heart. We thought it important to reconstitute the L-type Ca$^{2+}$ channel, which was composed of all four subunits cloned from heart of the same species. Otherwise, any different characteristics from native channels may have been the result of subunits other than those of $\beta$ subunits. We obtained three kinds of splice variants of $\alpha_1c$ subunit (i.e. $\alpha_1c$, $\alpha_1c12$, and $\alpha_1c15$; GenBank accession numbers AF394938, AF394939, and AF394940, respectively). The sequence of the cloned $\alpha_1c$ subunits consists of an ORF of 6513 bases that encodes 2171 amino acids. The calculated molecular masses are about 243 kDa.

When an ion channel protein is expressed in a heterologous expression system and the patch-clamp experiment is done, GFP is often attached to the C terminus of the channel protein, to choose the transfected cell easily. However, it has recently become known that this attached GFP changes the kinetics of the channel itself (15). To avoid these problems, we used pIRES that contained a pore-forming $\alpha_1c$ subunit along with one of the $\beta_2$ subunits. Besides this, we transfected cells with pIRES-hrGFP or pIRES-$\alpha_2\delta$-hrGFP simultaneously. These vectors contain the internal ribosome entry site (IRES) from the encephalomyocarditis virus, which allows translation of two consecutive open reading frames from the same mRNA (27). In this case, the single mRNA species supports translation of separate $\alpha_1c$ and one of the $\beta_2$ proteins or $\alpha_2\delta$ and hrGFP proteins. Thus, if we could record $I_{Ba}$ from a cell expressing hrGFP, it was highly possible that recombinant Ca$^{2+}$ channels contained $\alpha_1c$ and one of the $\beta_2$ subunits (along with the $\alpha_2\delta$ subunit when pIRES-$\alpha_2\delta$-hrGFP was used.). Fig. 5 (A and B) shows the representative traces of $I_{Ba}$ that we recorded from BHK cells transfected with pIRES-$\alpha_1c$-$\beta_2a$ and pIRES-$\alpha_2\delta$-$\beta_2a$, respectively. Ca$^{2+}$ channels were expressed in about 70–90% of the hrGFP-expressing cells. We used 10 mM Ba$^{2+}$ as charge carrier to circumvent the effect of Ca$^{2+}$ on inactivation kinetics and to make the difference in voltage-dependent inactivation kinetics clearer. $I_{Ba}$ was blocked by the dityrhodipryidine derivative, nifedipine (10 μM, data not shown). We judged the $\beta_2$ subunit to have functioned if the current was found to express $a2c$ and $a2d$ in approximately a 9:1 ratio, respectively. We had four clones that contained a full-length $\alpha_2\delta$ subunit. However, all of them corresponded with the mouse $a2d$ subunit.

Fig. 2. A, nucleotide sequence comparison between $\beta_2a$ and $\beta_2b$ subunits. 5'-RACE analysis of rat heart was performed as described under “Experimental Procedures,” and the sequence of $\beta_2b$ subunit was determined. Upper and lower lanes indicate the sequence of $\beta_2a$ and $\beta_2b$ subunits, respectively. A site of sequence identity is marked with an asterisk. The sequence of the $\beta_2b$ subunit was from Perez-Reyes et al. (GenBank accession number M80545). The nucleotide sequence of the rat $\beta_2a$ subunit can be retrieved using GenBank accession number AF137376, rabbit $\beta_2$ (GenBank accession number X64297), and rabbit $\beta_2$ (GenBank accession number X64298) are aligned for comparison. The potential sites for protein kinase C (●) and casein kinase II (○) phosphorylation are indicated above the line. Identical amino acids are indicated by vertical bars. Dashes represent gaps in the sequence.

| A | B |
|---|---|
| $\beta_2a$ | $\beta_2b$ |
| 1 | 1 |
| 2 | 2 |
| 3 | 3 |
| 4 | 4 |
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| 6 | 6 |
| 7 | 7 |
| 8 | 8 |
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Fig. 5. (A) shows the representative traces of $I_{Ba}$ that we recorded from BHK cells transfected with pIRES-$\alpha_1c$-$\beta_2a$ and pIRES-$\alpha_2\delta$-$\beta_2a$, respectively. Ca$^{2+}$ channels were expressed in about 70–90% of the hrGFP-expressing cells. We used 10 mM Ba$^{2+}$ as charge carrier to circumvent the effect of Ca$^{2+}$ on inactivation kinetics and to make the difference in voltage-dependent inactivation kinetics clearer. $I_{Ba}$ was blocked by the dityrhodipryidine derivative, nifedipine (10 μM, data not shown). We judged the $\beta_2$ subunit to have functioned if the current was
larger than 100 pA, because recombinant Ca²⁺ channel currents expressed without β subunits were very small (typically 10–50 pA, data not shown), as reported elsewhere (21, 22). It is apparent that the current with β₂c inactivated faster than that with β₂a. The fractions of current remaining at the end of 400-ms depolarization (r₄₀₀) derived from traces of β₂c-transfected cells were significantly smaller throughout the membrane potentials examined than those with β₂a, though I-V relations were not different (Fig. 5, C–E). The steady-state inactivation curves derived from records with β₂a and β₂c were not significantly different (Fig. 5F). The rates of inactivation with β₂c were apparently faster than those with β₂a even when we used COS-7 cells instead of BHK cells (data not shown). The r₄₀₀ values derived from COS-7 cells with β₂a and β₂c showed the same tendency as those from BHK cells (Fig. 5E). When pIRES-α₁c-hrGFP was transfected to BHK cells in addition to pIRES-α₁c-β₂a or pIRES-α₁c-β₂c, Iₘ₄ was larger with β₂c transfection also inactivated faster than Iₘ₄ with β₂a transfection (Fig. 6, A and B; charge carrier was 0.5 mM Ba²⁺ (see the reason below)). The same observation in different expression systems with or without other auxiliary subunits strongly suggests that the β₂a and the β₂c subunits function differently.

To compare the inactivation rates from recombinant Ca²⁺ channels with those from native cardiac myocytes, the concentration of charge carrier Ba²⁺ ion that flows through Ca²⁺ channel was changed from 10 to 0.5 mM. Otherwise, the peak amplitudes of Iₘ₄ recorded from native cardiac myocytes was
over 10 nA and it became difficult to voltage-clamp a cell. As seen in the representative traces (Fig. 6, upper traces), the inactivation rates from a native cardiac myocyte were very similar to those from a BHK cell with β2a, but different from those with β2c. A current decay was fitted to a two-exponential function significantly better than a single-exponential function. Fig. 7 shows comparisons of time constants (τfast and τslow) and their contribution (Δ(Δ)/Δt) calculated using traces from BHK cells with β2a, β2c, and native cardiac myocytes. τslow (Fig. 7A), τfast (Fig. 7B), and Δ(Δ)/Δt (Fig. 7C) obtained from native cardiac myocytes were significantly smaller than those from BHK cells with β2a (statistically significant by ANOVA and contrast test), whereas they were very similar to those with β2c (not significant by contrast test). However, we could not reconstitute Ca2+ channel expressed in native heart completely. The r400 values of native I400 were smaller than those of β2a as expected, but they were larger than those of β2c (Fig. 7D). Besides, the voltage dependence of the normalized I-V relationship from native cardiac myocytes was slightly but significantly different from that from BHK cells with β2a or β2c subunit (Fig. 6, D-F). However, these observations strongly suggest that the β2c subunit functions in cardiac myocytes.

To examine the inactivation kinetics in more physiological conditions, we changed the charge carrier from 0.5 mM Ba2+ to 1.8 mM Ca2+. The upper panel of Fig. 8 shows representative traces recorded from BHK cells expressing recombinant channels and a native cardiac myocyte. Ca2+ accelerated current decays in all traces compared with those recorded in the solution containing Ba2+. However, I400 through the recombinant Ca2+ channel with β2c (Fig. 8A) was, apparently, still slower to inactivate than with β2a (Fig. 8B) and the native cardiac channel (Fig. 8C). In contrast, it was difficult to distinguish between the traces recorded from the BHK cells transfected with β2c (Fig. 8B) and the native cardiac myocyte (Fig. 8C). When a decay of I400 was fitted to a two-exponential function, τslow (Fig. 8H) obtained from BHK cells with β2c was significantly slower than that from BHK cells with β2a and native cardiac myocytes, and τfast from recombinant channels containing β2c and native channels were not different statistically as expected. However,

DISCUSSION

In rat heart, the β2a subunit was reported to be expressed in a tissue-specific manner (8). However, recent reports cast doubt on the functional roles of β2a subunit in cardiac myocytes. Wei et al. (15) proposed the possibility that other β subunits function dominantly, because they could not reconstitute similar inactivation rates of I400 even when cardiac myocytes were transfected with the β2a subunit. Qin et al. (13) reported that PCR analysis could not detect any signal in rabbit heart with the rat β2c-specific primers. In an attempt to study cardiac Ca2+ channel using the RT-PCR method, we also failed to obtain the β2a subunit from rat heart with the rat β2a-specific primers, but obtained it from brain. Historically, the rat β2a subunit was first cloned from brain (8); then, Northern blot analysis demonstrated that this subunit was also expressed in heart (8). We were able to obtain a partial fragment of β2a subunit from heart. However, with the specific primer for the N-terminal region of the β2a subunit, although we were able to obtain a signal from brain, no signal could be procured from heart. From these observations, we believed that rat heart did not express the N-terminal region of the β2a subunit. Therefore, we were obliged to perform 5’-RACE. As a result, we cloned a splice variant of β2a subunit, called here the rat β2a subunit. Northern blot analysis demonstrated that the β2c subunit was expressed abundantly in heart and that a part of the β2a subunit considered to be expressed in heart might be, in fact,
the $\beta_{2c}$ subunit.

We could not conclude that the $\beta_{2a}$ subunit was not expressed in heart. In the present study, Northern blot analysis detected the 6- and 2-kb transcripts in heart with the $\beta_{2c}$-specific probe. The identities of these transcripts were not clear. Theoretically, they might have been fragments of the $\beta_{2a}$ subunit. If so, our PCR analysis should have detected some $\beta_{2a}$ signals not only from brain but also from heart. If the 6-kb transcript was the mRNA of the $\beta_{2a}$ subunit, which does not have the sequence of the forward primer, i.e. the total-beta-2-primer414f, it might be expressed in heart, although 5' RACE did not disclose any truncated forms of the $\beta_{2a}$ subunit. Otherwise, the 6- and 2-kb transcripts may be artifacts.

It is certain that the mechanism responsible for the slow inactivation occurring when the $\beta_{2a}$ subunit is expressed along with $\alpha_{1c}$ subunit in heterologous expression systems, if indeed the former is a cardiac subunit, has not yet been clarified. When the $\beta_{2c}$ subunit was expressed along with the $\alpha_{2c}$ subunit and the $\alpha_{2\delta}$ subunit, the inactivation rates of $I_{Ca}$ were comparable with those from cardiac myocytes, although those with the $\beta_{2c}$ subunit were significantly slower, as reported previously (15). These observations suggest that the $\beta_{2c}$ subunit may be one of the functional $\beta_{2}$ subunits in rat heart.

However, we cannot deny that other $\beta$ subunits and/or other unknown regulators may also function in cardiac myocytes. Indeed, we could not reconstitute the native Ca$^{2+}$ channel, although we used all four subunits, i.e. $\alpha_{1c}$, $\beta_{2a}$, and $\alpha_{2\delta}$ subunits, which were cloned from rat heart. The sustained components of $I_{Ba}$ recorded from native cardiac myocytes (the r400 values) were significantly greater than those from BHK cells with $\beta_{2c}$ subunit. Moreover, the I-V relationships between the recombinant Ca$^{2+}$ channels and the native cardiac ones are significantly different. Furthermore, the inactivation kinetics of $I_{Ca}$ derived from recombinant Ca$^{2+}$ channels containing $\beta_{2c}$ and native channels were significantly different, although those of $I_{Ba}$ were similar. These data strongly indicate the existence of other missing auxiliary subunits or modulators, some of which are Ca$^{2+}$-dependent. Alternatively, activity of modulators of Ca channel, such as calmodulin (28), in native cardiac myocytes might be different from that in BHK cells.

Cens et al. (29), using a series of deletion mutants and chimeric constructs of $\beta_{1}$ and $\beta_{2}$ subunits, showed that the N-terminal region of the $\beta_{2a}$ subunit was the major element slowing the inactivation. They found that a residual current at the end of a test pulse was large only if the N-terminal region of the chimeric constructs was derived from $\beta_{2a}$. Our findings also confirmed that the short N-terminal region was important for modification of inactivation kinetics, because $\beta_{2a}$ and $\beta_{2c}$ subunits were only different in this region.

FIG. 8. Ca$^{2+}$-dependent effects of inactivation kinetics. $I_{Ca}$ was recorded in the same experimental condition as in Fig. 6, except that charge carrier was Ca$^{2+}$ (1.8 mM) instead of Ba$^{2+}$ (0.5 mM). The upper panel shows representative traces recorded from BHK cells transfected with $\beta_{2a}$ (A), $\beta_{2c}$ (B), and a native rat cardiac myocyte (C). Vertical bar and horizontal bar indicate 1 nA and 50 ms, respectively. The middle panel shows I-V relationships obtained from BHK cells transfected with $\beta_{2a}$ (D, $n = 6$), $\beta_{2c}$ (E, $n = 6$), and native rat cardiac myocytes (F, $n = 6$). Two-way repeated measures of ANOVA detected significant differences in voltage dependence among the three groups. Contrast test demonstrated that voltage dependences of all three groups were significantly different from each other. Asterisks show statistical significance by contrast test. $A_{i}/A_{j}$ of all three groups were significantly different from each other by contrast test.
The N-terminal region of the rat \( \beta_{2a} \) subunit was identical to those of a Lambert-Eaton myasthenic syndrome antigen and the human \( \beta_{2a} \) subunit except for some amino acids considered to be species differences (23, 30). The similarity to the former causes (23, 30). The similarity to the latter, i.e. the human \( \beta_{2a} \) subunit, indicates that the expression of the \( \beta_{2a} \) subunit in heart is not specific to rat.

In human heart, mRNA expression of the \( \beta_{1b} \) (3, 10), \( \beta_{1c} \) (3, 10), and \( \beta_1 \) (11) subunits and protein expression of the \( \beta_2 \) subunit (12) have been demonstrated. In addition, the \( \beta_{2a} \) (32) and \( \beta_{2b} \) (23) subunits have been cloned from human heart. Among them, the N-terminal region of the rat \( \beta_{2a} \) subunit was identical to the human \( \beta_{2a} \) subunit except for some differences considered to be species-specific. However, the I-V relationship and the inactivation kinetics of the human \( \beta_{2a} \) subunit reported were quite different from those of the rat \( \beta_{2a} \) subunit (23). These differences may be caused by variations in the experimental conditions because Allen et al. (23) used Xenopus oocyte as the heterologous expression system and recorded \( I_{\text{Ba}} \) at 15, 20, and 25 °C, using 40 mM B_{2a} as charge carrier. To our knowledge, there are no reports of studies directly comparing the inactivation kinetics of \( I_{\text{Ba}} \) or \( I_{\text{Ca}} \) from the recombinant cardiac L-type Ca\(^{2+} \) channels composed of all four heteromultimeric subunits cloned from the heart of the same species, with those of native channels at a physiological temperature, i.e. 37 °C.

In summary, the result of Northern blot analysis and the similarity of inactivation kinetics of the recombinant Ca\(^{2+} \) channel using the \( \beta_{2a} \) subunit with those of native cardiac myocytes strongly suggest that \( \beta_{2a} \) subunit is one of the functional \( \beta_2 \) subunits expressed in heart. Our findings also support the belief that the short N-terminal region of the \( \beta_2 \) subunit is important for modification of Ca\(^{2+} \) channel function and that different splice variants of the \( \beta_2 \) subunit could modulate Ca\(^{2+} \) entry through L-type Ca\(^{2+} \) channels in different tissues and regions.
Cloning of a Functional Splice Variant of L-type Calcium Channel β2 Subunit from Rat Heart
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