Modified Cardiovascular L-type Channels in Mice Lacking the Voltage-dependent Ca\(^{2+}\) Channel β3 Subunit*

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EXPERIMENTAL PROCEDURES

Voltage-dependent calcium channels (VDCCs) are the main mediators of calcium entry in many cell types and play pivotal roles in the control of calcium-dependent cellular functions, such as smooth muscle contraction. These channels are classified electrophysiologically and pharmacologically into five groups (L, N, T, R, and P/Q). L-type calcium channels are crucial for excitation-contraction coupling as voltage sensors in skeletal muscle and as the main mediators of calcium entry in heart and smooth muscle tissues. VDCCs are composed of four subunits, α1, α2, β, and γ (1, 2). The α1 subunits are the pore-forming subunits and are targets of calcium channel blockers such as dihydropyridines (DHPs), phenylalkylamines, and benzothiazepines, which are widely used for the treatment of hypertension, angina pectoris, and cardiac arrhythmias. Ten genes encoding pore-forming α1 subunits have been reported to date (3). The β subunits accelerate activation and inactivation of calcium channel currents, increase the channel population, and influence the coupling of gating charge movement; in addition, the βγ dimer (Gβγ) is thought to be involved in G protein-mediated channel inhibition (4–8). The β subunits are non-glycosylated hydrophilic proteins located on the intracellular side of the membrane; four variants are known (β1, β2, β3, and β4). Due to its wide expression in the aorta, trachea, and lung, β3 is considered the β subunit of smooth muscle calcium channels; it is also thought to couple with α1C subunits and comprise L-type calcium channels in these cells (9, 10). Although the role of the β subunits has been investigated by heterologous co-expression studies and antisense experiments with cultured cells, little is known about the physiological importance of the β3 subunit in situ.

The purpose of the present study was to clarify the importance of the voltage-dependent calcium channel β3 subunit in the cardiovascular system by using gene-targeted mutant mice. We examined channel populations in the aorta, calcium channel currents in aortic smooth muscle cells, calcium sparks in response to high KCl, and expression patterns of calcium channel subunits in the cardiovascular system of β3-null mice. We also measured blood pressure and heart rate in β3-null mice and compared them with wild-type controls. β3-Null mice showed high blood pressure, hypertrophic changes in the aortic smooth muscle layer, and cardiac enlargement in response to a high salt diet.


data from in situ hybridization experiments showed that β3 subunit expression was significantly reduced in the aorta, trachea, and lung of β3-null mice. This reduction was confirmed by Western blot analysis. In contrast, β1, β2, and β4 subunits were not affected by the absence of β3 subunits.

Western blot analysis showed that the expression of α1C subunits in the aorta, trachea, and lung of β3-null mice was similar to that of wild-type mice. These results suggest that the absence of β3 subunits affects the expression of other subunits and that the reduction in calcium channel currents in the aorta, trachea, and lung of β3-null mice is due to the absence of the β3 subunit.

The purpose of the present study was to clarify the importance of the voltage-dependent calcium channel β3 subunit in the cardiovascular system by using gene-targeted mutant mice. We examined channel populations in the aorta, calcium channel currents in aortic smooth muscle cells, calcium sparks in response to high KCl, and expression patterns of calcium channel subunits in the cardiovascular system of β3-null mice. We also measured blood pressure and heart rate in β3-null mice and compared them with wild-type controls. β3-Null mice showed high blood pressure, hypertrophic changes in the aortic smooth muscle layer, and cardiac enlargement in response to a high salt diet.


developments in the pathophysiology and treatment of cardiovascular diseases, it is important to understand the role of β3 subunits in these diseases. The present study provides new insights into the physiological importance of β3 subunits in the cardiovascular system.
RNA Isolation and RT-PCR Analysis—Total RNA was isolated from the mouse aorta using the RNeasy extraction kit (Qiagen Inc., Valencia, CA). Reverse transcription reaction was performed in a solution of 10 pmol of oligo(dT) primer, 1 μg of RNA, 1× first strand cDNA buffer (Invitrogen), 10 mM dithiothreitol, 0.4 mM dNTPs, 40 units of RNAasin, and 200 units of Superscript II (Invitrogen), in a volume of 25 μl at 42 °C for 45 min. β2 and β2 subunit-specific sequences were amplified by PCR with the following primers: MB35 (5′-CTC AAA GAG CAG AAG GCC-3′) and MB3A (5′-CAT AGC TGT TCA GAG AGG GTC-3′), corresponding to the sequences of the murine β2 subunit LKQEQK-AR156 and 186PSLGYE192, MB35 (5′-CTA GAG AAC ATG AGG CTA CAT-3′) and MB3A (5′-CTC AGT GGT TGT TGA CAC-3′), corresponding to the sequences of the murine β2 subunit LENMLRQ157 and 197PKPSANS204. RT-PCR amplification of the α1C subunit was performed using the specific primers MA1C3 (5′-TTG GCC ATT GCG GTG AAC ACT CTG-3′) and MA1C4 (5′-CTG GAG TGC ATC ATG TAT CTT G-3′), which generated a PCR product of 237 bp.

Western Blot Analysis—For analysis of α1, β2a, and β3, the aorta was dissected from each mouse and homogenized in homogenization buffer containing 20 mM NaO4P2, 20 mM NaH2PO4, 1 mM MgCl2, 0.3 mM sucrose, 0.5 mM EDTA, and a mixture of protease inhibitors. Aliquots of 100 μg of the homogenate from each mouse were resolved by 10% gradient SDS-PAGE and subjected to Western blot analysis as described previously (15). The membranes (0.2 mg/ml) were incubated with Krebs solution containing 10 mM HEPES, 2.2 mM CaCl2, 1.2 mM MgCl2, 14 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.2 with 1 N NaOH. The bath solution contained 92 mM NaCl, 5.9 mM KCl, 30 mM BaCl2, 1.2 mM MgCl2, 14 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with 10 N NaOH. Whole-cell currents were elicited by 150-ms voltage clamp steps, from −50 to +60 mV, every 15 s, from the holding potential of −60 mV. Whole-cell currents were displayed without any filtering, and membrane potentials have been corrected for junction potentials according to the method of Neher (17). Cell capacitance was measured by dividing the charge (Q) during the capacitive surge elicited by 10 mV hyperpolarization (ΔV) from −60 mV, using the equation Cm = Q/ΔV. Data acquisition and analysis were done on a computer using software (Cell Soft) developed at the University of Calgary, Canada. Pooled data are shown as the mean ± S.E. The statistical significance of differences between groups was determined using Scheffe’s test after one-way analysis of variance. The statistical significance of differences between groups was determined using Scheffe’s test after one-way analysis of variance.

Measurement of Blood Pressure—Mice were age-matched between the groups and were trained several times before measurements. The heart rate and systolic and diastolic blood pressure were measured using an automated computerized system with the tail-cuff method (BP-98A; Softron, Tokyo, Japan). The animals were fed the high salt diet for 2 weeks before the experiments. Multivariate differences were analyzed by repeated measured analysis of variance. Results are given as means ± S.E. Tissue Preparation and Histology—Tissues of β3+/+ and β3−/− mice were excised under ether anesthesia. The tissues were fixed immediately in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2) overnight at 4 °C and embedded in paraffin wax. Histological examination of slides stained with hematoxylin-eosin was performed.

Immunohistology—Immunohistochemical analysis was performed using the streptavidin-biotin amplification method. After deparaffinization, slides were treated with Pronase. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine solution (10 mM diaminobenzidine, 50 mM Tris-HCl buffer (pH 7.6), and 0.006% H2O2), and counterstaining was performed with hematoxylin.

Normal goat serum (1%) was applied to the sections for 20 min at room temperature, followed by the primary antibody at a dilution of 1:200 for 18 h at 4 °C. Commercially available antibodies that specifically recognize the α1C subunit (Alomone, Jerusalem, Israel) were used. Sections were reacted with Envision plus (Dako, Copenhagen, Denmark) to visualize the antigen-antibody complex.

Whole-cell immunofluorescence labeling of isolated smooth muscle cells was tested with anti-β3 antibody at a dilution of 1:100 (Alomone) as the primary antibody, and further analyzed with fluorescein-conjugated polyclonal goat anti-rabbit IgG at a dilution of 1:200.

Electrophysiology with CHO Cells—Chinese hamster ovary (CHO) cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% dialyzed fetal bovine serum. For electrical recordings, CHO cells at 30–50% confluence were plated onto 22-mm coverslips. Some CHO cells were transfected with an expression vector carrying the cloned smooth muscle α1C subunit (Ca1.2b) in pcDNA3.1 (+) (Invitrogen) and/or the β2a in pTracer (Invitrogen) and/or the β2 subunit in pcDNA3 (Invitrogen) with or without a green fluorescent protein (GFP) expression vector (pTracer) by lipofection (Superfect Transfection Reagent; Qiagen, Tokyo, Japan) (18). Expression of the channel subunits was quantified by the emission of green light using an IX70 fluorescence microscope (Olympus, Tokyo, Japan). Whole-cell patch clamp recording was carried out 48–72 h after transfection. Whole-cell currents were elicited by 3000-ms voltage clamp steps, from the holding potential at −60 to +10 mV every 15 s.

Statistics—The data are expressed as the means ± S.E. Differences were evaluated using unpaired t tests, unless stated otherwise. The level of statistical significance was p < 0.05.

RESULTS

β3 and β2 Are Expressed in the Murine Aorta—RNA products derived from β3+/+ and β3−/− mice were analyzed using the β3-specific primers, MB3S and MB3A, and β2-specific primers, MB2S and MB2A. RT-PCR analysis of the wild-type aorta showed β3- and β2-specific bands of 187 and 222 bp, respectively (Fig. 1A), indicating the expression of both subunits in this tissue. We also performed the experiment with β1- and β4-specific primers, which yielded no PCR products (data not shown). The expression of the α1C subunit gene was confirmed in both wild-type and mutant mice. We also confirmed the expression of β3, β2, and α1C subunit genes with other primer sets. Moreover, we also performed the experiment for other α1C subunit genes to participate in the formation of high voltage-activated calcium channels, such as α1A, but PCR yielded no products with these primers.
analysis of the aortic plasma membranes. Membrane proteins (100 μg/lane) were probed with the following rabbit polyclonal antibodies. i, anti-β3 antibody; lane 1, β3−/−; lane 2, β3+/−; ii, anti-β2 antibody; lane 1, β3−/−; lane 2, β3+/−. iii, anti-α1C antibody; lane 1, β3−/−; lane 2, β3+/−. C, Representative binding curves for (+)-[^3]HPN 200-110 in aortic smooth muscle. Total binding (TB, large circles) and nonspecific binding (NSB, small circles) in β3+/− (open circles) and β3−/− (closed circles) mice. Inset, Scatchard transformation of equilibrium binding of β3+/− (open circles) and β3−/− (closed circles) mice.

Reduced Calcium Channel Population by Disruption of the β3 Subunit

Western Blot Analysis—To confirm the deficiency of the β3 subunit and the expression of the β2 subunit in the aortic tissue at the protein level, we performed Western blot analysis using anti-β2, anti-β3, and anti-α1C subunit antibodies. A single band corresponding to β3 was detected in β3+/− mice, whereas no corresponding band was detected in β3−/− mice (Fig. 1B). Anti-β2 antibody detected a single band of 84 kDa in β3+/− mice, indicating β2 subunit expression and suggesting that this subunit is a component of voltage-dependent calcium channels in the vascular tissue. There was no significant difference in the level of β2 subunit expression in the aortas of β3+/− mice, as compared with β3+/+ controls, suggesting that there was no compensatory increase in the level of β2 expression in this tissue. On Western blot analysis, a single band with a molecular mass of 210 kDa was detected in the aortas of β3+/− and β3−/− mice with the anti-α1C subunit antibody. We also performed Western blot analysis with anti-pan α1 antibody, with the same results, suggesting that the majority of calcium channels are encoded by a single α1C subunit gene that forms VDCs in the mammalian cardiovascular system. β3−/− mice showed a reduced amount of the α1C subunit in the aortic plasma membrane (−45% reduction as determined by densitometry), which suggested a decrease in the channel population in the smooth muscle cell membrane by the targeted disruption of the β3 subunit gene.

Binding Studies—To characterize further the decrease in the channel population, we next performed saturation binding analyses using the dihydropyridine derivative (+)-[^3]HPN 200-110, which binds to a saturable, non-interacting set of binding sites in aortic smooth muscle membranes. Fig. 1C shows typical results from a saturation experiment. The Kd values were 0.248 ± 0.072 nM (β3+/−, n = 4) and 0.257 ± 0.064 nM (β3−/−, n = 4), and the Bmax values were 8.27 ± 0.51 fmol/mg protein (β3+/−) and 3.68 ± 0.42 fmol/mg protein (β3−/−) (Fig. 1C, inset), suggesting a decrease in the number of DHP receptors in the cell membrane (−56% reduction) of the mutant mice, with no apparent changes in the channel affinities. This 56% reduction in the channel population coincided with a similar reduction in the level of expression of α1C subunits detected by Western blot analysis. The nonspecific binding values were quite high (about 30%), which made subsequent Scatchard analysis difficult in comparison to skeletal muscle membrane preparations, whose nonspecific binding value was about 10% (Kd = 0.287 nM, Bmax = 401.15 fmol/mg protein).

Reduction and Modulation of Voltage-dependent Calcium Channel Currents—Whole-cell patch clamping was used to examine the effect of β3 expression on voltage-dependent Ca2+ channel currents in freshly dissociated aortic smooth muscle cells. The mean cell capacitance was 26.9 ± 1.1 in β3+/− cells and 24.4 ± 1.0 pF in β3−/− cells (13 cells from 7 mice for each, p > 0.05), respectively. Rectangular pulses (150-ms duration) of various voltages were applied in the presence of 30 μM Ba2+, as a charge carrier. Fig. 2A shows examples of voltage-dependent Ca2+ channel currents. At positive test potentials, inward Ba2+ currents were observed in both β3+/− and β3−/− cells (Fig. 2A). In Fig. 2B, the density of the peak inward current was plotted against the test potential (I-V relationship). The density of the voltage-dependent inward current was, however, maximal at +10 mV irrespective of β3 expression. A reduction of about 30% in the Ca2+-channel current density was observed in β3−/− cells (0.84 ± 0.08 pA/pF, n = 12 versus β3+/−, 1.22 ± 0.10 pA/pF, n = 7, p < 0.05), in which the peak current was observed at +10 mV (Fig. 2B). Next, DHP sensitivity was examined with nicardipine. In both groups, the inward currents were effectively inhibited by 100 nM nicardipine, but a significant decrease was observed in the DHP-sensitive component in β3−/− cells (0.61 ± 0.10 pA/pF, n = 7, p < 0.05 versus β3+/−, 0.99 ± 0.13 pA/pF, n = 7, Fig. 2C).

By contrast, inactivation of the voltage-dependent Ca2+ channel current was significantly slower in β3−/− cells. The decay time constant of the voltage-dependent Ca2+ channel current evoked at +10 mV was well fitted by an exponential function; the time constant was 59.2 ± 6.2 and 109.1 ± 7.4 ms in β3+/− cells (n = 7) and β3−/− cells (n = 12), respectively (p < 0.01, Fig. 2D). This is consistent with the observation that the inactivation was altered by different β subunits (9).

Decreased Responsiveness to Diltiazem in Global [Ca2+]i, and No Changes in Ca2+ Sparks—It has been suggested that VDCs play an important role in coupling sparks and spontaneous transient outward currents, and that this coupling represents a negative feedback mechanism for regulating arterial tone. Therefore, Ca2+ sparks in intact mesenteric arteries were further examined.

Fig. 3A shows typical recordings of Ca2+ changes in the first branch of a wild-type mesenteric artery, in response to 30 mM KCl. This elevation of external K+ increased global [Ca2+]i, in both β3+/− and β3−/− mice (F/P0 increases of 1.34- and 1.21-fold in β3+/− and β3−/−, respectively; Fig. 3B). The decrease in the global [Ca2+]i, in β3−/− mice on application of an L-type calcium channel blocker, diltiazem, was significantly larger than in β3+/− mice (Fig. 3, B and C), suggesting decreased responsiveness to diltiazem in the mutants. However, there were no significant differences in the amplitudes of local [Ca2+]i transients (Ca2+ sparks, >100 ms in half-duration) between β3+/− (n = 18) and β3−/− (n = 24) from 5 mice each in either standard or high KCl solutions or in the presence
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Fig. 2. Calcium channel currents recorded in the aortic smooth muscle cells. A, representative traces of voltage-dependent 
Ca²⁺ channel currents in murine aortic smooth muscle from β3⁺⁺ (+/+) and β3⁻⁻ (−/−) mice. Cells were depolarized from the holding potential of −60 to −30, −10, and +10 mV for 150 ms. B, I-V curves generated by 150-ms depolarization pulses from a holding potential of −60 mV to potentials between −50 and +60 mV in β3⁺⁺ (n = 7, open circles) and β3⁻⁻ (n = 12, closed circles) cells. The current density was estimated by dividing the peak amplitude by the cell capacitance (pA/ pF). C, effects of nicardipine on I Na in β3⁺⁺ (n = 7, open bar) and β3⁻⁻ (n = 7, closed bar) cells. Cells were depolarized every 15 s from the holding potential of −60 to 0 mV for 150 ms. After stabilization of I Na, 100 µM nicardipine was applied. D, the time constants of the I Na decay in β3⁺⁺ (n = 7, open bar) and β3⁻⁻ (n = 12, closed bar) cells. The numbers of myocytes used are given in parentheses. The statistical significance of the difference between β3⁺⁺ and β3⁻⁻ mice is indicated by *, p < 0.05, or **, p < 0.01.

d of diltiazem (Fig. 3D). The frequency of Ca²⁺ sparks was also not affected significantly by β3 deficiency (0.22 ± 0.05 and 0.18 ± 0.04 Hz in β3⁺⁺ and β3⁻⁻ cells, respectively, in standard solution, p > 0.05). The characteristics of longer periodical Ca²⁺ events (Ca²⁺ waves) were also detected in these preparations. The amplitude (F/F₀) and frequency were 1.79 ± 0.03 and 0.13 ± 0.05 Hz, respectively, in β3⁺⁺ cells (13 cells from 5 mice) and were not significantly different from those in β3⁻⁻ cells (1.98 ± 0.16 and 0.15 ± 0.04 Hz, respectively, 14 cells from 5 mice).

β3 Subunit Expression Pattern

Immunohistological Examination of the α₁C Subunit in the Heart and Aorta—To evaluate the decrease in channel population identified by Western blot and DHP binding analyses, we examined the distribution of the α₁C subunit, of which the majority of L-type channels in the heart and aorta is comprised. The α₁C subunit is distributed homogeneously in the heart and aortic smooth muscle cell layer. There were no clear differences in the patterns of immunohistological labeling between wild-type and mutant mice (Fig. 4A). Conventional immunohistological labeling with streptavidin-biotin amplification did not allow us to detect the distribution of the β3 subunit, as the levels of background signals were high. Therefore, we examined expression of the β3 subunit by whole-cell fluorescence immunohistochemical analysis in isolated aortic smooth muscle cells (Fig. 4B, left panels). In the wild-type mice (Fig. 4Bi), a homogeneous distribution of the β3 subunit was observed, whereas only background signals were detected in β3⁻⁻ mice (Fig. 4Bii, inset has high signal intensification, resulting in high background signals). The corresponding phase contrast images are shown in the right panels.

Decreased Responsiveness to Amlodipine and Salt-sensitive Increase in Blood Pressure—The significance of the L-type voltage-dependent calcium channels in the cardiovascular system is widely known, and its blockers, such as DHP, are widely used in the treatment of hypertension. Therefore, decreased blood pressure was anticipated, but no significant differences were observed in systolic or diastolic blood pressure in β3⁻⁻ mice on normal diets, as compared with wild-type controls. We also measured blood pressure using arterial catheters. As there were no significant differences in the results obtained using the
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Intraperitoneal injection of amiodipine (1.0 mg/kg), a DHP analogue significantly lowered systolic and diastolic blood pressure in β3+/+ mice, whereas it had no significant effect in β3−/− mice (Table I).

As high dietary salt intake in humans is associated with high blood pressure (19), we next examined the effects of a high salt diet, containing 8% NaCl, on the blood pressure of β3+/+ and β3−/− mice. Loading with the high salt diet for 2 weeks increased systolic and diastolic blood pressure in β3−/− mice but not in β3+/+ mice (Table I). Loading with the high salt diet for 3 months induced hypertrophic changes in the aortic smooth muscle of β3−/− mice (Fig. 4C), whereas no significant changes were observed in β3+/+ mice. Mutant β3−/− mice on the high salt diet also showed an increase in heart weight (Fig. 4D). As these pathological changes are common features in hypertensive animals, they may have been a consequence of elevated blood pressure.

Electrophysiological Recordings of Transiently Transfected Cav1.2b-expressing CHO Cells—To analyze further the importance of β subunits in the voltage-dependent calcium channels in smooth muscle cells, we next analyzed transient expression of the Cav1.2b, β2, and β3 subunits in CHO cells. Fig. 5A shows representative whole-cell Ca2+ channel currents recorded using Ba2+ as the charge carrier. A rectangular 10-mV pulse (3000 ms in duration) was applied. The mean cell capacitance was 16.0 ± 0.4 pF.

**Table I.** Effects of disruption of the β3 subunit on systolic (SBP) and diastolic (DBP) blood pressure in mice on normal and high salt diets. Each group consisted of 35 mice from 9 to 12 weeks old.

|                | Heart rate | SBP     | DBP     |
|----------------|------------|---------|---------|
|                | beats/min  | mm Hg   |         |
| +/+            | 628 ± 8    | 98.5 ± 1.9 | 60.9 ± 1.6 |
| −/−            | 619 ± 14   | 97.0 ± 1.9 | 60.4 ± 1.6 |
| +/+ amlo       | 668 ± 9    | 87.2 ± 2.0* | 51.3 ± 2.4* |
| −/− amlo       | 622 ± 19   | 98.2 ± 2.1 | 57.8 ± 2.7 |
| +/+ NaCl       | 631 ± 13   | 97.9 ± 1.8 | 65.4 ± 1.1 |
| −/− NaCl       | 627 ± 11   | 113.1 ± 1.9** | 71.7 ± 1.4** |

*p < 0.01 versus +/+.

To examine whether transfection itself influences the Ca2+ channel current, we first transfected CHO cells with a GFP plasmid alone (pTracer). This mock transfection had no effect on voltage-dependent Ca2+ channel currents (Fig. 5Ai). The voltage-dependent inward Ca2+ channel current (Fig. 5Aii) was recorded from CHO cells transiently expressing α1C (Cav1.2b) with GFP plasmid (α1C/Mock). In the CHO cells expressing α1C, the current density estimated from the peak inward current at 10 mV (PCD10mV) was 10.7 ± 0.5 pA/pF (n = 8). The voltage-dependent inward current decayed very slowly during depolarization (time constant, 568.0 ± 11.8 ms, n = 8). These features are essentially the same as reported previously (20, 21).

The inward Ba2+ currents (Fig. 5A, iii–v) were recorded from CHO cells in which the β2a subunit alone (iii), the β2a and β3 subunits simultaneously (iv), or β3 alone (v) were transiently expressed with α1C. Fig. 5B summarizes the effects of expressing β subunits on PCD10mV. Expression of either β subunit significantly increased PCD10mV (ii, 20.0 ± 1.0 pA/pF, n = 8; iv, 21.6 ± 0.8 pA/pF, n = 8; v, 29.0 ± 1.0 pA/pF, n = 8). There were, however, no statistically significant differences (p > 0.05) between the three groups.

Expression of the β3 subunit significantly accelerated the rate of inactivation. The time constant (τ) for each combination is summarized in Fig. 5C. CHO cells, expressing α1C, with GFP plasmid (α1C/Mock), were inactivated very slowly. Of the three types of β-expressing CHO cells, the decay was fastest with β3 alone (time constant, 528.4 ± 18.2 ms, n = 8) and slowest with β2 alone (time constant, 975.1 ± 31.0 ms, n = 8). The latter is thought to mimic β3−/− aortic smooth muscle cells. The rate of inactivation for the transient expression of β2 alone significantly differed from the inactivation rates for the other two cases. The combined expression of the β2 and β3 subunits had an intermediate effect (time constant, 669.1 ± 19.9 ms, n = 8). Overall, β3 had qualitatively the same effects in this expression system (CHO cells) as in the isolated smooth muscle cells of β3-deficient mice.

In addition, to address whether transfection of β subunits invokes expression of some endogenous voltage-dependent Ca2+ channels, we transfected the β3 subunit in CHO cells. Even after expressing the β3 subunit, step depolarization did not evoke an inward Ca2+ channel current (data not shown). Essentially the same results were obtained on transfecting β2 instead of β3 (data not shown).

Table I. Effects of disruption of the β3 subunit on systolic (SBP) and diastolic (DBP) blood pressure in mice on normal and high salt diets. Each group consisted of 35 mice from 9 to 12 weeks old.
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In this study, we found a reduction of only 30% in the whole-cell Ca^2+ channel currents in β3−/− cells, whereas overexpression experiments demonstrated a more marked effect of the β3 subunit, as compared with the results obtained with α1C subunit-expressing cells (Fig. 5). As we found expression of the β2 subunit in the aorta, we further compared the effects of co-expression of the β2 and α1C subunits with co-expression of both the β2 and β3 subunits and the α1C subunit. The differences between these two conditions were apparently similar to those between β3−/− and β3−/+ cells. The expression of the β2 subunit, as revealed in the RT-PCR experiment (Fig. 1A), suggests that the β2 subunit also interacts with α1C subunits in the aortic smooth muscle cells in vivo. Of all known β subunits, recombinant rabbit β3 subunit has the strongest effect in accelerating the inactivation rate (9); the slow inactivation rate in β3−/− cells determined in the present study is also consistent with these observations. Therefore, our biochemical and electrophysiological results suggest that the heterogeneity of voltage-dependent calcium channels in vascular smooth muscle cells is at least partially due to the heterogeneous expression of the two β subunits, β2 and β3. It is likely that β2 is responsible for the relatively long-lasting L-type calcium channel currents in vascular smooth muscle cells. Furthermore, a recent study revealed an increase in a truncated form of the β3 subunit in human heart failure, suggesting the importance of the β3 subunit in cardiovascular function and the involvement of this subunit in pathological status (25).

We found no significant changes in local Ca^{2+} events, such as sparks and waves, in β3−/− mice, although significant differences in responsiveness to diltiazem were detected in global [Ca^{2+}], elevation induced by 30 mM K^+ as compared with wild-type controls. Local intracellular calcium transients, Ca^{2+} sparks, are caused and regulated by the combined actions of VDCC, ryanodine-sensitive calcium release (RyR) channels, and Ca^{2+} pumps in the sarcoplasmic reticulum (26). The major physiological role of Ca^{2+} sparks in smooth muscle is the regulation of membrane potential via the activation of large conductance Ca^{2+}-sensitive K^+ (BKCa) channels, which represent a tripartite functional unit with VDCC and RyR (26, 27). A single Ca^{2+} spark is capable of producing a large local increase in [Ca^{2+}], but has little effect on the global [Ca^{2+}]. As L-type calcium channel activation is known to increase the frequency and amplitude of Ca^{2+} sparks, our mouse model, in which the calcium channel current density is reduced by 30%, was used to examine Ca^{2+} sparks. Although we found no significant changes in Ca^{2+} sparks in β3−/− mice, L-type calcium channels play a significant role in the control of Ca^{2+} sparks, as diltiazem showed a significant inhibitory effect on their frequency and amplitude. This suggests that the enhanced Ca^{2+} influx through channels, including the β3 subunit during exposure to 30 mM K^+, neither contributes significantly to the amount of Ca^{2+} release per Ca^{2+} spark nor to the cycling rate of Ca^{2+} release from, and refilling of, the sarcoplasmic reticulum. The mutant mice used in the present study have a congenital 30% reduction in the calcium current density, and thus the regula-

![Fig. 5. A. representative current traces obtained from CHO cells expressing GFP alone (CHO/Mock) (i), α1C with GFP (α1C/Mock) (ii), and β2a (iii), β2a/β3 (iv), and β3 (v) transfected cells. The currents were evoked by depolarizing pulses to 10 mV from a holding potential of −60 mV (3000 ms in duration). Leak currents were subtracted using the P4 protocol. B, effects of β subunits on the peak currents in CHO cells. Each column and vertical bar represents the mean ± S.E. The β subunits transfected into the cells are indicated at the bottom. The numbers of cells analyzed in this experiment are as follows: α1C alone (n = 8), β2a (n = 8), β2a/β3 (n = 8), and β3 (n = 8). * p < 0.05 versus α1C, C, effects of different β subunits on the time constants of 1 ms decay in CHO cells. Data are expressed as means ± S.E. The numbers of cells used in this experiment are as follows: α1C alone (n = 8), β2a (n = 8), β2a/β3 (n = 8), and β3 (n = 8). The β subunits transfected into the cells are indicated at the bottom. * p < 0.05 versus α1C.](image)
tory mechanisms of [Ca^{2+}], in smooth muscle cells could be modified in a compensatory manner during development. Changes in expression levels of related molecules, including RyR, BK channels, Ca^{2+} pump, and Na^{+}/Ca^{2+} exchanger, remain to be determined. Alternatively, the functional coupling of α_{1C}/β3 channels with RyR may not be as tight as that of α_{1C}/β2 channels, although this is unlikely, based on the uniform distribution of α_{1C} in β3^{+/−} and β3^{−/−} mice. In addition, the technical limits of laser-confocal microscopy, especially its temporal resolution, would make it difficult to detect slight differences in spark parameters due to the 30% reduction in peak calcium channel current.

We found no significant differences in blood pressure between β3^{+/−} and β3^{−/−} mice on the normal diet. As calcium channels are thought to play important roles in maintaining the cardiovascular tonus by regulating calcium influx into smooth muscle cells, there must be a compensatory mechanism for the decreased channel population in β3^{−/−} mice. On the other hand, we detected decreased responsiveness to the DHP analogue amlodipine in the results of blood pressure measurement. As we found a significant reduction in the channel population, based on the results of biochemical analysis, and decreased DHP sensitivity in electrophysiological experiments, the decreased responsiveness to amlodipine in the blood pressure measurement clearly coincided with the decreased channel population and reduced electrophysiological DHP responsiveness in the patch clamp experiments, and was probably due to the same mechanism, i.e., the reduced channel population in the arterial smooth muscle cells.

The increase in blood pressure in response to salt loading is possibly related to sodium excretion and re-absorption in the kidney. Therefore, we examined calcium channel subunit gene expression in the kidney by *in situ* hybridization. We found no significant differences between β3^{+/−} and β3^{−/−} mice, except the lack of expression of the β3 calcium channel subunit in the mutants. There were also no morphological changes in the kidneys of β3^{−/−} mice. Furthermore, we examined plasma renin concentrations, but no clear changes were detected because of the high degree of variance in the results. To date, no genes related to salt-sensitive hypertension have been mapped to human chromosome 12q13, the locus of the human homologue of the high degree of variance in the results. To date, no genes

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**REFERENCES**

1. Hofmann, P., Biel, M., and Flockerzi, F. (1994) *Annu. Rev. Neurosci.* 17, 399–418
2. Catterall, W. A. (2000) *Annu. Rev. Cell. Dev. Biol.* 16, 521–555
3. Perez-Reyes, E., Criibs, L. L., Daud, A., Lacerda, A. E., Barclay, J., Williamson, M. P., Fox, M., Rees, M., and Lee, J.-H. (1998) *Nature* 391, 896–900
4. Pragnell, M., De Waard, M., Mori, Y., Tanabe, T., Snutch, T. P., and Campbell, K. P. (1994) *Nature* 368, 67–70
5. De Waard, M., Witzer, D. R., Pragnell, M., Liu, H., and Campbell, K. P. (1995) *J. Biol. Chem.* 270, 12056–12064
6. Singer, D., Biel, M., Letan, I., Flockerzi, V., Hofmann, F., and Dascal, N. (1991) *Science* 253, 1553–1557
7. Tareilus, E., Roux, M., Qin, N., Olesse, R., Zhou, J., Stefani, E., and Birnbaumer, L. (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94, 1708–1713
8. Varadi, G., Lory, P., Schultz, D., Varadi, M., and Schwartz, A. (1991) *Science* 332, 159–162
9. Hullin, R., Singer-Lahat, D., Freichel, M., Biel, M., Dascal, N., Hofmann, F., and Flockerzi, V. (1992) *EMBO J.* 11, 885–890
10. Collin, T., Lory, P., Taviaux, S., Courtieu, C., Guibault, P., Berta, P., and Nargeot, J. (1994) *Eur. J. Biochem.* 220, 257–262
11. Murakami, M., Yamamura, H., Murakami, A., Okamura, T., Nunoki, K., Mistui-Saito, M., Muraki, K., Kano, K., Imaiuzumi, Y., Flockerzi, V., and Yanagisawa, T. (2002) *J. Cardiovasc. Pharmacol.* 36, 569–573
12. Murakami, M., Fleischmann, B., DeFelipe, C., Freichel, M., Trest, C., Ludwig, A., Wissenbach, U., Schweger, H., Hofmann, F., Hescheler, J., Flockerzi, V., and Cavaille, A. (2002) *J. Biol. Chem.* 277, 40342–40351
13. Kang, M.-G., Chen, C. C., Felix, R., Letts, V. A., Frankel, W. N., Mori, Y., and Campbell, K. P. (2001) *J. Biol. Chem.* 276, 32917–32924
14. Witsch, D. R., De Waard, M., Sakamoto, J., Franzini-Armstrong, C., Pragnell, M., Kahl, S. D., and Campbell, K. P. (1995) *Science* 261, 486–489
15. Srinivasan, G., Joli, A., Mossburger, K., and Glassman, H. (1986) *FEBBS Lett.* 197, 204–210
16. Imaiuzumi, Y., Muraki, K., and Watanabe, M. (1990) *J. Physiol. (Lond.)* 427, 321–324
17. Neher, E. (1992) *Methods Enzymol.* 207, 123–131
18. Bosse, E., Bottleder, R., Kleppisch, T., Hescheler, J., Welgling, A., Hofmann, F., and Flockerzi V. (1992) *EMBO J.* 11, 2003–2008
19. John, S. W. M., Kregg, J. H., Oliver, P. M., Liu, H., and Campbell, K. P. (1994) *Science* 267, 679–681
20. Wellin, A., Bosse, E., Cabilio, V., Bottleder, B., Ludwig, A., Nastainczyk, W., Flockerzi, V., and Hofmann, F. (1995) *J. Physiol. (Lond.)* 471, 749–765
21. Aoyama, M., Murakami, M., Iwashita, T., Ito, Y., Yamaki, K., and Nakayama, S. (2003) *Biochem. J.* 10, 709–724
22. Bichet, D., Cornet, V., Geib, S., Carlier, E., Volson, S., Hoshi, T., Mori, Y., and Watanabe, M. (1993) *Science* 257, 219–220
23. Nishimura, S., Takekijima, H., Hofmann, F., Flockerzi, V., and Imoto, K. (1992) *FEBBS Lett.* 324, 283–286
24. Neely, A., Wei, X., Olesse, B., Birnbaumer, L., and Stefani, E. (1993) *Science* 262, 575–578
25. Hullin, R., Khan, I. F. Y., Wirtz, S., Mohacsi, P., Varadi, G., Schwartz, A., and Herzig, S. (2003) *J. Biol. Chem.* 278, 21623–21630
26. Jaggar, J. H., Porter, V. A., Lederer, W. J., and Nelson, M. T. (2000) *Am. J. Physiol.* 278, C235–C256
27. Ohi, Y., Yamamura, H., Nagano, N., Ohsii, S., Muraki, K., Watanabe, M., and Imaiuzumi, Y. (2001) *J. Physiol. (Lond.)* 534, 313–322
28. Welling, L., Lacinova, L., Donat, K., Ludwig, A., Bosse, E., Flockerzi, V., and Hofmann, F. (1995) *Pfluegers Arch.* 429, 400–410
29. Lacinova, L., Welling, A., Bosse, E., Ruth, P., Flockerzi, V., and Hofmann, F. (1995) *J. Pharmacol. Exp. Ther.* 274, 54–63