Sustained β -Adrenergic Stimulation Increased L-Type Ca^{2+} Channel Expression in Cultured Quiescent Ventricular Myocytes

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Abstract: The abundance of voltage-gated L-type Ca^{2+} channels is altered by β -adrenergic receptor (β -AR) stimulation and by an elevation of the intracellular Ca^{2+} concentration in cardiac myocytes. In whole animal, chronic β -AR stimulation or pacing heart results in various changes in the abundance of the channel, but it reduces the β -AR responsiveness of the L-type channel. Because β -AR stimulation facilitates the L-type calcium channels, it is difficult in the whole animal to study the effects of β -AR and Ca^{2+} influx on the upregulation of the L-type channel independently of each other, which makes the culture of nonbeating adult myocytes an attractive model. We found that culturing quiescent adult rabbit ventricular myocytes with isoproterenol (ISO, 2 μM) for 72 h or more caused a significant increase in the

expression of mRNA coding for the L-type channel α_{1C} subunit by approximately twofold as compared to time-matched controls, and it was followed by a 1.8-fold increase in the Ca^{2+} current density at 96 h. Somewhat surprisingly, an acute application of 1 μM ISO increased the current amplitude even in ISO-treated cells. The increase in the current density, induced by sustained β -AR stimulation, was blocked by a β -AR antagonist, propranolol (10 μM), but not by a Ca^{2+} antagonist, nitrendipine (10 μM). In addition, the effects were reproduced by forskolin (10 μM), but not by a Ca^{2+} agonist, Bay-K 8644 (2 μM). Taken together, these results suggest that sustained β -AR stimulation upregulates L-type channel expression, but does not alter the β -AR responsiveness of the channel in quiescent myocytes.

Key words: L-type calcium channel, upregulation, patch clamp, culture of adult myocytes, β -adrenergic receptor stimulation.

The voltage-gated L-type Ca^{2+} channels play a key role in the regulation of cardiac contractility by controlling Ca^{2+} entry into cardiac myocytes during action potentials. The abundance and function of these channels are modulated on a longer time scale, as exemplified by the changes seen in heart failure (see review [1]). The current density through L-type Ca^{2+} channels was reduced in end-stage heart failure and in a pacing-induced heart failure model of porcine, but not in the model of dog or rabbit [2–4]. In several heart failure models, biophysical properties of the L-type channel current remain unchanged, indicating that the number of functional channels is changed. In contrast, the responsiveness of the channel to β -AR agonists is significantly diminished: a high concentration of β -AR agonist (ISO, 1 or 10 μM) failed to increase the current amplitude [2, 3]. Similarly, a chronic administration of a β -AR agonist downregulated β -AR in rat heart [5] without affecting the number and function of channels [6, 7]. These results indicate that the changes in the number of functional L-type channels might vary depending on conditions, but the responsiveness to β -AR stimulation is re-

duced. In contrast to the reduction of responsiveness, in the failing human heart, a mechanical unloading [8] or support with a mechanical device [9] restored the depressed β -AR responsiveness.

Gene expression in cardiac myocytes is regulated either by Ca^{2+} or by β -AR stimulation. Electric stimulation (mechanical loading or pacing), which activates the L-type channels and then induces a robust elevation of intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), has been shown to cause hypertrophy and to regulate gene expression [4]. β -AR stimulation also caused hypertrophy [6], and an inhibition of β -AR affected gene expression in human failure heart [10]. Taken together, these findings suggest that in the whole system, the increase in $[\text{Ca}^{2+}]_i$ and the β -AR stimulation could both cause these changes. However, these factors are difficult to study independently of one another *in vivo*, since electric stimulation may affect the activity of the sympathetic nervous system, resulting in β -AR stimulation, and β -AR stimulation enhances the L-type channel current. Therefore the specific effects of mechanical loading, $[\text{Ca}^{2+}]_i$, and neuronal or hormonal stim-

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ulation on gene expression are often studied using primary cultured adult cardiac myocytes [11–16]. Culturing adult cardiac myocytes eliminates both the influence of Ca^{2+} entry through the L-type channels resulting from spontaneous contraction and of β -AR stimulation resulting from the sympathetic nervous system, since cultured adult myocytes are quiescent (not beating) and have no nervous system.

L-type Ca^{2+} channels in cultured adult cardiac myocytes are upregulated by electrical stimulation [13] or elevations in $[\text{Ca}^{2+}]_i$ [15]. Sustained β -AR stimulation has also been reported to upregulate L-type channels in spontaneously beating neonatal myocytes [17, 18]. However, the effects were transient: an increase in the mRNA level of L-type channels became maximal at 2 h after the application of ISO, and the effect was not sustained. Furthermore, it is difficult to exclude the influence of the increase in $[\text{Ca}^{2+}]_i$, since the rate of spontaneous beating and Ca^{2+} influx through the L-type channels might be increased by β -AR stimulation. Because increases in beating rate and $[\text{Ca}^{2+}]_i$ upregulates the L-type channels [13, 15], the role of cAMP *per se* in regulating the abundance of L-type Ca^{2+} channels remains largely unknown. Here we have investigated the effects of sustained β -AR stimulation on the functional L-type channel expression and the β -AR responsiveness of the L-type channel using quiescent ventricular myocytes. We found that chronic exposure to a β -AR agonist increased the current density of the L-type channel without altering the biophysical properties of the channel, including its β -AR responsiveness, which indicated that the sustained β -AR stimulation upregulates L-type channels. These effects were inhibited by a β -AR antagonist, but not by a Ca^{2+} antagonist, and were reproduced by forskolin, but not by a Ca^{2+} agonist.

MATERIALS AND METHODS

This study was conducted in accordance with the guiding principals for the care and use of animals in the field of physiological sciences set forth by the Physiological Society of Japan [19]. The protocol was approved by our university's Animal Experimental Committee.

Isolation and culture of rabbit ventricular myocytes. Single ventricular myocytes were isolated from the hearts of Japanese white rabbits (1.5 kg). The rabbits were anesthetized by pentobarbital (40 mg/kg, i.v.) and then the hearts were removed. The ventricular myocytes were isolated by collagenase treatment (Type I, Sigma Chemical, St. Louis, MO) with Langendorff perfusion at 37°C [20]. Isolated cells were plated onto laminin-coated glass coverslips (10 × 10 mm) for electrophysiology, or laminin-coated 35 mm culture dishes for RT-PCR, and allowed to attach for 2 h at 37°C under an atmosphere of 5% CO_2 –95% air. The cells were then washed with culture medium (medium 199, Sigma) and supplemented with 5% fetal bovine serum

(GIBCO BRL, Grand Island, NY), 0.25 $\mu\text{g}/\text{ml}$ amphotericin B, 0.1 mg/ml streptomycin, and 100 units/ml penicillin, then cultured for 3 days to eliminate unhealthy and injured cells [21]. Thereafter the cells were cultured with reagents that were added to the medium, and the reagent-containing solution was renewed daily.

Semiquantitative RT-PCR. Total RNA was extracted with 1 ml TRIzol[®] Reagent (GIBCO BRL). Samples of the extracted RNA (1 μg) were then incubated with 0.5 μg of pd(T) at 70°C for 10 min, and reverse transcription (RT) was carried out at 42°C for 60 min in 25 μl of reaction mixture containing 25 U of AMV reverse transcriptase, 50 mM Tris-HCl, pH 8.3, 100 mM KCl, 10 mM MgCl_2 , 1 mM deoxynucleotide mixture (dNTP), 10 mM DTT, and 12.5 U RNase inhibitor. The absence of contamination by genomic DNA was verified in each sample by PCR amplification without the RT step. The resultant cDNA (1 μl) was amplified in a thermal controller (PTC-100, MJ Research Inc., Waltham, MA) in 25 μl of reaction buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl_2 , 200 μM dNTP mix, 0.5 μM each specific oligonucleotide primer, and 1 U EX Taq DNA polymerase (Takara Co., Tokyo, Japan). The primers used were 5'-CAGGATGT-TAGTCTATATT-3' (sense) and 5'-GATGTTGAAG-TAGTTCCTGC-3' antisense for rabbit cardiac α_{1C} subunit and 5'-TGAACCACGAGAAGTATGAC-3' (sense) and 5'-TCACAAAGTGGTCATTGAGG-3' (antisense) for GAPDH. The PCR protocol entailed a denaturation at 4°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s. In preliminary studies, we determined that the amount of PCR product increased exponentially with 16 to 22 cycles for GAPDH and with 24 to 30 cycles for the L-type Ca^{2+} channel α_{1C} subunit; a plateau phase was reached after 22 and 30 cycles, respectively. An expression of the housekeeping enzyme GAPDH was determined in each sample as an internal standard for mRNA integrity.

PCR products were subjected to agarose gel electrophoresis, visualized with ethidium bromide (2 ng/ml), and transferred as an image file using a ZERO-Dscan[™] (Scanalytics, Billerica, USA), after which the relative intensities of the bands, expressed in optical density units, were quantitatively analyzed using BIO-PRINT[®] image software 1.61 (Vilber Lourmat, Cedex, France). An analysis of α_{1C} subunit mRNA was performed in parallel with GAPDH mRNA analysis to provide an internal standard [5, 10]. Each gel was photographed as shown in Fig. 1A.

Electrophysiological measurements. Prior to patch clamp experiments, cultured cells were washed three times with Tyrode solution containing (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl_2 , 1 MgCl_2 , 10 HEPES, 1 glucose, and 5.4 KCl; pH was adjusted to 7.3 with NaOH. After superfusing the cells for at least 30 min, the macroscopic currents were recorded using the whole-cell clamp method at room temperature (22–25°C) [22]. Patch pipettes, which were cou-

pled to an Axopatch 1-D patch-clamp amplifier (Axon Instruments, Foster City, CA, USA), were filled with pipette solution containing (in mM) 140 KCl, 1 MgCl₂, 5 HEPES, 1 EGTA, and 3 MgATP, and adjusted to pH 7.3 with KOH, which had a resistance of 2–5 MΩ. After whole-cell configuration was established, membrane capacitance (C_m) was estimated by analyzing the charging transients elicited by a 10 mV pulse from a holding potential (HP) of –50 mV. Membrane voltages were computer software controlled (pCLAMP ver. 6, Axon Instruments), and currents were filtered at 2 kHz, digitized at 10 kHz, and stored on computer for offline analysis. L-type Ca²⁺ channel currents were measured as Ba²⁺ entry through the channel (I_{Ba}) to eliminate Ca²⁺-dependent inactivation of the channel and Ca²⁺-dependent reduction of responsiveness of the channel to protein kinase A [23]. For recording the I_{Ba} , external Na⁺ was substituted by isomolar tetraethylammonium (TEA) and external Ca²⁺ by 1.8 mM Ba²⁺. Membrane currents were elicited with a series of 500 ms depolarizing pulses to 40 mV from HP of –80 mV in 10 mV increments applied at a frequency of 0.1 Hz. I_{Ba} amplitudes were estimated as the maximal inward deflection from the current obtained in the presence of 500 μM Ni²⁺. Voltage dependence of steady-state inactivation was determined from the peak amplitude of I_{Ba} during depolarization to a test potential of 10 mV after 2 s prepulses (V). Normalized peak current amplitudes were plotted against membrane voltage (V) and fitted by a Boltzman equation: $I/I_{max} = 1/(1 + \exp[(V_{1/2} - V)/k])$, where $V_{1/2}$ is midvoltage of inactivation and k is the slope of the linear portion of the inactivation curve.

Data analysis. To minimize individual differences, currents were recorded from 3–6 cells in each treatment group and each day after treatment. The data are presented as means ± standard error (SE). Statistical significance between the 2 groups or among various groups was evaluated by unpaired Student's *t*-tests or by Bonferroni's *t*-tests, respectively. The values of $p < 0.05$ were deemed significant.

RESULTS

Effects of sustained β-AR stimulation on levels of L-type Ca²⁺ channel expression and Ca²⁺ currents

The cultured adult cardiac myocytes were quiescent under control conditions and after chronic exposure to ISO (2 μM), as previously reported [21]. Membrane capacitance declined with time in culture [24] (Table 1), while β-AR stimulation induced significant increases in the capacitance at 96 h (1.3-fold vs. time-matched controls, $p < 0.05$, Table 1), which reflects an induction of cellular hypertrophy [11]. Accompanying the increases in capacitance were increases in the relative levels of mRNA coding for the L-type channel α_{1C} subunit. Figure 1A

Table 1. Changes in membrane capacitance elicited by culture in the presence and absence of isoproterenol (2 μM).

	Control (pF)		ISO treated (pF)	
Fresh	91.0 ± 7.8 $n = 15$			
0 h	52.2 ± 5.0 $n = 43$			
48 h	41.7 ± 5.6	$n = 24$	46.1 ± 5.6	$n = 21$
72 h	34.0 ± 3.9	$n = 29$	42.8 ± 8.6	$n = 31$
96 h	39.7 ± 1.9	$n = 55$	52.7 ± 3.2*	$n = 50$

Data are presented as means ± SE (n = number of cells from multiple cultures). * $p < 0.05$ vs. control.

shows a representative semiquantitative RT-PCR experiment in which the primers yielded products corresponding to the predicted sizes for GAPDH (522 bp; lanes 1 and 2) and the α_{1C} subunit (740 bp; lanes 3 and 4). Note that after 96 h, the intensity of the band corresponding to the α_{1C} subunit from cells exposed to ISO (Fig. 1A, lane 4) was stronger than that from control cells (Fig. 1A, lane 3). Under control conditions, the relative level of α_{1C} mRNA was quite stable during the 96-h culture period (Fig. 1B, white column). In the presence of ISO, however, the levels of α_{1C} mRNA approximately doubled at 72 h ($p < 0.05$, Fig. 1B).

Following the increase in α_{1C} mRNA expression was an increase in peak I_{Ba} density. To account for the changes in cell surface area during culture with or without ISO (2 μM), the amplitude of I_{Ba} was normalized to individual cell capacitance. The peak I_{Ba} density in control cells declined slightly during the 96-h culture period, but the effect was not significant (Fig. 1C; 0 h vs. 96 h). On the other hand, after 96 h of β-AR stimulation, I_{Ba} density was increased by approximately twofold as compared to control (control, 7.0 ± 1.0 pA/pF, $n = 27$; ISO, 13.0 ± 1.4 pA/pF; $n = 37$) (Fig. 1C). Thus sustained β-AR stimulation leads to an upregulation of the mRNA of the α_{1C} subunit and subsequent increases in current density.

Properties of L-type Ca²⁺ channels in cells cultured with or without sustained β-AR stimulation

Although sustained β-AR stimulation increased peak I_{Ba} density, neither the current–voltage (I – V) relationship nor the time course of the current decay was altered by time either in culture or in the 96-h ISO treatment (Fig. 2, A and B). In all cases, I_{Ba} were first detected near –30 mV and reached a peak at about –10 mV (Fig. 2A). Because no inward current was observed at potentials more negative than –40 mV indicates that the contribution of T-type currents to I_{Ba} density was negligible [22].

Voltage-dependent steady-state inactivation was determined as the channel availability after 2 s prepulses (Fig. 2C). Consistent with previous findings [24], steady-state inactivation curves were shifted toward negative poten-

Fig. 1. Sustained β -AR stimulation upregulates the expression of L-type Ca^{2+} channels. **A:** PCR products coding for GAPDH- (lanes 1 and 2) and the L-type Ca^{2+} channel α_{1C} subunit (lanes 3 and 4) obtained after 96 h of culture in the absence (lanes 1 and 3) and presence of 2 M ISO (lanes 2 and 4). Shown is an ethidium bromide-stained agarose gel; DNA markers are on the left. **B:** Relative levels of mRNA coding for the L-type channel α_{1C} subunit in control (white bar) and ISO-treated (black bar) cells are plotted against treatment time. The bars represent means SE from 4–6 samples; * $p < 0.05$ control vs. ISO-treated cells. **C:** I_{Ba} density in control and ISO-treated cells are plotted vs. treatment time. Note that I_{Ba} density declined slightly with time in culture (compare freshly isolated [triangles] and control cells [open circles]), but was significantly increased by ISO treatment (solid circles). Symbols represent means SE of 21–37 cells in each treatment group; ** $p < 0.01$ control vs. ISO-treated cells.

tials with the elapsed time of culture. $V_{1/2}$ in 96-h control cells was shifted by about -5 mV as compared to freshly isolated cells ($p < 0.05$; Table 2), and ISO treatment had no additional effect (96-h control, $V_{1/2} = -39.2 \pm 0.4$ mV, $n = 17$; 96 h ISO $V_{1/2} = -38.1 \pm 0.5$ mV, $n = 23$). Apparently, the β -AR-mediated enhancement of peak I_{Ba} density was not the result from a modulation of the channel availability.

Sensitivity to β -AR signaling of L-type Ca^{2+} channels in control and ISO-treated cells

Acute potentiation of the L-type Ca^{2+} channel currents mediated by β -AR stimulation persists in cells in culture [25]. When we examined the effect of an acute application of 1 μM ISO in cells cultured for 96 h in the presence or absence of 2 μM ISO, the cells were washed three times with Tyrode solution and superfused for at least 30 min. We were somewhat surprised to find that acute ISO increased the peak amplitude of I_{Ba} to a similar degree in both the control and the ISO-treated cells (2.2-fold in 96 h ISO-treated cells, $n = 24$; 2.6-fold in 96-h control, $n = 23$) (Fig. 3A). An acute application of ISO also shifted the $I-V$ and inactivation curves toward more negative potentials

Fig. 2. Voltage-dependent activation and inactivation are not affected by sustained β -AR stimulation. **A:** Representative I_{Ba} currents recorded from a control cell at 0 h (left panel) and after 96 h (center panel) and a cell treated for 96 h with 2 M ISO (right panel). Currents were elicited by 500 ms depolarizing pulses from an HP of -80 mV to 40 mV in 10-mV increments. **B:** $I-V$ relationship. Peak I_{Ba} density is plotted against membrane potential. I_{Ba} appeared at -30 mV and peaked at -10 mV in all groups. (open circles), 0 h control; (open squares), 96 h control; (solid squares), 96 h ISO-treatment. The I_{Ba} density in ISO-treated (96 h) cells was significantly greater than in time-matched controls (Table 2); * $p < 0.05$. **C:** Voltage-dependent steady-state inactivation was determined as the channel availability after 2 s prepulses from -110 to 20 mV. The inactivation curve was unaffected by sustained exposure to ISO.

(Fig. 3, B and C). In contrast to the *in vivo* effects [5], sustained β -AR stimulation did not attenuate the responsiveness of L-type Ca^{2+} channels to β -AR signaling in cultured adult quiescent myocytes.

Table 2. Properties of I_{Ba} inactivation ($V_{0.5}$ and k) in ventricular myocytes cultured with or without isoproterenol (2 μ M).

	Control			ISO treated		
	$V_{0.5}$ (mV)	k (mV)	n	$V_{0.5}$ (mV)	k (mV)	n
Fresh	-33.0 ± 0.5	5.4 ± 0.4	12			
0 h	-35.9 ± 0.3	5.6 ± 0.3	13			
48 h	-35.6 ± 0.5	5.7 ± 0.4	11	-34.9 ± 0.8	6.0 ± 0.7	8
72 h	-41.3 ± 0.4	7.1 ± 0.4	14	-40.9 ± 0.5	7.1 ± 0.4	14
96 h	-39.2 ± 0.4	6.9 ± 0.4	17	-38.1 ± 0.5	6.7 ± 0.5	23

Data are presented as means $\pm$ SE (n = number cells from multiple cultures).

Fig. 3. Effects of acute application of ISO on I_{Ba} in control and ISO-treated myocytes. **A:** Representative I_{Ba} recorded before (open symbols) and after (closed symbols) the acute application of 1 μ M ISO to cells cultured for 96 h in the absence (circles) or presence (squares) of ISO. The currents were elicited by 500 ms depolarizing pulses to -10 mV from a holding potential of -80 mV. **B:** $I-V$ relationship. Maximal I_{Ba} density was increased about 2.6- and 2.2-fold, respectively, by the acute application of 1 μ M ISO to control (left panel) and ISO-treated cells (right panel). ISO elicited a negative shift in the $I-V$ curve in both groups (closed symbols). **C:** Effect of acute stimulation with ISO on steady-state inactivation. ISO shifted the inactivation curve by 7 mV toward more-negative potentials in both groups: control ($-ISO$), $V_{1/2}$, -40.2 ± 0.4 mV vs. ($+ISO$) -47.2 ± 0.8 mV ($n = 17$); ISO treated ($-ISO$), $V_{1/2}$, -38.1 ± 0.3 mV vs. ($+ISO$) -44.6 ± 1.0 mV ($n = 21$).

Upregulation of L-type channels by sustained β -AR stimulation is caused by an elevation of cAMP, but not by an elevation of $[Ca^{2+}]_i$

An up-regulation of the L-type Ca²⁺ channels induced by sustained ISO treatment was inhibited by propranolol (10 μ M), a β -AR antagonist (Fig. 4A), indicating that the effects of ISO were the results of the β -AR stimulation. The inhibitory effect of propranolol was consistent with a previous report that propranolol (10 μ M) inhibited the hypertrophy induced by ISO (0.01–10 μ M) [11]. These effects might result from a possible increase in Ca²⁺ influx; β -AR stimulation shifted the $I-V$ toward more negative potentials (Fig. 3 and Table 3). Previous studies have reported that an elevation of $[Ca^{2+}]_i$ increases the L-type channel expression [15], and the L-type channel blocker inhibited gene expression induced by an insulin-like growth factor (IGF) in rat cardiac myocytes [26]. There-

fore we examined whether the possible increases in Ca²⁺ influx through L-type channels upregulate the expression of the channel. The cells were cultured for 96 h with ISO and the dihydropyridine Ca²⁺ channel blocker nitrendipine (Nit, 10 μ M) (Fig. 4B). Nit did not inhibit the β -AR-induced hypertrophy (Nit+ISO, 49.2 ± 4.6 pA/pF, n.s. vs. 96 h ISO). Furthermore, the upregulation of the L-type Ca²⁺ channel was not inhibited by coculturing the cells with ISO and Nit, as shown in Fig. 4; 96 h ISO, 12.8 ± 1.0 pA/pF, $n = 55$; 96 h ISO+Nit, 11.4 ± 2.1 pA/pF, $n = 33$ (n.s.). An influx of Ca²⁺ through the L-type channel may not contribute to the L-type channel upregulation induced by the sustained β -AR stimulation. This was also supported from the results that the forskolin treatment of cells for 96 h increased I_{Ba} density, but the Bay-K 8644 treatment failed to increase it (Fig. 4C).

DISCUSSION

In the present study, we have been able to show that sustained β -AR stimulation increases the levels of mRNA coding for the L-type Ca²⁺ channel α_{1C} subunit in cultured adult rabbit ventricular myocytes, as well as the L-type current density. Neither the biophysical properties nor the β -AR responsiveness of the channel was affected

Fig. 4. Effects of β -AR- and Ca^{2+} antagonist on the ISO-induced increase in I_{Ba} density. **A–C:** The upper panel shows the I - V relationships for cells cultured for 96 h with or without reagents, and the lower shows average peak density. **A:** Cells were cultured for 96 h under control conditions (open circles, $n = 16$), with $2 \mu\text{M}$ ISO (open squares, $n = 16$) or with ISO plus propranolol (solid squares, $n = 38$). **B:** Cells were cultured for 96 h under control conditions (open circles, $n = 55$), with $2 \mu\text{M}$ ISO (open squares, $n = 50$) or with ISO plus $10 \mu\text{M}$ nitrendipine (solid triangles, $n = 38$). **C:** I - V relationships for cells cultured for 96 h under control conditions, with $2 \mu\text{M}$ BayK 8644 (open diamonds, $n = 12$) or with $10 \mu\text{M}$ forskolin (solid diamonds, $n = 5$). * $p < 0.05$. n.s.: not significant.

Table 3. Modulation of I_{Ba} ($V_{0.5}$ and k) activation properties by application of isoproterenol ($1 \mu\text{M}$) to control and ISO-treated cells.

	Control		+ ISO ($1 \mu\text{M}$)		
	$V_{0.5}$ (mV)	k (mV)	$V_{0.5}$ (mV)	k (mV)	n
0 h, control	-15.6 ± 0.5	4.1	-21.2 ± 0.5	3.6	17
96 h, control	-17.8 ± 0.6	4.6	-21.3 ± 0.5	4.5	23
96 h, ISO treated	-17.5 ± 0.7	4.9	-21.0 ± 0.7	4.7	23

Data are presented as means $\pm$ SE (n = number cells from multiple cultures).

by the sustained β -AR stimulation, and the upregulation of the L-type channel induced by the sustained β -AR stimulation was independent of the increase in Ca^{2+} influx through the channel.

The L-type Ca^{2+} channel is composed of the $\alpha_{1\text{C}}$ subunit and auxiliary β and α_2 subunits, which are known to alter the biophysical properties of the L-type channel [22, 27]. Especially in failure heart, a downregulation of the β subunit has been reported [28] and suggested the differential regulation of the L-type channel by various β subunits [29]. In contrast to the failure heart, sustained β -AR stim-

ulation did not affect the biophysical properties of the channel, suggesting that under the primary culture of the myocytes, sustained β -AR stimulation increases the expression of all three subunits, as in neonatal myocytes [18]. On the other hand, the effects of β -AR stimulation on L-type channel expression developed much slower in adult cells than in neonatal cells (for mRNA, 72 h vs. 2 h; current level, 96 h vs. 24 h) [17]. This difference may result from absence of the spontaneous contractile activity of the cultured myocytes, which reflects a repetitive transient in $[\text{Ca}^{2+}]_i$ [30] and a regulation of gene expression in

cultured myocytes [15]. About a 24-h delay from the increase in α_{1C} mRNA to that in I_{Ba} density is considered necessary to produce responsible proteins because the cellular hypertrophy occurred simultaneously with I_{Ba} density. A similar delay has also been reported in neonatal beating myocytes with a β -AR modulation of L-type abundance [17].

The increase in intracellular cAMP concentration is considered to play an important role in the upregulation of the L-type channel in cultured neonatal myocytes [17, 18], resulting in the activation of cAMP responding element binding protein (CREB), which regulates gene expression in myocytes [31, 32]. In fact, the cAMP responding element has a consensus sequence to regulate the 5'-flanking sequence in the α_{1C} subunit gene promoter region [33]. In that regard, the role of CREB has been described in the development and failure of the heart [34], and the role of Ca²⁺ influx through the L-type channels in the regulation of CREB activity has been reported [35]. In the present study, sustained β -AR stimulation caused the cellular hypertrophy and upregulation of the L-type channel, despite the presence of a Ca²⁺ channel blocker (Fig. 4). Moreover, the effects were mimicked by an adenylate cyclase activator, forskolin, but not by an L-type Ca²⁺ channel agonist, BayK8644. Therefore the upregulation of the L-type channel by β -AR stimulation is considered to be due to an increase in [cAMP]_i and not by an increase in Ca²⁺ influx through the channel in cardiomyocytes, and may follow the CREB activation.

In vivo studies have shown that the chronic administration of a β -agonist downregulates β -AR [5] and disrupts β -AR-mediated signal transduction leading to L-type channel modulation [6, 7]. In contrast, we found that the sustained β -AR stimulation did not cause a desensitization of the signal transduction: the application of ISO increased I_{Ba} amplitude and shifted $I-V$ and steady-state inactivation curves to more-negative potentials in both the control and the ISO-treated cells. This may reflect the quiescence of the cultured cells because mechanical unloading reportedly restores β -AR responsiveness in human failure heart [8].

In summary, the abundance of L-type Ca²⁺ channels is increased by sustained β -AR stimulation in cultured adult ventricular myocytes without affecting the biophysical properties or β -AR responsiveness of the channels. Although it is difficult to completely exclude the influence of an increase in [Ca²⁺]_i, this is the first report that describes L-type channel upregulation without an increase in Ca²⁺ current. Cultured adult and quiescent cardiac myocytes are thus a useful tool with which to investigate the mechanisms governing gene expression induced by Ca²⁺ influx, by mechanical stimulation, and by receptor-mediated signal transduction.

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