Involvement of CaV3.1 T-type calcium channels in cell proliferation in mouse preadipocytes

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Am J Physiol Cell Physiol 298: C1414–C1423, 2010. First published March 24, 2010; doi:10.1152/ajpcell.00488.2009.—Voltage-gated Ca2+ channels (CaV) are ubiquitously expressed in various cell types and play vital roles in regulation of cellular functions including proliferation. However, the molecular identities and function of CaV remained unexplored in preadipocytes. Therefore, whole cell voltage-clamp technique, conventional/quantitative real-time RT-PCR, Western blot, small interfering RNA (siRNA) experiments, and immunohistochemical analysis were applied in mouse primary cultured preadipocytes as well as mouse 3T3-L1 preadipocytes. The effects of CaV blockers on cell proliferation and cell cycle were also investigated. Whole cell recordings of 3T3-L1 preadipocytes showed low-threshold Cav, which could be inhibited by mibebradil, Ni2+ (IC50 of 200 μM), and NNC55-0396. Dominant expression of α1G mRNA was detected among CaV transcripts (α1A–α1H), supported by expression of Cav3.1 protein encoded by α1G gene, with immunohistochemical studies and Western blot analysis. siRNA targeted for α1G markedly inhibited Cav. Dominant expression of α1G mRNA and expression of Cav3.1 protein were also observed in mouse primary cultured preadipocytes. Expression level of α1G mRNA and Cav3.1 protein significantly decreased in differentiated adipocytes. Mibebradil, NNC55-0396, a selective T-type Cav blocker, but not diltiazem, inhibited cell proliferation in response to serum. NNC55-0396 and siRNA targeted for α1G also prevented cell cycle entry/progression. The present study demonstrates that the Cav3.1 T-type Ca2+ channel encoded by α1G subtype is the dominant Cav in mouse preadipocytes and may play a role in regulating preadipocyte proliferation, a key step in adipose tissue development.

3T3-L1; adipose; α1G; cell cycle

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Thus CaV appear to provide a novel therapeutic target for the treatment of obesity. However, the molecular identities and physiological roles of CaV expressed in preadipocytes remained unexplored. Therefore, to clarify the characteristics of expression of CaV in preadipocytes, whole cell voltage-clamp technique, conventional/quantitative real-time RT-PCR, Western blot, small interfering RNA (siRNA) experiments, and immunohistochemical analysis were applied to mouse 3T3-L1 preadipocytes, a subclone of 3T3 Swiss mouse embryo fibroblasts (11, 12), mouse primary cultured preadipocytes, and their differentiated adipocytes. Here we provide direct evidence that the CaV3.1 T-type Ca\(^{2+}\) channel encoded by α\(_{1G}\) subtype is the dominant CaV in mouse preadipocytes and plays a role in the regulation of their proliferation.

### Table 1. PCR primers used for amplification of voltage-gated Ca\(^{2+}\) channel genes

<table>
<thead>
<tr>
<th>Channel (gene symbol)</th>
<th>Current</th>
<th>Size, bp</th>
<th>Sequence (5'–3')</th>
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<tr>
<td>Cav2.1(α1A)</td>
<td>P/Q-type</td>
<td>544</td>
<td>Sense CCGAGAACACGGTTATCGT</td>
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<td></td>
<td></td>
<td></td>
<td>Antisense GCACTGACACCATGAAAGTC</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>Antisense CACTGACACGATGGAAGTC</td>
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<tr>
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<td>L-type</td>
<td>568</td>
<td>Sense AGAGGCTGCTACTGATGCG</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Antisense CAGTCTCTGAGCCACTGAG</td>
</tr>
<tr>
<td>Cav1.3(α1D)</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>Antisense AGGCTGAGGATGCTCAGGG</td>
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<tr>
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<td>927</td>
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<td></td>
<td>Antisense CAAAGCAGGAAGAAATAGAC</td>
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<tr>
<td>Cav3.1(α1G)</td>
<td>T-type</td>
<td>455</td>
<td>Sense GATGCCTGATGCAGTACG</td>
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<td></td>
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<td></td>
<td>Antisense TGACAGGAGCTGAATACAG</td>
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<td>Cav3.2(α1H)</td>
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<td>Sense AGCAACTCCTGCTGATCAG</td>
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<td>Antisense GAGAGATATTGACAGTGAG</td>
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<td>GAPDH</td>
<td></td>
<td>259</td>
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<td>siRNA Cav3.1(α1G)</td>
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<td></td>
<td>Antisense AACAGGGAGGCCATGCCAG</td>
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<td>Negative control</td>
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<td></td>
<td>Antisense UUGGUAGUCUAAGACGUC</td>
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siRNA, small interfering RNA.

Fig. 1. Voltage-gated Ca\(^{2+}\) currents (I\(_{Ca}\)) expressed in 3T3-L1 preadipocytes. A: cells were held at −100 mV, and command voltage pulses to +100 mV (400 ms in duration) were applied. The bath solution contained 10 mM Ca\(^{2+}\) in Aa and Ab. In Ac, 10 mM Ba\(^{2+}\) was replaced by 10 mM Ba\(^{2+}\). Effects of TTX (10 μM) and mibefradil (30 μM) and nicardipine (1 μM) are illustrated in Aa and Ab, respectively. Note that the transient inward current was blocked by mibefradil but not TTX and nicardipine. B: current voltage (I-V) relations measured at the peak of the transient inward current and the effects of holding potential (V\(_{h}\)). The current traces at various command voltage pulses were elicited from a V\(_{h}\) of −100 mV, but not at −40 mV. D: steady-state activation (□) and inactivation (●) curves for I\(_{Ca}\). Data obtained from 4 cells were fitted by Boltzmann equation.
MATERIALS AND METHODS

The Animal Care and Use Committee of the University of Tokyo approved the present study.

Cell culture of 3T3-L1 preadipocytes and adipocyte differentiation method. 3T3-L1 preadipocytes were obtained from the Japanese Collection of Research Bioresources (JCRB) Bank (Osaka, Japan) as previously described (35). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) containing 100 μg/ml penicillin and 100 μg/ml streptomycin. Cells were plated and grown until 2 days after confluence and then induced to differentiate into adipocytes by change of the culture medium to DMEM containing 0.5 mM methylisobutyloxanthine, 0.25 μM dexamethasone, and 10 μg/ml insulin for 48 h (36). With this protocol, >95% of the cells were differentiated into the adipocyte phenotype 3–4 days after initiating differentiation. The differentiated 3T3-L1 adipocytes were switched to fresh DMEM containing 10% FBS.

Isolation and culture of preadipocytes and adipocytes from mouse white adipose tissue. Mouse primary cultured preadipocytes were obtained from Primary Cell (Hokkaido, Japan). The isolation method was similar to that in previous reports (1, 18). Briefly, isolated epididymal white adipose tissue obtained from ICR mice at 2–4 days after birth was rinsed in collagenase solution. The mixture was then allowed to digest with gentle shaking. The resulting cell suspension was allowed to settle in order to separate into a supernatant containing adipocytes and an inferior layer composed mainly of preadipocytes. The primary cultured preadipocytes were used for later experiments. The preadipocytes were then seeded in a plate (2.4 × 10⁶ cells/plate) in DMEM supplemented with 10% fetal calf serum containing penicillin (10 U/ml), streptomycin (10 μg/ml), 4-Aminopyridine (4-AP, 10 mM), octanoic acid (1 mM), ascorbic acid (100 μM), acetic acid (100 μM), octanolic acid (1 mM), and 3,5,5′-triodothyronine (T₃; 50 nM). The medium was replaced with differentiating medium consisting of the above medium with dexamethasone (2.5 μM) and insulin (10 μg/ml). Under these conditions, adipocyte differentiation occurred a few days later (7), and differentiated adipocytes were maintained in the differentiation medium without dexamethasone. Full differentiation developed at 8–10 days after induction of differentiation.

Solutions and drugs. The composition of control Tyrode solution was as follows (in mM): 136.5 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.53 MgCl₂, 5.5 glucose, and 5.5 HEPES-NaOH buffer (pH 7.4). To block K⁺ currents, the patch pipette contained (in mM, 4-AP, 10 mM), ascorbic acid (100 μM), acetic acid (100 μM), octanolic acid (1 mM), and 3,5,5′-triodothyronine (T₃; 50 nM). The medium was replaced with differentiating medium consisting of the above medium with dexamethasone (2.5 μM) and insulin (10 μg/ml). Under these conditions, adipocyte differentiation occurred a few days later (7), and differentiated adipocytes were maintained in the differentiation medium without dexamethasone. Full differentiation developed at 8–10 days after induction of differentiation.

Fig. 2. Effects of various types of Ca²⁺ channel blockers on I_Ca, expressed in 3T3-L1 preadipocytes. Cells were held at −100 mV, and command voltage pulses to +0 mV (400 ms in duration) were applied at 0.1 Hz. A–D: concentration-dependent inhibition of I_Ca by mibefradil (A and B) and Ni²⁺ (C and D). Original current traces are shown in A and C, and the inhibitory effect of these agents on the current amplitude measured at the peak is plotted against various concentrations of mibefradil (B) and Ni²⁺ (D). Data are shown as means ± SE (n = 5) and fit by a Michaelis-Menten simple bimolecular model: % inhibition = 100/[1 + (IC₅₀ [mibefradil or Ni²⁺])]. Where IC₅₀ is 50% inhibitory concentration for drugs. The data were best fit with an IC₅₀ value of 3 μM for mibefradil and 200 μM for Ni²⁺. E: effects of NNC55-0396 (10 μM) on I_Ca. F: % inhibition of I_Ca by various Ca²⁺ channel blockers: mibefradil (Mib, 10 μM), NNC55-0396 (NNC, 10 μM), SNX-482 (SNX, 100 μM), omega-agatoxin IVA (ω-agatoxin, 100 nM), omega-conotoxin GVIA (ω-conotoxin, 500 nM), and nicardipine (Nicard). Data are shown as means ± SE (n = 5).
SNX-482, ω-agatoxin IVA, ω-conotoxin GVIA, diltiazem, and nicardipine) were obtained from Sigma. All experiments were performed at room temperature (20–25°C).

Recording technique and data analysis. Membrane currents were recorded with tight-seal whole cell clamp techniques and a patch-clamp amplifier (EPC-7, List Electronics, Darmstadt, Germany) as previously described (14, 38). Steady-state inactivation was estimated with a double-pulse protocol. Conditioning voltage pulses (500 ms in duration) of various membrane potentials were applied from a holding potential of −100 mV. At 10 ms after the end of each conditioning pulse, a test pulse of +0 mV was applied. The ratio of $I_{Ca}$ amplitude with and without conditioning pulses was plotted against each conditioning voltage. From current-voltage ($I-V$) data, the steady-state activation curve was derived by using the following equation: 

$$g_{Ca} = \frac{I_{Ca}}{\left(V_m - E_{Ca}\right)}$$

where $I_{Ca}$ is the peak current amplitude at each membrane potential ($V_m$), $g_{Ca}$ is the chord conductance, and $E_{Ca}$ is the $Ca^{2+}$ equilibrium potential. $E_{Ca}$ was obtained from the $I-V$ curve, where the $I-V$ curve crossed over the zero line.

RNA extraction, reverse transcriptase-polymerase chain reaction, and real-time quantitative reverse transcriptase-polymerase chain reaction. Total cellular RNA was extracted from preadipocytes and differentiated adipocytes with the RNeasy mini kit (Qiagen, Cambridge, MA). For reverse transcriptase-polymerase chain reaction (RT-PCR), complementary DNA (cDNA) was synthesized from 1 μg of total RNA with reverse transcriptase and random primers (Toyobo, Osaka, Japan). The reaction mixture was then subjected to PCR amplification with specific forward and reverse oligonucleotide primers for 35 cycles consisting of heat denaturation, annealing, and extension. PCR products were size-fractionated on 2% agarose gels and visualized under UV light. Primers were chosen on the basis of the sequence of mouse α-subunit genes of CaV ($\alpha_{1A}$–$\alpha_{1H}$) as shown in Table 1. Table 1 also shows the CaV protein created by each gene.

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Fig. 4. Cav3.1 T-type CaV expression in 3T3-L1 preadipocytes, mouse primary cultured preadipocytes, and differentiated adipocytes. 

Aa: microscopic image of 3T3-L1 preadipocytes. Ab: staining (green) for Cav3.1 in 3T3-L1 preadipocyte. Double staining of nuclei with Hoechst 33258 to visualize nuclei and anti-Cav3.1 is illustrated. Ac: negative controls without the antibody (anti-$\alpha_{1G}$) in 3T3-L1 preadipocytes. Cells were counterstained with Hoechst 33258 to visualize nuclei. Ad: transfection of small interfering RNA (siRNA) for $\alpha_{1G}$ mRNA. The transfected siRNA conjugated with rhodamine is visualized under fluorescent microscopy. Ae: Oil Red O staining of differentiated 3T3-L1 adipocytes. Af: staining (green) for Cav3.1 in mouse primary cultured preadipocyte. Bb: differential interference contrast (DIC) image of mouse differentiated adipocytes. The differentiated adipocytes have lipid deposits. Bc: staining (green) for Cav3.1 in differentiated 3T3-L1 adipocytes (Af) and mouse differentiated adipocytes (Bc). Scale bars, 50 μm.
Total RNA of mouse adult brain was used for positive control. Real-time quantitative RT-PCR was performed with the use of real-time TaqMan technology and a sequence detector (ABI PRISM 7000, Applied Biosystems, Foster City, CA) (22). Gene-specific primers and TaqMan probes were used to analyze transcript abundance. The 18S ribosomal RNA level was analyzed as an internal control and used to normalize the values for transcript abundance of Cav family genes. The probes used in this study were purchased as Assay-on-Demand from Applied Biosystems: assay ID Mm00432190_ml for α1A, Mm00432226_ml for α1B, Mm00437917_ml for α1C, Mm00494444_ml for α1E, Mm01299131_ml for α1G, Mm00445369_ml for α1H, Mm01299033_ml for α2A, Mm01295675_ml for adioprotein 2 (aP2), Mm00494477_ml for Pref-1, and 4310893E for 18S rRNA endogenous control. We performed six independent experiments.

Oil Red O staining. Differentiated 3T3-L1 adipocytes were rinsed in PBS before being fixed with 10% formaldehyde. Approximately 15 min after the fixation, Oil Red O stain was used to stain for lipid accumulation (20 min). After being rinsed with distilled water, photomicrographs were taken with a digital camera to document staining.

Immunocytochemistry. Immunocytochemical analyses were performed on preadipocytes and differentiated adipocytes of 3T3-L1 and mouse primary cultured preadipocytes with anti-CaV3.1 (Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit polyclonal antibody against channel isoforms diluted to 1:100. The cells were cultured on Lab-Tek Chamber Slide Glass (Nalge Nunc International, Naperville, IL), fixed with 2% paraformaldehyde in PBS for 30 min, and then blocked for 10 min with 2% horse serum in PBS. The cells were incubated overnight with the primary antibodies diluted with 0.1% Triton X-100 and 0.01% NaN3 in PBS. For negative controls, cells were treated without antibody. Alexa Fluor 488-labeled donkey anti-rabbit IgG antibody diluted 1:1,000 (Molecular Probes, A21206) was used to visualize channel expression. The cells were also stained with Hoechst 33258 (Sigma Aldrich) to visualize nuclei. A confocal laser scanning microscope (Olympus Fluoview FV300, Olympus, Tokyo, Japan) was used for observations.

Transfection of synthetic small interfering RNA. α1G siRNA and nonsilencing (negative control) siRNAs as described in Table 1 were purchased from Qiagen (Cambridge, MA). They were transfected into 3T3-L1 preadipocytes to a final concentration of 5 nM with Lipofectamine 2000 (5 μl/ml culture; Invitrogen) according to the instructions of the manufacturer. Transfected cells were incubated for 48 h in an atmosphere of 5% CO2 and 95% air at 37°C before each experiment. Analysis of mRNA and protein by real-time RT-PCR and Western blot was then performed. Rhodamine-conjugated siRNA was used to confirm the transfection of siRNA with Nikon ECLIPSE TE2000-u. The density of IC50 and the effects of siRNA on cell cycle were also investigated in the transfected cells.

Western blotting. Proteins were separated on a 7.5% polyacrylamide gel for 70 min at 200 V and then transferred to Amersham Hybond-P (GE Healthcare UK, Little Chalfont, UK) for 75 min at 72 mA. The membrane was blocked with 3% skim milk in PBS (0.01 M phosphate buffer, 0.138 M NaCl, 0.0027 M KCl, pH 7.4) with 0.1% Tween 20 (PBS-T) for 1 h. It was probed with anti-CaV3.1 polyclonal antibody diluted to 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit polyclonal antibody against CaV3.1 protein between preadipocytes and differentiated adipocytes of 3T3-L1 and mouse primary cultured preadipocytes and adipocytes. The membrane was blocked with 3% skim milk in PBS (0.01 M phosphate buffer, 0.138 M NaCl, 0.0027 M KCl, pH 7.4) with 0.1% Tween 20 (PBS-T) for 1 h. It was probed with anti-CaV3.1 polyclonal antibody diluted to 1:200 (Santa Cruz Biotechnology) and anti-β-actin monoclonal antibody (1:10,000; Sigma) overnight at 4°C. Washed three times in PBS-T for 15 min each, and subsequently incubated with anti-rabbit IgG linked to peroxidase (Millipore) diluted to 1:1,000 with blocking buffer for 1 h at room temperature. After three additional washes, bound antibodies were detected with Amersham ECL-plus (GE Healthcare UK) and analyzed with an LAS-3000 mini image analyzer (Fuji-Film, Tokyo, Japan).

Proliferation assay. Cell proliferation was assessed with the Cell Titer 96 Aqueous kit (Promega, Madison, WI). The preadipocytes were plated in 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 8 × 104 cells/well with experimental media with or without Ca2+ channel blockers. These plates were incubated for 24 h, after which the tetrazolium salt and dye solution were added and color development was allowed to proceed for 3 h at 37°C and 5% CO2. Each plate was then read at an absorbance of 490 nm.

Flow cytometry. 3T3-L1 preadipocytes were plated at ~5,000 cells/cm2 onto 10-cm plates and allowed to sit for 2 nights in FBS-free medium, and then cells were perfused into 5% FBS-containing medium in the presence or absence of CaV blockers. In siRNA experiments, cells treated with α1G siRNA and nonsilencing (negative control) siRNAs were plated at ~5,000 cells/cm2 onto 10-cm plates and allowed to sit for 24 h in FBS-free medium, and then cells were perfused into 5% FBS-containing medium. Cell cycle data were determined after 24-h treatment by flow cytometry in propidium iodide-stained mouse 3T3-L1 preadipocytes.

Data analysis. All values are expressed as means ± SE. Differences between multiple groups were compared by ANOVA. Two-group analysis was performed with a Student’s t-test. Differences were considered significant if P < 0.05.

RESULTS

T-type CaV in 3T3-L1 preadipocytes. Figure 1, A–C, show IC50 expressed in 3T3-L1 preadipocytes where the bath solution contained 10 mM Ca2+. The cells were held at −100 mV, and command voltage pulses (400 ms in duration) were applied to +0 mV. A fast transient inward current was elicited during the depolarizing pulse. TTX (10 μM, Fig. 1Aa) or replacement of extracellular Na+ with N-methyl-D-glucamine+ (data not shown), an impermeant cation, did not affect the transient inward current at all. However, mibefradil (30 μM, Fig. 1Ab), a potent inhibitor of T- and L-type currents (24), but not

Fig. 5. Comparison of CaV3.1 protein expression between preadipocytes and differentiated adipocytes. Western blot analysis shows the marked difference of CaV3.1 protein expression between 3T3-L1 preadipocytes and differentiated adipocytes (Ab) and between mouse primary cultured preadipocytes and differentiated adipocytes (Ba). In Ab and Bb, the relative abundance of CaV3.1 protein between preadipocytes and differentiated adipocytes is considered as 100%. Note that the expression level of CaV3.1 protein in differentiated adipocytes significantly decreased compared with 3T3-L1 preadipocytes and mouse primary cultured preadipocytes. **P < 0.01 vs. preadipocytes.
nicardipine (1 μM, Fig. 1Ab), a potent L-type Ca2+ channel blocker, completely abolished it. With replacement with 10 mM Ba2+ instead of Ca2+, the amplitude of the inward current slightly decreased (Fig. 1Ac). In addition, 1 μM Bay K 8644 did not enhance it (data not shown), suggesting that high-threshold L-type Cav or voltage-gated Na+ channels do not contribute to form the inward current expressed in 3T3-L1 preadipocytes but non-L-type Cav do form it.

Typical current data recorded at each membrane potential and I-V relations measured at the peak of I\textsubscript{Ca} are shown in Fig. 1, B and C. Cells were held at -40 mV (Fig. 1B) or -100 mV (Fig. 1Bb) in the same cell. When the cell was held at -100 mV, the transient inward currents were elicited at potentials more positive than -50 mV. The peak amplitude of I\textsubscript{Ca} was observed at approximately -20 mV and reached zero at potentials of approximately +50 to +60 mV. On the other hand, I\textsubscript{Ca} was not elicited during the depolarizing pulses at a holding potential of -40 mV. Figure 1D shows the steady-state activation curve for I\textsubscript{Ca}. The inactivation data were fitted to the following equation (Boltzmann equation) by using least-squares methods: \( (V/V_{\text{max}}) = 1/[1 + \exp(V - V_{\text{h}}/k)] \), where \( V \) is the membrane potential in millivolts, \( V_{\text{h}} \) is the membrane potential at half-maximum, and \( k \) is the slope factor. The values of \( V_{\text{h}} \) and \( k \) were -51.7 ± 0.9 mV and 8.4 ± 0.8 (n = 5), respectively. The steady-state activation curves were obtained from the conductance and also fitted by Boltzmann equation. The values of \( V_{\text{h}} \) and \( k \) were -30.0 ± 0.3 and -7.3 ± 0.3 (n = 5), respectively. The overlap of the steady-state activation and inactivation curves at potentials more positive than -50 mV determines the window currents.

Figure 2, A and B, shows the concentration-dependent effects of mibebradil on I\textsubscript{Ca}. Mibebradil inhibited I\textsubscript{Ca} with an IC\textsubscript{50} value of 3 μM (Fig. 2B), and mibebradil at concentrations >10 μM nearly completely abolished I\textsubscript{Ca}. Next, we investigated the Ni2+ sensitivity of I\textsubscript{Ca} (Fig. 2, C and D). Ni2+ concentration-dependently inhibited I\textsubscript{Ca} with an IC\textsubscript{50} value of 200 μM, and 1 mM Ni2+ almost completely abolished it. Inhibitory effects were also observed with NNC55-0396 (10 μM; Fig. 2E), a selective inhibitor of T-type Cav (21, 37).

Furthermore, to determine which types of Cav are involved in I\textsubscript{Ca} expressed in 3T3-L1 preadipocytes, we examined responses to a test stimulus applied in the absence or presence of various agents. The Ca2+ channel blockers used here were as follows: mibebradil (10 μM), NNC55-0396 (10 μM), SNX-482 (a R-type Ca2+ channel blocker; 100 nM), ω-agatoxin IVA (a P-type Ca2+ channel blocker; 100 nM), ω-conotoxin GVIA (a N-type Ca2+ channel blocker; 500 nM), and nicardipine (10 μM). The percent inhibition of various Ca2+ channel blockers is illustrated in Fig. 2F. Mibebradil and NNC55-0396 inhibited I\textsubscript{Ca} by 91 ± 3% and 67.7 ± 4.6%, respectively, but nicardipine inhibited it only by 3 ± 1%. SNX-482, ω-agatoxin IVA, and ω-conotoxin GVIA were ineffective.

Dominant expression of Cav3.1 encoded by \( \alpha_{1G} \) mRNA in 3T3-L1 preadipocytes. We investigated the expression of α-subunit genes of Cav family members (\( \alpha_{1A} - \alpha_{1H} \) mRNA).
By RT-PCR analysis, the transcripts of $\alpha_{1A}$, $\alpha_{1B}$, $\alpha_{1C}$, $\alpha_{1E}$, $\alpha_{1G}$, and $\alpha_{1H}$ were detected (Fig. 3A) and the transcripts of $\alpha_{1D}$ and $\alpha_{1F}$ were not detected compared with control mouse brain. The amplitude of cDNA fragments was of predicted molecular size, identical to cDNA fragments amplified from reversely transcribed mRNA. Expression of CaV family members was also investigated by real-time quantitative RT-PCR (Fig. 3B). Transcript levels were normalized to 18S ribosomal housekeeping gene. Thus $\alpha_{1G}$ appeared to dominantly encode for $\alpha$-subunit gene of CaV in 3T3-L1 preadipocytes.

Expression of CaV3.1 protein was confirmed by immunostaining (Fig. 4Ab) and Western blots using anti-CaV3.1 (Fig. 5, Aa and Ab) in 3T3-L1 preadipocytes. The cells were also counterstained with Hoechst 33258 to visualize nuclei, and the double staining of nucleus and CaV3.1 is shown in Fig. 4Ab. No expression was detected in negative controls without the antibody (Fig. 4Ac).

We next investigated the effects of siRNA for $\alpha_{1G}$. Transfection of siRNA conjugated with rhodamine was confirmed by fluorescent microscopy (Fig. 4Ad). After siRNA treatment the inhibitory effect on $\alpha_{1G}$ mRNA expression was analyzed by real-time RT-PCR. The expression level of $\alpha_{1G}$ mRNA in 3T3-L1 preadipocytes transfected with siRNA was significantly decreased compared with nonsilencing (negative control) siRNA (Fig. 6A; $P < 0.01$, $n = 6$). Western blot analysis showed that the protein expression level of CaV3.1 in 3T3-L1 preadipocytes transfected with siRNA was also decreased compared with nonsilencing (negative control) siRNA ($P < 0.01$, $n = 3$; Fig. 6, Ba and Bb). Next, $I_{Ca}$ was recorded with tight-seal whole cell clamp techniques in the transfected cells (20 cells, siRNA for $\alpha_{1G}$ and negative control, respectively). The current density of $I_{Ca}$ adjusted by cell capacitance was significantly reduced in cells transfected with siRNA for $\alpha_{1G}$ (Fig. 6C; $P < 0.01$), compared with nonsilencing (negative control) siRNA.

CaV in mouse primary cultured preadipocytes. Expression of $\alpha$-subunit genes of CaV family members ($\alpha_{1A}$–$\alpha_{1H}$) was also investigated by real-time quantitative RT-PCR (Fig. 3C) in mouse primary cultured preadipocytes, and $\alpha_{1G}$ appeared to encode for $\alpha$-subunit gene of CaV in a similar way to that in mouse 3T3-L1 preadipocytes. Expression of CaV3 protein was confirmed by immunostaining (Fig. 4Ab) and Western blot analysis using anti-CaV3.1 (Fig. 5, Ba and Bb), which was similar to 3T3-L1 preadipocytes. The cells were also counterstained with Hoechst 33258 to visualize nuclei, and the double staining of nucleus and CaV3.1 is shown in Fig. 4Bb.

Comparison of $\alpha_{1G}$ mRNA expression between preadipocytes and differentiated adipocytes. We examined the effects of differentiation on $\alpha_{1G}$ mRNA expression in both 3T3-L1 preadipocytes and mouse primary cultured preadipocytes. After differentiation, lipid accumulation stained by Oil Red O stain could be observed in 3T3-L1 cells (Fig. 4Ae). Similarly, lipid accumulation was observed in differentiated mouse adipocytes (Fig. 4Bb). Figure 7 compares the expression levels of $\alpha_{1G}$ and p2 mRNA, a differentiated marker gene of adipocytes, in both cell types. The expression level of p2 mRNA was notably increased in 3T3-L1 adipocytes and mouse adipocytes, while the expression level of $\alpha_{1G}$ mRNA was significantly decreased during the differentiation. In addition, the mRNA expression level of Pref-1 (also known as DLK-1), a marker of preadipocytes, was significantly high in both preadipocytes (Fig. 7). Similarly, Western blot analyses showed that the protein expression level of CaV1 in adipocytes was significantly decreased compared with 3T3-L1 preadipocytes ($P < 0.01$, $n = 4$; Fig. 5, Aa and Ab) and mouse preadipocytes ($P < 0.01$, $n =
4; Fig. 5, Ba and Bb). Immunohistochemical staining using anti-CaV3.1 showed the expression of α1G in both differentiated 3T3-L1 adipocytes (Fig. 4f) and mouse differentiated adipocytes (Fig. 4Bc), but compared with preadipocytes, weak staining or no staining was observed in both differentiated adipocytes.

T-type CaV blockers and siRNA for α1G mRNA inhibit cell proliferation in 3T3-L1 preadipocytes. Figure 8 shows the effects of various Ca2+ channel blockers on cell proliferation and cell cycle in 3T3-L1 preadipocytes. Mibefradil (10 μM; Fig. 8Aa) and NNC55-0396 (1–10 μM; Fig. 8Ab) significantly inhibited cell proliferation in response to FBS, while diltiazem and nicardipine (10 μM; Fig. 8Aa) had little effect. In addition, we investigated the effects of NNC55-0396 (Fig. 8B) and diltiazem (Fig. 8C) on cell cycle. Cells were treated in the absence or presence of these drugs for 24 h and analyzed by flow cytometry. Treatment with NNC55-0396 (10 μM; Fig. 8, B and D) showed a smaller proportion of cells entering S and G2/M and a higher proportion arrested in G0/G1, while diltiazem [10 μM (Fig. 8, C and D) and 30 μM (data not shown)] did not mimic the effects of NNC55-0396. The effects of siRNA targeted for α1G mRNA on cell proliferation and cell cycle in the response to serum were also investigated. As shown in Fig. 9A, siRNA targeted for α1G mRNA significantly inhibited cell proliferation in response to FBS compared with control siRNA. In addition, compared with nonsilencing (negative control) siRNA-treated cells (Fig. 9, Ba and Bb), cells treated with siRNA targeted for α1G mRNA showed a much smaller proportion of cells entering G2/M and a much higher proportion arrested in G0/G1 in the presence of FBS (Fig. 9, Ba and Bc). On the other hand, siRNA did not affect the cell cycle in the absence of FBS (data not shown). Thus it is likely that siRNA targeted for α1G mRNA inhibited cell cycle entry/progression, but not because of toxic effects.

**DISCUSSION**

The major findings of the present study are as follows. 1) T-type Ca2+ channel encoded by α1G subtype, blocked by mibefradil, Ni2+, NNC55-0396, and siRNA for α1G, was the dominant CaV in mouse 3T3-L1 preadipocytes. 2) RT-PCR, Western blot, and immunohistochemical analysis also showed the dominant expression of CaV3.1 T-type CaV in mouse primary cultured preadipocytes as well as 3T3-L1 preadipocytes. 3) The expression level of CaV3.1 encoded by α1G was significantly down-regulated in differentiated adipocytes by decreasing the transcription level. 4) Mibefradil, NNC55-0396, but not diltiazem, and siRNA for α1G inhibited cell proliferation in response to serum. NNC55-0396 and siRNA for α1G inhibited cell cycle entry/progression. These results demonstrate for the first time that the T-type Ca2+ channel encoded by α1G subtype is the dominant CaV in mouse preadipocytes and plays a role in the regulation of their proliferation.

CaV are ubiquitously expressed in various cell types and play vital roles in regulation of cellular functions such as proliferation. The existence of CaV has also been described in preadipocytes and adipocytes (4, 5, 10, 32), but the molecular identities and function of CaV remained quite unexplored. Recently, Diaz-Velasquez et al. (8) showed the presence of α1G-subtype T-type CaV in 3T3-F442A preadipocytes, a sub-clone of 3T3 Swiss mouse embryo fibroblasts (11, 12), by using RT-PCR analysis and immunohistochemical studies. The present study showed the presence of CaV in mouse 3T3-L1 preadipocytes by using patch-clamp experiments. Mibefradil, a T- and L-type Ca2+ channel blocker (24), inhibited it with an IC50 value of 3 μM, and NNC55-0396, a selective T-type Ca2+ channel blocker (21, 37), also inhibited it. Also, the other Ca2+ channel blockers, SNX-482, ω-agatoxin IVA, ω-conotoxin GVIA, and nicardipine, were less or only a little effective to inhibit CaV, compared with mibefradil or NNC55-0396. Ni2+ inhibited it with an IC50 value of 200 μM. In a heterologous expression system, the α1H subtype has been reported to exhibit a greater sensitivity to inhibition by NiCl2 (IC50 ~10 μM) than either the α1G or α1S subtype (IC50 ~200–300 μM) (23, 27). Thus, from the sensitivity to Ni2+ and the other Ca2+ channel blockers, it is likely that T-type Ca2+ channel encoded by α1G subtype was the dominant CaV in 3T3-L1 preadipocytes. These results were supported by RT-PCR analysis, immunohistochemical studies, and Western blot analysis. The dominant expression of α1G mRNA was detected by conventional/quantitative real-time RT-PCR among α-subunits of CaV transcripts (α1A–α1I), and the expression of CaV3.1 protein encoded by α1G gene was detected by immunohistochemical studies and Western blot analysis. In addition, siRNA targeted for α1G markedly inhibited CaV expressed in 3T3-L1 preadipocytes.
The present study also provides the first evidence showing the expression of Cav3 transcripts in mouse primary cultured preadipocytes as well as 3T3-L1 preadipocytes. The dominant expression of α1G mRNA was detected by conventional/quantitative real-time RT-PCR among Cav3 transcripts (α1A-α1H), and the expression of Cav3.1 protein encoded by α1G gene was detected by immunohistochemical studies and Western blot analysis in both mouse preadipocytes. In addition, the protein level of Cav3.1 encoded by α1G mRNA was significantly downregulated in differentiated adipocytes by decreasing the transcription level. Thus T-type Ca$$^{2+}$$ channel encoded by α1G subtype does not appear to function in mature white adipocytes, but alternatively it seems to play a vital role in the function of mouse preadipocytes. However, in contrast with the data of immunohistochemical studies and Western blot analysis, we have not been able to detect the obvious T-type Cav3 by patch-clamp experiments. Probably T-type Cav in mouse primary cultured preadipocytes may be very small for detection. Alternatively, it may be quickly decreased or run down under the culture conditions. Therefore, further studies using freshly isolated preadipocytes are needed to clarify the existence of T-type Cav3 in mouse preadipocytes.

Díaz-Velasquez et al. (8) reported that T-type Cav3 may be involved in adipocyte differentiation, where mibebradil and Ni$$^{2+}$$ partially inhibited staurosporine-induced preadipocyte conversion to adipocytes. They proposed that Ca$$^{2+}$$ mobilization by at least T-type Cav3 is involved in adipocyte differentiation, where GSK3β activation depends on transient increase in [Ca$$^{2+}$$], (13, 16). Proliferation of preadipocytes is also an important key step in adipose tissue development since it conditions the number of potential new adipocytes in the adipose tissue. Electrophysiological studies have shown the expression of K$$^{+}$$ channels in isolated cultured brown fat cells from neonatal rats and human preadipocytes, which may control membrane potential and then cellular proliferation and differentiation of preadipocytes. Hu et al. (20) reported that K$$^{+}$$ channel blockers inhibited human preadipocyte proliferation by accumulating cells at G0/G1 and reducing S-phase population. In the present study, we showed that mibebradil inhibited 3T3-L1 proliferation in response to serum, while diltiazem and NNC55-0396 or siRNA also appears to inhibit progression of S phase into G2/M phase. The reason for the toxic effects of siRNA cannot be ruled out in this study. Further studies are needed to clarify the involvement of T-type Cav3 in the cell cycle. However, the present study showed that T-type Cav3 encoded by α1G subtype appears to play a role in the regulation of cell cycle entry/progression, and subsequently cell proliferation, in mouse preadipocytes. T-type Ca$$^{2+}$$ channels may play a vital role in proliferation when activated by transient membrane depolarization or when they are open at resting membrane potentials as judged from window current, thereby promoting a steady-state calcium entry. Consistent with a role for Cav3.1 in controlling the cell cycle, similar effects on proliferation have been reported in cultured human pulmonary arterial cells and also in nonexcitable cells such as tumor cells (28, 34). Thus it is very likely that T-type Ca$$^{2+}$$ channels play a role in regulating [Ca$$^{2+}$$], and subsequently preadipocyte proliferation.

Recent in vivo studies have shown that mice lacking Cav3.1 T-type Cav3 or mice treated with TTA-A2, a selective T-type Cav3 blocker, are resistant to high-fat diet-induced weight gain and fat increase and exhibit improved body composition (39). These effects likely result from better alignment of diurnal feeding patterns with daily changes in circadian physiology, although the involvement of T-type Cav3 on adipose tissues has not been investigated. The present study showed that the T-type Ca$$^{2+}$$ channel plays a role in the regulation of cell proliferation in mouse preadipocytes. Thus T-type Ca$$^{2+}$$ channel blockers appear to be interesting new drugs for obesity.

In conclusion, the present study demonstrates that the T-type Ca$$^{2+}$$ channel encoded by α1G subtype is the dominant Cav3 in mouse preadipocytes and plays a role in the regulation of their proliferation, a key step in adipose tissue development.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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