Ribozyme Ablation Demonstrates That the Cardiac Subtype of the Voltage-sensitive Calcium Channel Is the Molecular Transducer of 1,25-Dihydroxyvitamin D₃-stimulated Calcium Influx in Osteoblastic Cells*

(Received for publication, July 12, 1999, and in revised form, November 24, 1999)

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1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) stimulates transmembrane influx of Ca²⁺ through L-type voltage-sensitive Ca²⁺ channels (VSCCs) in ROS 17/2.8 osteoblastic cells. Ca²⁺ influx modulates osteoblastic activities including matrix deposition, hormone responsiveness, and Ca²⁺-dependent signaling. 1,25(OH)₂D₃ also regulates transcript levels encoding VSCCs. L-type VSCCs are multisubunit complexes composed of a central pore-forming α₁ subunit and four additional subunits. The α₁ subunit is encoded by one gene in a multigene family, defining tissue-specific subtypes. Osteoblasts synthesize two splice variants of the α₁C cardiac VSCC subtype; however, the molecular identity of the 1,25(OH)₂D₃-regulated VSCC remained unknown. We created a ribozyme specifically cleaving α₁C mRNA. To increase target ablation efficiency, the ribozyme was inserted into U1 small nuclear RNA (snRNA) by engineering the U1 snRNA expression cassette, conferring the ribozyme transcript with stabilizing stem-loops at both sides and the Sm binding site that facilitates localization into nucleoplasm. After transfection of ROS 17/2.8 cells with U1 ribozyme-encoding vector, stable clonal cells were selected in which the expression of α₁C transcript and protein were strikingly reduced. Ca²⁺ influx assays in ribozyme transfectants showed selective attenuation of depolarization and 1,25(OH)₂D₃-regulated Ca²⁺ responses. We conclude that the cardiac subtype of the L-type VSCC is the transducer of stimulated Ca²⁺ influx in ROS 17/2.8 osteoblastic cells.

Various Ca²⁺ channels transduce signals that control the physiological activities of target cells. Voltage-sensitive calcium channels (VSCCs)¹ are classified as L, T, N, P/Q, and R type according to their voltage sensitivity, single-channel properties, ion selectivity, and pharmacological properties (1, 2). A high threshold for voltage-dependent activation and sensitivity to dihydropyridine type organic channel blockers characterize L-type VSCCs that consist of five distinct subunits (α₁, α₂, β, γ, and δ) encoded by four genes (3). The ion translocating function of the L-type channel is conferred by its pore forming α₁ subunit that contains four homologous repeats, each having six putative transmembrane segments (S1–S6) (1–4). At least four subtypes have been identified and ascribed to coding gene differences; α₁C, α₁D, α₁P, and α₁S correspond to cardiac muscle, neuroendocrine tissue, retina, and skeletal muscle, respectively. The diversity of the channel is further generated by alternative splicing into various isoforms, as is the case for the α₁C subunit (5).

L-type VSCCs have been identified in osteoblasts of various origins (6–9), where they are thought to play important roles in regulating osteoblast responses to external stimuli. In particular, L-type VSCCs transduce the rapid effects of 1,25(OH)₂D₃ and depolarization, leading to Ca²⁺ influx in various osteoblastic cells (6, 7, 10). In the longer term, 1,25(OH)₂D₃ treatment alters gene expression in osteoblastic cells, resulting in increased expression of matrix proteins including osteopontin and osteocalcin and reduced expression of the VSCC α₁C subunit (11, 12). However, the molecular identity of the VSCC involved in Ca²⁺ flux induced by 1,25(OH)₂D₃ and depolarization has never been demonstrated conclusively.

Recent developments in ribozyme technology provide powerful means to selectively inhibit expression of specific target genes. Ribozymes are a class of small catalytic RNA molecules that recognize specific substrate RNA molecules by their complementary nucleotide sequence, cleaving the substrate RNA as an endoribonuclease at enzymatic rates (13). Ribozyme expression in transfected target cells thus can inhibit the functional expression of a specific gene. Factors that contribute to ribozyme efficiency in transfected cells are expression levels, stability against rapid degradation, correct folding for exposure to the target, and co-localization and processing at sites where target transcripts are produced and accumulated. By all of these criteria, the U1 small nuclear RNA (snRNA) expression cassette provides an excellent vehicle for ribozyme delivery and expression (14–16). This cassette has been successfully used to introduce a ribozyme to inhibit fibrillin 1 expression in a human osteosarcoma cell line (16).

U1 snRNA is a highly expressed, stable small RNA (164 nucleotides) involved in both splicing and catalytic processing during pre-mRNA splicing (14). The U1 snRNA expression cassette has potent, constitutive upstream elements comparable in strength with the SV40 early promoter (15). At the 5'-end, there is a trimethylguanosine 5' cap. At both ends,
PCR products were subjected to electrophoresis in 1.2% (w/v) agarose cycles of 1 min at 95 °C, 1 min at 55 or 65 °C, and 2 min at 72 °C. The TECH, Palo Alto, CA).

a sequence, and orientation of the insert. A ligated plasmid, termed DNA and ligation junctions were sequenced to verify the identity, for amplification. At least 10 colonies were selected, and the cloned ligated product was transformed into JM109 competent cells (Promega)

734-base pair fragment corresponding to bases GGGTGGAAGAGTAGTC-3 and random hexamer priming (Advantage TM RT for PCR Kit, CLON-TECH, Palo Alto, CA).

EXPERIMENTAL PROCEDURES

Generation of Partial cDNA Encoding α1C mRNA Target—For selecting and testing ribozyme activity in vitro, a cDNA copy of partial α1C mRNA was ligated into pGEM®-3Z vector (Promega, Madison, WI). Total RNA was extracted from cultured ROS 17/2.8 cells with RNA STAT-60TM (Tel-Test, Inc., Friendswood, TX). The upstream primer CCC1 (5'-GCTAACCCCGGAAGGACG-3') is located in the untranslated region and the downstream primer CCC3 (5'-ATCGGCTGGTCCTTGGCTGGA-3') represents a region corresponding to those in ribozyme 6 were randomly selected. Oligonucleotides in vitro cleavage reaction mixture containing 2.5 µg of linearized plasmid or 1.5 µg of ribozyme DNA templates and 25 µM each ATP, CTP, GTP, and UTP. In addition, a prescribed amount of SP6 RNA polymerase and buffers was added as suggested by the manufacturer's instructions in a total volume of 100 µl. Following transcription, the DNA templates were removed from the reaction mixture with RQ1-RNAase-Free DNase. Unincorporated nucleotides were removed from the RNA transcript by size exclusion chromatography through a small prepacked MicroSpin G-50 column (Amersham Pharmacia Biotech). The target RNA and ribozyme 6 were mixed, and the in vitro cleavage reaction was performed in Tris (pH 8.0), 20 mM MgCl₂, at 75 °C for various times. Prior to the addition of MgCl₂, the mixture was heated at 95 °C for 2 min and then quickly chilled on ice. The cleavage reaction was terminated by the addition of a 3-fold (v/v) excess of Ambion (Austin, TX) loading buffer containing formaldehyde, formamide, and 6 µM EDTA. Cleavage products were resolved by electrophoresis in 1.9% (w/v) agarose/formaldehyde gels and then stained with ethidium bromide and visualized after destaining with RNAase-free water.

Construction of the Eukaryotic Expression Vector Encoding Ribozyme—The α1C ribozyme used for transfection was designed and modified from the ribozyme tested in vitro. The vector for expressing this ribozyme in transfected cells was constructed from the plasmid pZeoU1EcoSpe (16), pZeoU1EcoSpe contains the pZeoSV plasmid DNA modified by excising the SV40 promoter, SV40 polyadenylation site, and polylinker at the BamHI sites. In constructing the pZeoU1EcoSpe, a U1 snRNA expression cassette in pUC13 (20, 21) was excised with BamHI digestion and ligated into the BamHI sites of the modified pZeoSV. Two rounds of site-directed mutagenesis were then performed to change 4 nt flanking the Sm protein binding site of U1 snRNA, see below for restriction sites. The 5'-flanking region of the inserted U1 snRNA expression cassette possesses a promoter/enhancer comparable in strength to the SV40 early promoter (15). The transfection vector expressing ribozyme 6 was termed pU1RBα1C, and the vector expressing the control RNA was designated pU1control. In construction of pU1RBα1C and pU1control, the Sm binding sequence AATTTGGG (AAUUUGGU) in mRNA was in- vestigated in the sequence. These two constructs were linearized with SsII and pU1control differs from that of pU1RBα1C only in that the nucleotides corresponding to those in ribozyme 6 were randomly selected. Oligonucleotides were synthesized and chemically phosphorylated at the 5'-end (Integrated DNA Technologies, Inc., Coralville, IA). The single-stranded oligonucleotides were annealed and ligated into the EcoRI and SpeI sites of pZeoU1EcoSpe to create pU1RBα1C and pU1control. The sequence and orientation of the inserts as well as the U1 snRNA expression cassette were confirmed by DNA sequencing.

Cell Culture—ROS 17/2.8 rat osteosarcoma cells were cultured in Ham's F-12 medium/Dulbecco's modified Eagle's medium-high glucose (1:1) containing 10% fetal calf serum at 37 °C in a humidified incubator containing 5% CO₂ as described previously (11). Zeocin-resistant clones, into which an anti-α1C ribozyme construct or control plasmid had been transfected (see below) were maintained in the same medium supplemented with 50–150 µg/ml Zeocin (Invitrogen Inc., Carlsbad, CA). The cell lines were weaned from an initial selection concentration of 225 µg/ml (see below).

Transfection of ROS 17/2.8 Cells—pU1RBα1C and pU1control were linearized with ApaI and cleaned with Wizard DNA Cleanup Resin (Promega). The linearized plasmids were transfected into ROS 17/2.8 cells and a site at nt 6 was selected for this study. The target sequence (25 nt) of ribozyme 6 was compared with available sequences in GenBank™ and found to be unique to α1C.

Ribozyme Design—A hammerhead ribozyme targeting α1C mRNA was designed according to published procedures (13). The ribozyme contained a 24-nt hammerhead domain flanked by 10–12 nt complementary to the targeted region of the α1C mRNA. To construct the ribozymes in vitro, two strands of DNA template containing a SP6 RNA polymerase promoter sequence and ribozyme sequence were synthesized. The ribozyme was targeted to a GUC at nt 6 of α1C mRNA, relative to the translational site, and was thus named ribozyme 6.


Ribozyme Ablation of 1,25(OH)₂D₃-induced Ca²⁺ Influx

G
U
A
G
G-C
G-C
A-U
G-G
G-U
A-C
3'–AACCGGUAACCA UUACUUUUGGCG–5'

Ribozyyme 6

α₁C mRNA (ROS 17/2.8) 5′–UCUUUUUGGCGUCCCUCUCCACGACGACGUGGAGU GCAAAAGGAACCGGAGGAGGUGUACGUUCCAGAGGAA–3′
α₁B mRNA 5′–AGUUG. U.U.U. – A.GAU. U.U.G. – G.G. – 3′
α₁S mRNA 5′–CGAGG–3′

FIG. 1. Ribozyme 6 is designed to specifically cleave the α₁C subtype of the VSAC. Sequences encoding α₁C, α₁B, and α₁S subtypes of the L-type VSAC were obtained from GenBank™ (accession numbers M67516, M57682, and X05921, respectively). The rat osteoblastic α₁S sequence present in ROS 17/2.8 cells was deduced from a cloned cDNA produced using primers CCC1 and CCC3 (see “Experimental Procedures”) and found to match the published sequence for α₁C in the complementary region to ribozyme 6. The GUC site chosen for ribozyme targeting is located at nt 6 (relative to start codon AUG designated nt 1–3) and contains a sequence unique to the α₁C, VSAC subtype. Because this sequence is not present in either α₁B or α₁S subtypes, the ribozyme will not hybridize with or cleave these transcripts if present.

 wells containing 0.15 ml of trypsin EDTA (22). After 15 min, the cell clumps were dispersed by pipetting, and the trypsin reaction was terminated with 1 ml of medium containing 10% (v/v) serum and 225 μg/ml Zeocin. The medium was replaced with fresh medium after the cells had attached firmly. To ensure plasmid retention, the Zeocin concentration was maintained with 1 ml of medium containing 10% (v/v) serum and 225 μg/ml Zeocin. The medium was replaced with fresh medium after the cells had attached firmly. To ensure plasmid retention, the Zeocin concentration was maintained with 1 ml of medium containing 10% (v/v) serum and 225 μg/ml Zeocin. The medium was replaced with fresh medium after the cells had attached firmly.

RT-PCR Analysis of Ribozyme 6 and α₁C Expression—Total RNA extraction and reverse transcription with random hexamer were performed as described above. For detection of ribozyme expression, PCR was performed with primers PR₁C₆(5′–CATTCGAAGGACCCCTGG- GAGG–3′) and PRU₁(5′–GGAGGCGGCAAGCAGT–3′). For detection of control plasmid expression, a nucleotide sequence termed PRcntr (5′–GACCTGGACCGGAAGTGTTG-GAATA–3′) and PRB₁(5′–GACCGCTTAGATGATCCTTGCCA–3′) was used with PRU₁. The amplification conditions were as follows: 45 s at 95 °C, 45 s at 58 °C, and 45 s at 72 °C for 30–35 cycles. PCR products were electrophoresed in 1.4% agarose gels and visualized with ethidium bromide.

For detection of the mRNA encoding α₁C, PCR was performed with primers CCC1 and CCC3 as described above. PCR also was performed with a downstream primer pair near the 3′-end used previously in our laboratory (23) and termed PR₁C₆(5′–CGAAGGACCCCTGG-GAGG–3′) and PRU₁(5′–GGAGGCGGCAAGCAGT–3′). For detection of control plasmid expression, a nucleotide sequence termed PRcntr (5′–GACCTGGACCGGAAGTGTTG-GAATA–3′) and PRB₁(5′–GACCGCTTAGATGATCCTTGCCA–3′) was used with PRU₁. The amplification conditions were as follows: 45 s at 95 °C, 45 s at 58 °C, and 45 s at 72 °C for 30–35 cycles. PCR products were electrophoresed in 1.4% agarose gels and visualized with ethidium bromide.

Confocal, Immunofluorescent Analysis of α₁C Expression—Cells were grown overnight, rinsed with Hank’s balanced salt solution three times, and fixed with 2.5% paraformaldehyde in phosphate-buffered saline for 10 min. Fixed cells were blocked for 20 min with blocking solution (1% gelatin, 0.05% casein, and 0.2% donkey serum) and then rinsed three times with phosphate-buffered saline. Commercial rabbit anti-α₁C primary antibody that does not react with either α₁B or α₁S subtypes (Alomone Laboratories, Jerusalem, Israel) were applied at a dilution of 1:500 in blocking solution for 1 h. A second antibody solution containing donkey anti-rabbit IgG-Texas Red (Amersham Pharmacia Biotech) at a dilution of 1:10 in blocking solution was applied for 1 h. Prior to visualization, the cells were rinsed three times in buffer containing 0.1% (w/v) gelatin and 0.05% (w/v) casein for 10 min each. Finally, the cells were prepared with Fluoromount G (Fisher) and observed with a confocal microscope.

Calcium Uptake Assays—Cultured cells were assayed for Ca²⁺ uptake using previously published procedures (25). Cells were seeded at a density of 50,000 cells/ml onto 3.5-cm dishes and grown to approximately 50% confluency. Culture medium was aspirated, and the cells were washed with restering buffer (132 mM NaCl, 5 mM KCl, 1.3 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, and 25 mM Tris-HCl, pH 7.4). The first cells were incubated for 2 min with either 37 °C or room temperature restering buffer, resting buffer plus 1 μM 1,25(OH)₂D₃, or depolarizing buffer (5 mM NaCl, 132 mM KCl, 1.3 mM MgCl₂, 1.2 mM CaCl₂, 10 mM glucose, and 25 mM Tris-HCl, pH 7.4). 1.25(OH)₂D₃ from stock solution (1 μM) in absolute ethanol was added into the resting buffer, and the same amount of ethanol was added into all other test tubes. All uptake solutions contained 12.5 μCi/ml ⁴⁵Ca²⁺ (NEN Life Science Products). Uptake was terminated by aspiration of the labeling solution followed by three washes with ice-cold restering buffer. Cell-associated ⁴⁵Ca²⁺ was extracted by a 2-h incubation with 0.5 N NaOH and measured by liquid scintillation counting. Two-tailed t tests were performed to assess the significance of the difference between each stimulated group compared with its control in restering buffer.

RESULTS

Identification of Subtype-specific Ribozyme Cleavage Sites in the Osteoblastic α₁C Transcript—The first objective was to verify the sequence of α₁C transcript produced in ROS 17/2.8 cells for identification of ribozyme cleavage sites. The cDNA corresponding to the 5′ region of α₁C in ROS 17/2.8 cells that encompasses the initiating AUG codon was cloned and sequenced. This region was chosen for ribozyme targeting because the mRNA near the translational start site is more likely to be exposed on the outer surface of the three-dimensional structure of the folded transcript. Only a few nucleotide discrepancies upstream of the translational start site were found in a comparison of the α₁C mRNA from ROS 17/2.8 osteoblastic sarcoma cells with the α₁C mRNA sequence in GenBank™ (26); the coding sequences were identical. For selection of subtype-specific ribozyme cleavage sites in the α₁C mRNA in ROS 17/2.8 cells, the mRNA sequence of α₁C mRNA was compared with those of α₁B from rat brain mRNA (27) and α₁S from rabbit skeletal muscle mRNA (28) obtained from GenBank™. No corresponding rat sequence of α₁S was found in the GenBank™ data base. Both Kᵩᵣ and Kᵩᵣ/Kᵩᵣ for GUC and CUC cleavage sites are comparably higher than for other potential sites. Because α₁C mRNA is not an abundant transcript in ROS 17/2.8 cells, both Kᵩᵣ and Kᵩᵣ/Kᵩᵣ were considered (29). GUC at nt 6, relative to the translational site of α₁C, was selected as the best potential ribozyme target site. Fig. 1 shows the design and specificity of the ribozyme that was termed ribozyme 6. MFold program analysis of the α₁C transcript further showed that the ribozyme 6 cleavage site was likely to be in a region of the predicted folded structure accessible for efficient ribozyme cleavage (Fig. 2A).

In Vitro Testing of Ribozyme Cleavage of the Substrate α₁C mRNA—To assess the ability of ribozyme 6 to cleave the α₁C transcript, reactions were performed in vitro with cloned products. To create a synthetic α₁C mRNA substrate, a region of α₁C mRNA that encompasses the selected subtype-specific target sites was inserted into the pGEM3 vector. This vector contains a SP6 promoter directly upstream of the cloned α₁C cDNA that corresponds to nt −160 to 566 relative to the translational start site. Transcription of the sense RNA construct by SP6 RNA

2 K. Brubaker and R. Duncan, personal communication.
polymerase yielded a 783-nt RNA substrate that contains 726 nt from the \( \alpha_{1c} \) mRNA, plus 57 nt at the ends (46 plus 11nts) contributed by the pGEM3 vector. Ribozyme 6 was synthesized from double-stranded synthetic DNA oligonucleotide as described (30).

The synthetic \( \alpha_{1c} \) mRNA substrate and ribozyme were mixed as described in the legend to Fig. 2B. Cleavage was assessed after various incubation times in the cell-free system. As shown in Fig. 2B, cleavage of the \( \alpha_{1c} \) target by ribozyme 6 occurred efficiently within the first 1.5 h (lane 3). The 783-base substrate was cleaved by ribozyme 6 into limit 571-base and 212-base fragments that appeared to be fairly stable for at least 6 h. Two other ribozymes targeting \( \alpha_{1c} \) sites were also designed and cloned (data not shown). Ribozyme 6 was the most efficient, consistent with its target being located in the proximity of the start site.

Expression of Ribozyme 6 mRNA in Transfected Cells—Ribozyme 6 and control double-stranded DNA oligonucleotides were inserted separately into a modified U1 snRNA expression vector as described under “Experimental Procedures.” The predicted secondary structures of both transcripts of U1-ribozyme and U1-control chimeric RNA were compared with the parent U1 snRNA using the RNA Mfold program. As shown in Fig. 3, the U1-ribozyme 6 chimeric transcript was predicted to preserve the 5′ and 3′ stem-loop structures of U1 snRNA. The Sm binding site (AAUUUGUGG) of U1-ribozyme also is predicted to maintain its single-stranded character seen in U1 snRNA. While these models do not necessarily reflect the actual structure of the RNA molecules under study, they provide useful models for the design of active ribozymes.

The ribozyme-encoding and control plasmids were transfected into ROS17/2.8 cells using LipofectAMINE™ and cloned as described under “Experimental Procedures.” Multiple, stable, and Zeocin-resistant clones transfected with ribozyme 6 were screened for \( \alpha_{1c} \) mRNA expression by RT-PCR analysis. The clone that demonstrated the most efficient ablation of \( \alpha_{1c} \) mRNA as revealed by PCR was selected for further study.

For detecting the transcripts of U1-ribozyme and U1-control chimeric genes, specific primers were designed as described under “Experimental Procedures.” The RT-PCR analysis shown in Fig. 4 demonstrates that ribozyme 6 was expressed only in the selected clonal cells transfected with ribozyme 6-encoding plasmids, while control ribozyme transcripts were found only in clonal cells transfected with control plasmids. As expected, both ribozyme and control transcripts were absent in the parental ROS 17/2.8 cells. The CDNA bands migrate at a size corresponding to the expected 66 base pairs, demonstrating that the ribozyme and control RNA constructs were expressed in the respective clonal cells.

Inhibition of \( \alpha_{1c} \) Expression in ROS 17/2.8 Cells—Selected clonal cells were analyzed by RT-PCR and immunofluorescence microscopy for \( \alpha_{1c} \) transcript and protein expression. As shown in Fig. 5, the \( \alpha_{1c} \) mRNA levels were strikingly reduced in cell lines transfected with ribozyme-encoding plasmids, while the \( \alpha_{1c} \) mRNA levels in cell lines transfected with control plasmids were comparable with those found in the original ROS 17/2.8 cells. The primers (CC1C1 and CC3C3) employed in Fig. 5, A and B, amplified \( \alpha_{1c} \) transcripts that spanned the predicted ribozyme cleavage site shown in Fig. 1. It was considered that short versions of \( \alpha_{1c} \) transcripts containing 3′ sequences might be present in cells transfected with active ribozyme. To address this, a downstream primer pair (PR11 and PR12) that amplifies region IV of \( \alpha_{1c} \) transcripts corresponding to bases 3551–4276 relative to the translational start site of rat \( \alpha_{1c} \) was used in RT-PCRs. No products were detected, suggesting that 5′-targeted, ribozyme ablation of \( \alpha_{1c} \) transcript leads to complete degradation. The amplimer size of the PR11/PR12 product has been revised from earlier estimates (11) of 740 nt to a more precise determination of 726 nt, shown in Fig. 5C, lanes 1 and 3. Similar amounts of glyceraldehyde-3′-phosphate dehydrogenase were amplified in reactions from all three cell lines (Fig. 5C, lanes 1–3), indicating that \( \alpha_{1c} \) targets were specifically affected. In Fig. 5B, additional nonspecific DNA bands were observed when the annealing temperature was decreased from 65 to 55 °C during the PCR process, but \( \alpha_{1c} \) transcripts remained undetectable. The inability to detect \( \alpha_{1c} \) transcript in ribozyme 6-transfected cells, even under lower stringency PCRs, points to the efficiency of the pU1RB\( \alpha_{1c} \)6 construct.

The selected clonal cells were further characterized by im-
munofluorescence using specific antibodies to α1C protein and confocal microscopy. The antibodies are specific to the α1C subtype of the VSCC as determined by the manufacturer. As shown in Fig. 6, A–C, the number of detectable immunoreactive cell surface α1C VSCCs was substantially reduced in clonal cells harboring ribozyme-coding pU1RBα1C6 (Fig. 6B). In contrast, clonal cells transfected with pU1control (Fig. 6C) showed a pattern and density of α1C protein expression similar to that shown in Fig. 6A. As determined by the manufacturer, the antibodies are specific to the α1C subtype of the VSCC.

**Fig. 3.** Sequence and predicted secondary structure of chimeric ribozyme transcript of pU1RBα1C6. Ribozyme 6 DNA was inserted into a modified U1 snRNA expression vector to produce a modified anti-α1C ribozyme-producing vector termed pU1RBα1C6. The hammerhead ribozyme is located at the center of the transcript flanked by stem structures at both ends. The 5' cap structure was methylated by 1 or 3 methyl (m) groups. A Sm binding site sequence (AAUUUGUGG) was included. Primers PRα1C6 (5'-CATTCTGATGACTCTCGTGAGG-3') (sense) and PRU1 (5'-GGAAAGCGCGACACGCACTG-3') (antisense) were designed to amplify the ribozyme transcripts. The number at α1C mRNA indicates the position relative to the translational start site. An arrow indicates the cleavage site.

**Fig. 4.** Ribozyme 6 and control chimeric RNA are expressed in transfected ROS 17/2.8 cells as detected by RT-PCR. Total RNA was extracted from ROS 17/2.8 parental cells (lanes 1 and 1'1) and subclones were transfected with ribozyme encoding pU1RBα1C6 (lanes 2 and 2') or with pU1control (lanes 3 and 3'). Preparation of the ribozyme and control constructs and transfection and subcloning were as described under “Experimental Procedures.” Reverse transcription reactions were performed with random hexamer primers. PCR was performed using ribozyme-specific primers (lanes 1, 2, and 3) as shown in Fig. 3. The control chimeric RNA was detected using control chimeric RNA-specific 5' primer (lanes 1', 2', and 3') and the same 3' primer as that used to detect the ribozyme.

**Fig. 5.** Ribozyme 6 inhibits the expression of α1C-encoding mRNA as detected by RT-PCR. Total RNA was extracted from non-transfected ROS 17/2.8 cells (lanes 1 and 1') or subclones transfected with ribozyme encoding pU1RBα1C6 (lanes 2 and 2') or with control pU1control (lanes 3 and 3'). Reverse transcriptase reactions were performed with random hexamer primers. PCR reactions shown in lanes 1, 2, and 3 were performed using primers CCC1 and CCC3 (see “Experimental Procedures”) located near the 5' end or subtype-nonspecific primers PR11 and PR12 (23) located closer to the 3' end of the α1C transcript. The annealing temperature of the PCR shown in A was 10 °C higher than that used in the reaction shown in B and C. Only α1C subtype products were detected in all cases. The PCRs shown in lanes 1', 2', and 3' were performed using glyceraldehyde-3'-phosphate dehydrogenase (G3PDH)-specific primers and provided a loading assessment.
found in the parental ROS 17/2.8 cells (Fig. 6A). This finding demonstrated that ribozyme 6 successfully inhibited cell surface expression of \( \alpha_{1C} \) protein as well as transcript in transfected cells.

**ROS 17/2.8 Cells Ablated of \( \alpha_{1C} \) Transcript Do Not Respond to 1,25(OH)\(_2\)D\(_3\) and Depolarization—**

\( \text{\textsuperscript{45}}\text{Ca}^2\) influx assays were performed using subconfluent cell monolayers as described under “Experimental Procedures.” As shown in Fig. 7, cells in which \( \alpha_{1C} \) of VSCC was ablated by active ribozyme 6 lost the ability to respond to either depolarization or 1,25(OH)\(_2\)D\(_3\). In contrast, cells transfected with control plasmids continued to express \( \alpha_{1C} \) and maintained their sensitivity to 1,25(OH)\(_2\)D\(_3\) and high K\(^+\)-stimulated depolarization reflected by a statistically significant 2–3-fold increase in \( \text{\textsuperscript{45}}\text{Ca}^2\) influx. As seen previously (6), slightly higher levels of stimulated \( \text{\textsuperscript{45}}\text{Ca}^2\) influx were seen when cells were depolarized. Overall, treatment with 1,25(OH)\(_2\)D\(_3\) increased \( \text{\textsuperscript{45}}\text{Ca}^2\) influx 2.2-fold, compared with 2.8-fold after depolarization. Together, these results revealed that the \( \alpha_{1C} \) subtype of VSCC is responsible for 1,25(OH)\(_2\)D\(_3\)-stimulated influx in ROS 17/2.8 cells.

**DISCUSSION**

\( \text{Ca}^{2+} \) influx induced by various stimuli, including depolarization and treatment with calcitropic hormones such as 1,25(OH)\(_2\)D\(_3\), plays an important role in signaling processes related to bone growth, differentiation, and remodeling. For example, recent work demonstrated that 1,25(OH)\(_2\)D\(_3\)-dependent \( \text{Ca}^{2+} \) influx modulates the post-translational state of the extracellular bone matrix phosphoprotein, osteopontin (31).
Ribozyme Ablation of 1,25(OH)₂D₃-induced Ca²⁺ Influx

The VSCC represents a major pathway for Ca²⁺ entry into bone cells (6, 32). Several laboratories, including our own, have demonstrated the presence and activation of VSCCs in osteoblasts treated with 1,25(OH)₂D₃ (6), parathyroid hormone (8), and epidermal growth factor (32) and during depolarization (6). The development of 1,25(OH)₂D₃ analogs with differential actions on nuclear receptor-mediated or membrane-initiated pathways provided a powerful approach to dissect the dual effects of 1,25(OH)₂D₃, demonstrating conclusively that the rapid activation of cell surface VSCCs is independent of the classical nuclear receptor (12, 33). Vitamin D₃ analogs that bind to the nuclear receptor but fail to stimulate Ca²⁺ influx have clinical utility because they stimulate bone formation with a lesser tendency to produce hypercalcemia compared with 1,25(OH)₂D₃ (34). This implies that activation of VSCCs may be of pathophysiological importance in the development of hypercalcemia.

Early studies established that depolarization and hormone-stimulated Ca²⁺ influx into osteoblasts could be inhibited by dihydropyridines (6), indicating that the VSCCs involved in this influx are of the high threshold, or L-type. Since the first cDNA encoding the α₁₃ skeletal muscle Ca²⁺ channel subunit was cloned (28), two major additional subtypes of L-type VSCCs were discovered: the cardiac muscle subtype, α₁₁C, and the neuroendocrine subtype, α₁D. Recently, a new L-type subtype (α₁D) from retina also was reported (35). Major subtypes of VSCCs are frequently found outside the excitatory tissue where they were first discovered; for example, the expression of the α₁₁C subtype was detected in rat brain as well as cardiac tissue (26).

Since the initial discovery that Ca²⁺ influx by 1,25(OH)₂D₃ was transduced by L-type VSCCs in ROS 17/2.8 osteoblastic cells (6), many efforts have been made to discern the exact molecular nature of the Ca²⁺ channel(s) present in osteoblasts. L-type VSCCs are the best studied Ca²⁺ channel type and appear to be the major VSCC in osteoblasts, although T-type channels may be expressed transiently during development (36, 37). Three types of cDNA sequences corresponding to α₁₁C, α₁₁C, and α₁₁C were detected by Barry et al. (8) in the rat UMR-106 osteosarcoma cell line. Two alternate splice isoforms of the α₁₁C subtype were identified in rat ROS 17/2.8 osteoblastic sarcoma cells and primary osteoblasts in our laboratory, but the other two subtypes were undetected (11). The molecular identification of the cardiac subtype was confirmed recently in primary cultures of rat calvarial osteoblasts, where it was proposed that the α₁₁C is involved in development of epidermal growth factor responsiveness of these cells (32). This finding was consistent with our previous report (11) that the α₁₁C subtype was present in primary cultures of cells grown from neonatal rat calvaria. Taken together, these studies indicate that the α₁₁C subtype of the VSCC is the major subtype expressed both in primary cultures and cell lines of osteoblastic phenotype. Nonetheless, a collective limitation of all prior studies is that they rely upon circumstantial evidence to predict relationships between structure and function. No evidence has been presented to definitively link the presence of α₁₁C VSCC subtype to a demonstrated function.

The approach taken in this study involved ribozyme-mediated ablation of the α₁₁C transcript in transfected osteoblastic cells, followed by functional assays using stable transfectants. For the study to be definitive, it was essential that α₁₁C transcript levels be reduced to negligible levels. Antisense approaches have been used previously in this system (38), but we believed more efficient ablation would be achieved using a ribozyme approach with catalytic efficiency. Considering that targeting the initiating AUG codon or adjacent sequences may increase the antisense effect of ribozymes (39, 40), ribozyme-6 was chosen for transfection and expression in ROS 17/2.8 cells. Because the alternative splice that occurs in the osteoblast α₁₁C mRNA is downstream in the coding region at transmembrane segments IVS3–IVS4 (11), ribozyme-6 was expected to cleave transcripts encoding both of these isoforms. We also engineered a ribozyme construct in which ribozyme 6 was inserted into a U1 snRNA expression cassette (16). This cassette offered the advantage of producing a ribozyme that should be highly expressed, resistant to exonucleases, and capable of concentrating in the nucleoplasms, where newly transcribed α₁₁C transcripts should be most abundant.

In this study, we prepared stable sublines of ROS 17/2.8 cells in which α₁₁C expression is highly inhibited by expression of active ribozyme-6. Control cell lines expressing inactive ribozyme constructs also were prepared. The selected clonal cells showing most efficient ribozyme-mediated ablation of α₁₁C transcripts, as well as clonal control transfectants, were employed to investigate the relationship between α₁₁C and Ca²⁺ influx modulated by 1,25(OH)₂D₃ and depolarization. ⁴⁵Ca²⁺ uptake assays were performed in subconfluent cell monolayers of sublines expressing active and inactive ribozymes, as well as in the parental line. As presented under “Results,” cells expressing active ribozyme-6 lost their rapid Ca²⁺ response to both 1,25(OH)₂D₃ and depolarizing high K⁺ buffer. In contrast, cells transfected with control plasmids maintained their sensitivity to 1,25(OH)₂D₃ and high K⁺ buffer, demonstrated by rapid ⁴⁵Ca²⁺ influx at levels comparable with nontransfected cells. Taken together, these data establish that the α₁₁C subtype of the L-type channel is the depolarization and 1,25(OH)₂D₃-responsive VSCC in osteoblasts.

The results presented here integrate and extend previous findings regarding the nature of the osteoblast VSCC. For the first time, we definitively demonstrated that the rapid membrane-initiated effects of 1,25(OH)₂D₃ on Ca²⁺ influx in osteoblasts are transduced by the α₁₁C subtype of the VSCC. By defining the cardiac subtype to be the molecular form of VSCCs responsive to 1,25(OH)₂D₃ and depolarization, we are now in a position to further explore the physiological importance of the VSCC in osteoblast function and to begin to define the signaling pathways activated directly by the VSCC.

Acknowledgments—We thank Drs. William T. Butler, Zhou (Georgia) Chen, and Dan Carlson for many wonderful ideas and enthusiastic support of this project. We are grateful to Dr. Harry C. Dietz at The Johns Hopkins University for providing the plasmid used to create the ribozyme vector and Dr. Kirk Czymek for assisting with the confocal microscopy. We thank all of the members of the Farach-Carson laboratory and Dr. Gary Meszaros for many useful discussions. We especially thank Dr. Kamil Akbari for assistance with sequence analysis. Finally, we are grateful to Sharron Kingston and Margie Barrett for assistance in the preparation of the manuscript.

REFERENCES

1. Catterall, W. A. (1995) Annu. Rev. Biochem. 64, 493–531
2. Tsien, R. W., and Tsien, R. Y. (1990) Annu. Rev. Cell Biol. 6, 715–760
3. Perez-Reyes, E., and Schneider, T. (1995) Kidney Int. 48, 1111–1124
4. Singer, D., Biel, M., Lotan, I., Flockerzi, V., Hofmann, P., and Dascal, N. (1991) Science 253, 1553–1557
5. Perez-Reyes, E., Wei, X. Y., Castellano, A., and Birnbaumer, L. (1990) J. Biol. Chem. 265, 30430–30436
6. Caffrey, J. M., and Farach-Carson, M. C. (1989) J. Biol. Chem. 264, 20265–20274
7. Lieberher, M. (1987) J. Biol. Chem. 262, 13168–13173
8. Barry, E. L. R., Gese, F. A., Proehner, S. C., and Friedman, P. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10914–10918
9. Duncan, R. L., Akbari, K. A., and Farach-Carson, M. C. (1998) Semin. Nephrol. 18, 178–190
10. Yukihiro S., Posner, G. H., Guggino, S. E. (1994) J. Biol. Chem. 269, 23889–23893
11. Meszaros, J. G., Karin, N. J., Akanbi, K., and Farach-Carson, M. C. (1996) J. Biol. Chem. 271, 32881–32885
12. Norman, A. W., Nemere, I., Zhou, L-X., Bishop, J. E., Lowe, K. E., Mayer, A. C., Collina, E. D., Tsaoa, T., Sergeant, I., and Farach-Carson, M. C. (1992) J. Steroid Biochem. Mol. Biol. 41, 231–240
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13. Haseloff, J., and Gerlach, W. L. (1988) Nature 334, 585–591
14. Green, M. R. (1991) Annu. Rev. Cell Biol. 7, 559–599
15. Skuzeski, J. M., Lund, E., Murphy, J. T., Steinberg, T. H., Burgess, R. R., and Dahlberg, J. E. (1984) J. Biol. Chem 259, 8345–8352
16. Montgomery, R. A., and Dietz, H. C. (1997) Hum. Mol. Genet. 6, 519–525
17. Ruffner, D. E., Stormo, G. D., and Uhlenbeck, O. C. (1990) Biochemistry 29, 10695–10702
18. Koizumi, M., Hayase, Y., Iwai, S., Kaniya, H., Inoue, H., and Ohtsuka, E. (1989) Nucleic Acids Res. 17, 7059–7071
19. Christoffersen, R. E., McSwiggen, J., and Konings, D. (1994) J. Mol. Struct. 311, 273–284
20. Manser, T., and Gesteland, R. F. (1981) J. Mol. Biol. Genet. 1, 117–125
21. Zhuang, Y., and Weiner, A. M. (1986) Cell 46, 827–835
22. Karin, N. J. (1999) BioTechniques 27, 681–682
23. Meszaros, J. G., Karin, N. J., and Farach-Carson, M. C. (1996) Connect. Tissue Res. 35, 107–111
24. French, M. M., Smith, S. E., Akanbi, K., Sanford, T., Hecht, J., Farach-Carson, M. C., and Carson, D. D. (1999) J. Cell Biol. 145, 1103–1115
25. Meszaros, J. G., and Farach-Carson, M. C. (1997) Methods Enzymol. 282, 236–243
26. Snutch, T. P., Tomlinson, W. J., Leonard, J. P., and Gilbert, M. M. (1991) Neuron 7, 45–57
27. Hui, A., Ellinor, P. T., Krizanova, O., Wang, J.-J., Diebold, R. J., and Schwartz, A. (1991) Neuron 7, 35–44
28. Tanabe, T., Takeshima, H., Mikami, A., Floketeri, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hiruse, T., and Numa, S. (1987) Nature 332, 313–318
29. Birikh, K. R., Heaton, P. A., and Eckstein, F. (1997) Eur. J. Biochem. 245, 1–16
30. Gaur, R. K., and Krupp, G. (1997) Methods Mol. Biol. 74, 69–78
31. Safran, J. B., Butler, W. T., and Farach-Carson, M. C. (1998) J. Biol. Chem. 273, 29955–29961
32. Loza, J. C., Carpio, L. C., Bradford, P. G., and Dziak, R. (1999) J. Bone Miner. Res. 14, 386–396
33. Khoury, R., Bidall, A. L., Norman, A. W., and Farach-Carson, M. C. (1994) Endocrinology 135, 2446–2453
34. Farach-Carson, M. C., and Devoil, R. E. (1995) News Physiol. Sci. 10, 198–204
35. Strom, T. M., Nypakatura, G., Apfelstedt-Sylla, E., Hellebrand, H., Lorenz, B., Weber, B. H., Wutz, K., Gutwillinger, N., Rutherford, K., Drescher, B., Sauer, C., Zrenner, E., Meitinger, T., Rosenthal, A., and Metzdorf, A. (1998) Nat. Genet. 19, 260–263
36. Chesnoy-Marchais, D., and Fritsch, J. (1988) J. Physiol. 398, 291–311
37. Loza, J., Stephane, E., Dolce, C., Dziak, R., and Simasko, S. (1994) Calcif. Tissue Int. 55, 128–133
38. Duncan, R. L., Kirner, N., Barry, E. L. R., Friedman, P. A., and Hruska, K. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1864–1869
39. Coleman, J., Green, P. J., and Inouye, M. (1984) Cell 37, 429–436
40. Hirashima, A., Sawai, S., Inokuchi, Y., and Inouye, M. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 7728–7730
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J. Biol. Chem. 2000, 275:8711-8718.
doi: 10.1074/jbc.275.12.8711

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