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TRPC3-Like Protein Is Involved in the Capacitative Cation Entry Induced by 1α,25-Dihydroxy-Vitamin D_3 in ROS 17/2.8 Osteoblastic Cells

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Abstract In ROS 17/2.8 rat osteoblastic-like cells a capacitative Ca^{2+} entry (CCE) pathway operates which is activated by either 1α,25-dihydroxy-vitamin D_3 (1α,25$(OH)_2D_3$) or thapsigargin (Tpg)-induced depletion of Ca^{2+} stores (Baldi et al. [2002]: J. Cell. Biochem. 86:678–687). In view of recent evidence favoring a role for transient receptor potential (TRP) proteins in mediating CCE, we investigated if channels involved in the 1α,25$(OH)_2D_3$-sensitive CCE in rat osteoblasts were related to an endogenous TRP-canonical (TRPC) isoform homologue. By reverse transcription (RT)-PCR using mRNA from ROS 17/2.8 cells and primers based on conserved regions within the mammalian TRPC3/6/7 subfamily, two fragments were amplified of 390 and 201 bp with 100 and 94% sequence identity, respectively, with human TRPC3. Northern blot analysis showed the presence of a 3.5 kb transcript and both immunobloting and immunocytochemistry using a specific anti-TRPC3 antibody confirmed endogenous expression of a TRPC3-like protein (~110 kDa) with membrane localization. In ROS 17/2.8 cells intranuclearly microinjected with anti-TRPC3 antisense oligodeoxynucleotides (ODN), both the initial rate and magnitude of CCE activated by either 1α,25$(OH)_2D_3$ or Tpg were markedly reduced, whereas no changes were detected in control-injected cells. The present findings constitute the first evidence to date suggesting that an endogenous TRPC3-like protein is functionally involved in the CCE route activated by 1α,25$(OH)_2D_3$ in a secosteroid target cell. We anticipate TRPC3 as a candidate for mediating store-operated non-selective cation entry into osteoblasts. J. Cell. Biochem. 90: 197–205, 2003.

Key words: vitamin D_3; osteoblasts; capacitative calcium entry; TRP proteins

Changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in osteoblasts play a crucial role in the ability of these cells to respond to osteotropic hormones [Said Ahmed et al., 2000]. As in other cell types [Putney and Bird, 1993; Clapham, 1995], receptor-activated Ca^{2+} signaling in osteoblasts involves a rapid and transient Ca^{2+} release from IP_3-sensitive stores and sustained Ca^{2+} entry from the outside through Ca^{2+} channels located in the plasma membrane, the latter apparently acting in concert with microenvironmental bone stimuli to regulate diverse osteoblast functions [Van Leeuwen et al., 1990; Duncan et al., 1998].

It is well documented that the osteoblastic cells, either primary cultures or established cell lines, respond to hormonally active form of vitamin D_3, 1α,25-dihydroxy-vitamin D_3 (1α,25$(OH)_2D_3$), with a fast and sustained increment in $[Ca^{2+}]_i$ which depends on both IP_3-mediated mobilization of Ca^{2+} from endoplasmic reticulum, and cation influx from the outside mainly through voltage-dependent Ca^{2+} channels (VDCCs) from the L-type as part of the non-genomic mechanism of action of the hormone in these cells [Lieberherr, 1987;

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Caffrey and Farach-Carson, 1989; Civitelli et al., 1990]. In a more general view, VDCCs are believed to account for most of the Ca^{2+} entry phase observed upon stimulation of osteoblasts with hormones coupled to activation of the PLCβ/IP_3 pathway [Duncan et al., 1998; Farach-Carson and Ridall, 1998]. However, we recently reported the existence of a store-operated capacitative Ca^{2+} entry (CCE) route in the rat osteosarcoma-derived osteoblastic-like cell line ROS 17/2.8, which is non-genomically activated by 1α,25$(OH)_2D_3$ [Baldi et al., 2002], suggesting that the VDCCs may not represent the sole Ca^{2+} entry route in osteoblasts, and pointing out the putative relevance of CCE as an alternative via through which osteotropic agents may influence osteoblast Ca^{2+} homeostasis.

In non-excitable cells the capacitative or store-operated Ca^{2+} entry pathway is activated as the result of a decrease and/or depletion of Ca^{2+} content of the endoplasmic reticulum [Putney, 1997]. Neither the precise mechanism underlying activation of this Ca^{2+} entry pathway nor the molecular identity of the channels involved has been yet elucidated. Studies on the molecular nature of CCE channels deal mainly with mammalian homologues of the *Drosophila* transient receptor potential (TRP) channel, designated as the TRP-canonical (TRPC) subfamily of the larger TRP superfamily [Zhu and Birnbaumer, 1998; Vennekens et al., 2002; Montell et al., 2002a,b]. Some of these proteins form non-selective cation channels [see Vazquez et al., 2001 and references therein] when expressed in cell lines and thus could be candidates for this mode of store-operated Ca^{2+} entry.

In continuing our earlier studies on 1α,25$(OH)_2D_3$-activated CCE in osteoblastic cells [Baldi et al., 2002], we here present evidence suggesting that a TRPC3 homologue is involved in mediating the steroid-induced CCE in ROS 17/2.8 rat osteoblasts.

MATERIALS AND METHODS

Materials

Molecular biology reagents were from GIBCO BRL-Life Technologies (Rockville, MD) and Promega Corp. (Madison, WI). Primers for PCR and antisense oligodeoxynucleotides (ODN) were synthesized by DNAgency (Malvern, PA). Fura-2/AM, nifedipine, verapamil, Ham F-12 medium, $CaCl_2$, $MnCl_2$, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was from Nutrientes Celulares (Buenos Aires, Argentina). [α-^{32}P]dCTP was from NEN Life Science Products, Inc., (Boston, MA); protein size markers were from New England Biolabs (Beverly, MA). Microspin G-50 columns, horse-radish peroxidase (HRP)-coupled anti-rabbit polyclonal antibody, nitrocellulose (Hybond ECL) and nylon (Hybond-N^+) membranes were from Amersham Life Sciences (Piscataway, NJ). All other reagents used were of analytical grade.

RNA Isolation and RT-PCR

Total RNA was extracted from ROS 17/2.8 cells as described elsewhere [Chomzcynski and Sacchi, 1987] and mRNA obtained using PolyATract system (Promega Corp.). RT-PCR was performed following manufacturer's instructions (Promega Corp.). The following primers were used: upstream F1 (1178) 5′-GAGAA(A/G)GAGTTCAAGAATGACTA-3′ (1200), downstream R1 (1593) 5′-GC(A/G)TGTGCTACAAACTTCAT-3′ (1574); upstream F2 (2345) 5′-AA(A/G)TTCAT(A/T)GA(A/G)AA(C/T)AT(C/T)GG-3′ (2364), and downstream R2 (2566) 5′-TTTTGGACTAGGAACTAGAC-3′ (2547). Numbers in parenthesis refer to nucleotide positions of cDNA clone NM_003305 from human TRPC3. PCR conditions were: an initial cycle of 94°C for 3 min, then 30 cycles of 94°C for 60 s, 53°C for 60 s, 70°C for 90 s, followed by 70° C for 5 min. Amplified fragments were purified by electroelution from agarose gels and sequenced by the University of Chicago Cancer Research Center DNA Sequence Facility (Chicago, IL). Sequences were used to search the National Center for Biotechnology Information (NCBI, Bethesda, MD) using the Advanced BLAST program [Altschul et al., 1997].

Northern Blot Analysis

A hybridization probe was synthesized (Prime A Gene, Promega Corp.) using the 201 bp PCR fragment obtained from ROS 17/2.8 cells as template and [α-^{32}P]dCTP, and purified with a Microspin G-50 column. mRNA was fractionated by electrophoresis on formaldehyde agarose gels, transferred onto nylon membranes and UV-cross-linked. Membranes were 3 h prehybridized in 0.25 M Na_2HPO_4 pH 7.2, 1% BSA, 1.27 mM EDTA, 7% SDS, then hybridized overnight in the same buffer with labeled probe,

washed, and exposed to Kodak X-Omat films (7 days at −80°C).

Cell Culture and Microinjection of ODN

ROS 17/2.8 rat osteoblastic-like cells were cultured as described [Baldi et al., 2002]. For microinjection, cells were plated (5.7×10^3 cells/cm^2) onto glass coverslips imprinted with squares for localization of injected cells. After 48 h, microinjection was performed as indicated below. ODNs used for intranuclear microinjection were partially protected from nuclease action with phosphorothioate linkages (5′, 3′ ends). Injection (100 hPA, 0.4 s) was performed with a manual injection system (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). Ten to 30 fl of indicated ODN in sterile deionized water were injected with microcapillaries (Femtotips, outlet diameter of 0.5 μm; Eppendorf-Netheler-Hinz GmbH). After injection, cells were cultured for an additional 48 h before fluorimetric measurements. The following ODN antisense sequences (AS) were used: AS_1, 5′-CATGCGTCTCAGGGATGGGCTTCC-CTCCAT-3′ (against the region surrounding the translation starting codon for human TRPC3 mRNA); AS_2, 5′-TTAATCATAGCAAT-TAGCATGTTGAG-3′ (against the 201 bp PCR fragment); AS_3, 5′-GTTGCTGCATCATTCA-CATCT CAGCATGCT-3′ (against the region containing the stop codon for human TRPC3 mRNA); as a control, a scrambled (Scra) ODN was designed: 5′-ATCCTTAGAGTCGCG-TACTT-3′. Final ODN concentration in the injection solution was 100 μM, for either Scra or AS pool; for the latter, equimolar concentrations of AS_1, AS_2, and AS_3 were mixed to give a final concentration of 100 μM.

Immunoblotting

Lysates were prepared from ROS 17/2.8 cells and rat brain in buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 2 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml aprotinin, and cleared by centrifugation (3,000 rpm, 4°C); samples were treated with 2× Laemmli sample buffer [Laemmli, 1970] and resolved (36 μg/lane) by SDS–polyacrylamide gel electrophoresis (7.5% acrylamide). Proteins were electrotransferred onto nitrocellulose membranes. After blocking with 5% non-fat dried milk, in TBST buffer (50-mM Tris pH 7.4, 200 mM NaCl, 0.05% Tween-20), the membranes were incubated 1 h with rabbit anti-TRPC3 (3 μg/ml; Alomone Labs, Jerusalem, Israel) antibody in TBST containing 3% BSA. In parallel experiments, anti-TRPC3 antibody was preincubated 1 h with the antigenic peptide used for anti-TRPC3 generation following manufacturer's instructions. Membranes were finally incubated with HRP-coupled goat anti-rabbit polyclonal antibody to 1:1,000, developed by enhanced chemiluminescence, and exposed to Kodak X-OMAT films for detection of immunoreactive proteins.

Immunocytochemistry

ROS 17/2.8 cells grown onto glass coverslips were fixed in 100% ethanol (30 min, 4°C) and non-specific sites blocked with normal goat serum. Samples were then incubated with rabbit polyclonal anti-TRPC3 antibody (1:50, 1 h, room temperature). In parallel experiments, anti-TRPC3 antibody was pre-incubated 1 h with antigenic peptide before being added onto fixed cells. All samples were incubated (1 h, room temperature) with secondary Oregon Green-conjugated goat anti-rabbit antibody (1:500; Molecular Probes, Eugene, OR) and observed under an Axioskop 40 microscope (UV filter, 100X/1.3 oil immersion Plan-neofluor objective). Images were captured with an Axio Cam high resolution color camera and analyzed with AxioVision 3.1 software (Zeiss, Buenos Aires, Argentina).

Intracellular Calcium Measurements

Intracellular Ca^{2+} changes in osteoblastic cells were monitored by using the Ca^{2+}-sensitive fluorescent dye Fura-2 as described [Baldi et al., 2002]. Mn^{2+} influx was assayed as the quenching of Fura-2 fluorescence by the cation entering the cell measured at 360 nm (the Ca^{2+}-independent, isoemissive wavelength for Fura-2). Briefly, Fura-2 loaded cells were incubated in nominally Ca^{2+}-free medium and then exposed to either $1\alpha,25(OH)_2D_3$, thapsigargin (Tpg), or vehicle; variations in $[Ca^{2+}]_i$ were monitored from the change in fluorescence when exciting at 380 nm. When $[Ca^{2+}]_i$ stabilized, wavelength excitation was changed to 360 nm, and Mn^{2+} influx was initiated (zero time for cation influx) by addition of 100 μM $MnCl_2$ to the medium. Nifedipine (2 μM) and verapamil (5 μM) were included in all incubating media to inhibit Ca^{2+} influx through VDCCs; at these concentrations, both inhibitors are highly specific for VDCCs

and do not interfere with Ca^{2+} fluxes through the CCE pathway, providing a pharmacological way to functionally isolate CCE from VDCCs when these channels coexist [Vazquez et al., 1998; Broad et al., 1999; Baldi et al., 2002].

RESULTS

In ROS 17/2.8 rat osteoblastic cells, CCE is activated upon challenging the cells with the secosteroid hormone $1\alpha,25(OH)_2D_3$ or by depletion of Ca^{2+} stores with the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor Tpg [Baldi et al., 2002]. In these cells, CCE is a nonselective cation entry route which allows passage of either Mn^{2+}, Sr^{2+}, or Ca^{2+}.

Recent evidence indicates that the members of the TRPC3/6/7 family, particularly TRPC3, cloned from rat tissues may be involved in mediating CCE [Harteneck et al., 2000; Montell, 2001; Vennekens et al., 2002]. We used here a combination of molecular and functional studies to obtain some insight regarding the putative identity of the channels involved in the $1\alpha,25(OH)_2D_3$-sensitive CCE in rat osteoblasts, specifically, if this could be related to expression of a TRPC homologue.

We first utilized RT-PCR to see if mammalian TRPC transcripts were expressed in ROS 17/2.8 cells. Using mRNA from cultured ROS 17/2.8 cells as template and primers based on two short peptides highly conserved among the TRPC3/6/7 subfamily in mammals, we amplified two fragments of 390 and 201 bp (GeneBank accesion numbers AF313482 and AF313481, respectively) showing 100 and 94% sequence identity, respectively, with human TRPC3 (GeneBank number NM_003305). These fragments were found to encode continuous reading frames of 131 and 67 amino acids. The 131 amino acid fragment exhibited 100, 98, and 97% sequence identity with rat [Ohki et al., 2000], human [Zhu et al., 1996], and mouse [Mori et al., 1998] TRPC3, respectively, whereas the 67 amino acid fragment showed about 80% identity with TRPC3 from these three species. According to the proposed topological model for mammalian TRPC3 [Birnbaumer et al., 1996; Vannier et al., 1998], the 131 and 67 amino acid fragments matched, respectively, a cytosolic channel region close to the first transmembrane domain and a region containing the putative sixth transmembrane domain of the channel plus the invariant C-terminal sequence shared by all of the TRPC members (EWKFAR domain) [Philipp et al., 1999; Vennekens et al., 2002]. When the presence of a TRPC3-like transcript was evaluated by Northern blot analysis using the 201 bp PCR fragment as hybridization probe, a transcript of about 3.5 kb was found in osteoblastic cells (Fig. 1); a band around 4.3 kb corresponding to TRPC3 was detected in rat brain, used as a positive control. This suggested the existence of a TRPC3-like message in ROS 17/2.8 cells. We next examined the expression of a TRPC3-like endogenous protein by using an anti-TRPC3 antibody raised against a peptide region of mouse TRPC3 highly conserved in rat and human TRPC3 sequences [Zhu et al., 1996; Preuss et al., 1997; Ohki et al., 2000]. Immunobloting experiments showed a band of about 110 kDa molecular weight detectable in lysates from both ROS 17/2.8 osteoblasts and rat brain (Fig. 2), compatible with the apparent molecular weight observed for TRPC3 in other cell types and tissues [Zhu et al., 1998; Boulay et al., 1999]. The TRPC3 immunoreactive band was competed out by the antigenic peptide (Fig. 2, lane 3), evidencing specificity of the bands recognized by the antibody. The ability of this antibody to specifically recognize both endogenous and heterologously overexpressed TRPC3 has also been shown in other

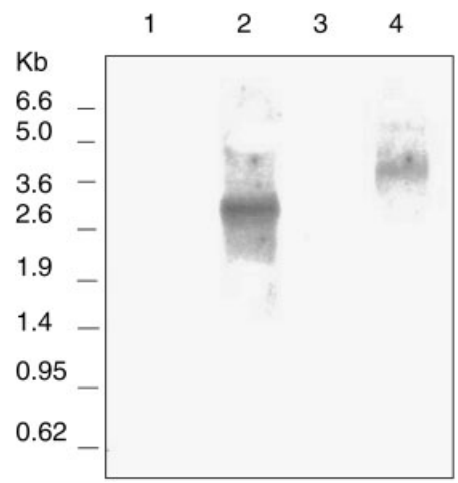

Fig. 1. Northern blot showing the expression of endogenous transient receptor potential-canonical (TRPC)3 in ROS 17/2.8 cells. mRNA was prepared from ROS 17/2.8 cells and rat brain tissues, and loaded in a 1% denaturant agarose gel. After transfer, the membrane was hybridized with the 201 bp ^{32}P-labeled PCR fragment amplificated from ROS 17/2.8 cells. **Lane 1**: 0.3 μg of ROS 17/2.8 mRNA. **Lane 2**: 3 μg of ROS 17/2.8 mRNA. **Lane 3**: 0.15 μg rat brain mRNA. **Lane 4**: 1.5 μg rat brain mRNA. Conditions for hybridization are given under ''Materials and Methods''. The positions of RNA size markers are indicated.

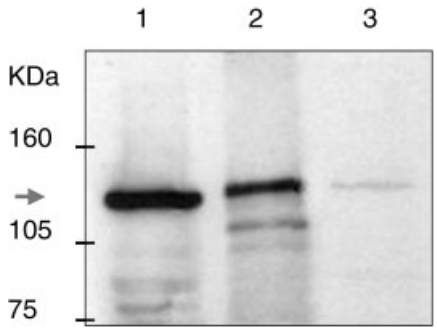

Fig. 2. Western blot showing the expression of endogenous TRPC3 in ROS 17/2.8 cells. Equivalent samples (36 μg protein) from ROS 17/2.8 cells and rat brain tissue lysates were resolved by SDS–PAGE followed by immunoblot analysis using an anti-TRPC3 antibody. **Lane 1**: ROS 17/2.8 cells. **Lane 2**: Rat brain. **Lane 3**: ROS 17/2.8 cells using anti-TRPC3 antibody preincubated with antigen peptide. The positions of the protein size markers are indicated.

cell types [Wu et al., 2002]. Additionally, as shown in Figure 3, immunocytochemical staining of ROS 17/2.8 cells with this antibody resulted in specific immunolabeling with both plasma membrane and reticular pattern distribution, which was not observed in osteoblasts incubated in the absence of anti-TRPC3 (not shown).

Finally, the role of TRPC3-like in CCE in ROS 17/2.8 cells was examined. Antisense ODNs directed against specific regions of human TRPC3 mRNA (see "Materials and Methods") were intranuclearly microinjected into individual osteoblasts to target TRPC3-like expression. A Scra ODN sequence was used as negative control. CCE was then assayed onto injected cells by fluorimetric monitoring of the store-operated cation entry induced by either $1\alpha,25(OH)_2D_3$ or Tpg. As in earlier studies [Vazquez et al., 1998; Baldi et al., 2002], Mn^{2+}, a poor substrate for Ca^{2+} transport mechanisms [Byron and Taylor, 1995], was used as a surrogate for Ca^{2+}, avoiding complications derived of Ca^{2+} transport and feedback mechanisms directly affecting channel regulation.

Stimulation of ROS 17/2.8 cells with 10 nM $1\alpha,25(OH)_2D_3$ in Ca^{2+}-free medium results in rapid but transient cytosolic Ca^{2+} rise due to activation of the PLC/IP_3 pathway [Civitelli et al., 1990; Baldi et al., 2002]. Mn^{2+} addition after this Ca^{2+} transient results in fast quenching of cytosolic Fura-2 fluorescence, revealing activation of the Mn^{2+}-permeable CCE route (Fig. 4); see also [Baldi et al., 2002]. In cells injected with anti-TRPC3 antisense ODN pool,

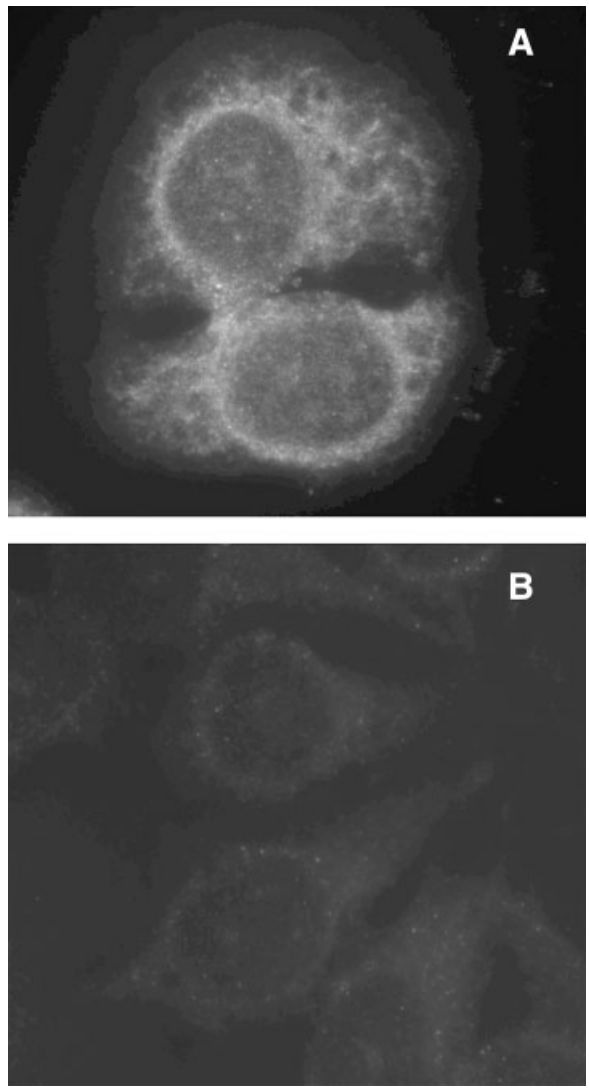

Fig. 3. Immunofluorescence of endogenous TRPC3 in ROS 17/2.8 cells. **A**: Cells were fixed and incubated with anti TRPC3 antibody. **B**: As a control the reaction was blocked by preincubation of the TRPC3 antibody with the control antigen.

sterol-dependent Mn^{2+} influx was reduced by 30–35% at steady-state respect to Scra-injected cells (Fig. 4A). Also, the initial rate of Mn^{2+} entry was reduced about 2-fold in anti-TRPC3 antisense injected cells. Mn^{2+} entry induced by $1\alpha,25(OH)_2D_3$ was not significantly different when non-injected versus Scra cells were compared (not shown). Microinjection of either Scra or anti-TRPC3 antisense ODNs did not affect neither basal rates of Mn^{2+} entry nor the transient Ca^{2+} release induced by the steroid (not shown).

Similarly, CCE activation by depleting calcium stores with the SERCA inhibitor Tpg was significantly affected in osteoblasts injected

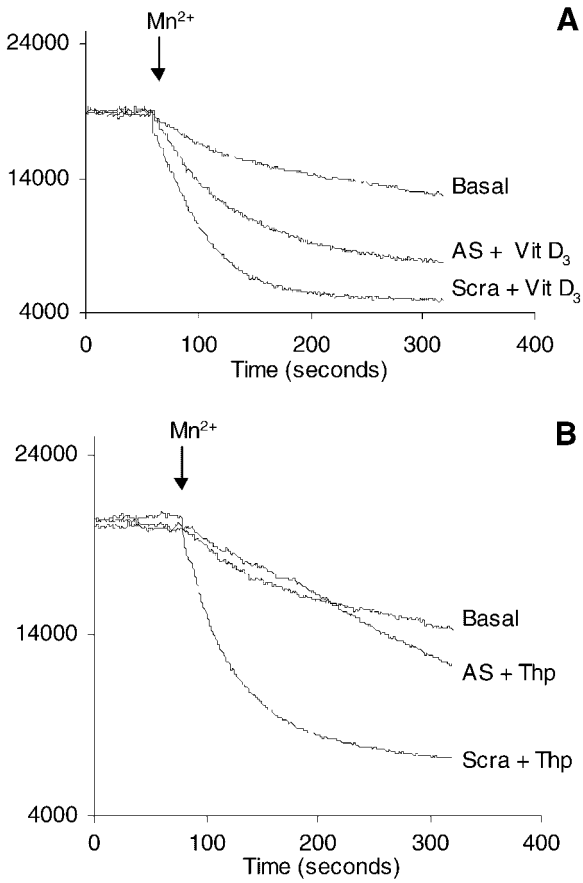

Fig. 4. Capacitative cation entry evoked by 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) and thapsigargin (Tpg) in ROS 17/2.8 cells is inhibited by hTRPC3 antisense oligonucleotides. Cells were cultured onto glass coverslips and 48 h before measurement were transfected with antisense sequences $(AS)_{1-3}$ hTRPC3 mixture or scrambled (Scra) oligodeoxynucleotides (ODN) as described in ''Materials and Methods''. **A**: Fura-2 loaded transfected cells were incubated in Ca^{2+}-free medium and then exposed to vehicle (ethanol <0.1%; basal) or 10^{-8} M 1α,25$(OH)_2D_3$. Once the transient Ca^{2+} rise that followed Ca^{2+} mobilization from stores occurred (monitored from the change in Fura-2 fluorescence when excited at 380 nm; for simplicity, not shown), Mn^{2+} (100 μM) was added to the medium. Mn^{2+} entry was monitored as the quenching of Fura-2 fluorescence measured at an excitation wavelength of 360 nm (see ''Materials and Methods''). **B**: Same as for (A) but 500 nM Tpg or its vehicle (dimethylsulfoxide <0.1%, basal) was used instead of 1α,25$(OH)_2D_3$. Shown are time traces representative from three independent experiments, each of them performed on 40 transfected cells.

with anti-TRPC3 antisense pool (Fig. 4B). The profile of the Tpg-induced Ca^{2+} rise in both non-injected and injected cells was identical, indicating that the transfection did not alter Ca^{2+} store content or kinetics of Ca^{2+} transport. Mn^{2+} addition to Scra-injected cells after Tpg-induced store-depletion, showed increased cation entry which reached a steady-state level within 2–4 min. However, store-operated Mn^{2+} influx in anti-TRPC3 injected cells was significantly reduced (50% decrease at steady-state). Again, the rate of Mn^{2+} entry was 3- to 3.5-fold slower in antisense-treated cells than in controls. These results strongly suggest that in ROS 17/2.8 cells, the endogenous TRPC3-like protein takes part in the CCE route activated by either the secosteroid 1α,25$(OH)_2D_3$ or mere store-depletion.

DISCUSSION

Accumulated evidence indicates that the expression of certain mammalian TRP proteins results in appearance of store-regulated non-selective cation channels, making them good candidates for mediating this class of CCE [Zhu et al., 1996; Harteneck et al., 2000; Zitt et al., 2002]. This might be particularly relevant to the osteoblastic cell line ROS 17/2.8 where CCE is a non-selective cation pathway [Baldi et al., 2002]. There is substantial evidence for store-operated non-selective cation channels in cell types other than osteoblasts [Vazquez et al., 2001; Trebak et al., 2002] and members of the TRPC3/6/7 subfamily may serve as components of these channels. Recently, TRP homologues from the TRPC3/6/7 group have been cloned from rat tissues, the heterologous expression of TRPC3 in COS-1 cells enhancing CCE, leading to the proposal that the rat TRPC3 may be involved in CCE under physiological circumstances [Preuss et al., 1997; Mizuno et al., 1999; Ohki et al., 2000]. In the present study we extended our earlier studies on 1α,25$(OH)_2D_3$-induced CCE in osteoblastic cells [Baldi et al., 2002] presenting evidence on the putative identity of the channels involved in that pathway.

First, two nucleotide fragments with sequence highly identical to mammalian TRPC3 were obtained by RT-PCR using mRNA from ROS 17/2.8 osteoblasts, and Northern blot analysis showed the presence of a transcript of about 3.5 kb in these cells, smaller in size compared to that expressed in rat brain, suggesting that a splice variant of TRPC3 might be expressed in osteoblastic cells. In fact, alternatively spliced variants of TRPC3 are known to exist in rat brain and heart tissues [Mizuno et al., 1999; Ohki et al., 2000]. However, as the full sequence of the osteoblastic TRPC3 is not known, the possibility that the other TRPs exist in these cells cannot be excluded. Additionally, using a

specific anti-TRPC3 antibody, the expression of a protein with both apparent molecular weight and cellular distribution compatible with those expected for TRPC3 was obtained for ROS 17/2.8 cells. The anti-TRPC3 antibody used in our studies was raised against amino acids 822–835 of mouse TRPC3 [Mori et al., 1998], a peptide highly conserved in both rat and human TRPC3 [Zhu et al., 1996; Preuss et al., 1997; Ohki et al., 2000]. This fits with the molecular evidence and favors the TRPC3-like nature of the novel endogenous osteoblastic protein.

We next examined if in ROS 17/2.8 osteoblasts this TRPC3-like protein was involved in CCE induced by either $1\alpha,25\text{-}(OH)_2D_3$-dependent stimulation of the PLC pathway and/or mere store-depletion. By using an anti-TRPC3 antisense strategy, the endogenous expression of TRPC3-like was specifically targeted with three different antisense ODNs matching conserved regions of the human TRPC3 mRNA. In previous studies, using the antisense technique in combination with fluorimetric detection of changes in cytosolic cation levels, we successfully addressed the role of calmodulin and protein kinase C in $1\alpha,25(OH)_2D_3$-induced Ca^{2+} influx in avian skeletal muscle cells [Capiati et al., 2001; Vazquez et al., 2000]. We directly monitored the impact of the anti-TRPC3 antisense ODNs on the store-operated cation entry phase induced by either agonist ($1\alpha,25(OH)_2D_3$) or Tpg. The resulting data showing that the $1\alpha,25(OH)_2D_3$ stimulation of the CCE pathway in ROS 17/2.8 cells was markedly reduced in cells transfected with anti-TRPC3 antisense pool (Fig. 4A) strongly suggest that the endogenous TRPC3-like protein takes active part in the CCE entry route activated by the steroid in these cells. Additionally, the fact that the CCE activation by store-depletion with Tpg was also significantly reduced in osteoblasts injected with anti-TRPC3 antisense pool points for a more general role of the endogenous TRPC3 protein in mediating CCE in osteoblasts. Interestingly, anti-TRPC3 ODNs had a more pronounced impact onto CCE when it was activated by Tpg rather than with the hormone. This is quite unexpected, as (a) either $1\alpha,25(OH)_2D_3$-dependent activation of the PLC/IP_3 cascade or Tpg treatment of ROS 17/2.8 cells results in complete depletion of calcium stores and full CCE activation [Baldi et al., 2002], and (b) CCE in these cells seems to be mediated by the same population of non-selective cation channels regardless the mode of stimulation. As it is known that the $1\alpha,25(OH)_2D_3$ stimulation of osteoblasts triggers a complex signaling involving not only the PLC/IP_3 cascade but also stimulation of kinases [Le Mellay et al., 1997; Farach-Carson, 2001; Boyan et al., 2002], and considering that the Tpg acting directly onto SERCAs bypasses any putative membrane-delimited and/or receptor-coupled signaling step, it is obvious that the quite different scenarios are involved in CCE regulation following either of these two modes of channel activation. Although not addressed here, it is tempting to speculate that the receptor signaling-related events involved in channel modulation may account for the aforementioned quantitative differences in the antisense effect when $1\alpha,25(OH)_2D_3$ versus Tpg-induced CCE are compared.

TRPC3 is a well characterized member of the TRPC superfamily, although there is controversy regarding its mechanism of activation. In many instances, TRPC3 behaves as a receptor-activated channel not activated by mere store-depletion (see [Harteneck et al., 2000; Putney et al., 2001; Vennekens et al., 2002]). However, transient heterologous expression of rat TRPC3 in COS-1 cells enhances cation entry upon store-depletion with Tpg [Preuss et al., 1997; Mizuno et al., 1999]. Similarly, either transient or stable expression of human TRPC3 in different cell lines increased CCE induced by both receptor-dependent and store-operated pathways [Zhu et al., 1996; Kiselyov et al., 1998; Vazquez et al., 2001]. These contradictory results might be related in some way to the differential degree of expression levels between endogenous and heterologously overexpressed channels [Vazquez et al., 2001, Yue et al., 2001]. As TRP channels may form tetramers [Birnbaumer et al., 1996], endogenous TRP-encoded channels most likely exist as heterotetramers while heterologously overexpressed TRPs form homotetramers [Wu et al., 2000; Vazquez et al., 2001]. It is possible that the prevailing mode of channel operation depends upon the final oligomeric composition. Besides the fact that the antisense efficiency is far from being total, a heterotetrameric structure for the endogenous TRPC3-like channel may also account for the non-complete inhibition of CCE in anti-TRPC3 antisense-transfected osteoblasts. Alternatively, non TRPC3-encoded channels may contribute to total CCE in osteoblasts; in fact, pharmacological evidence

indicates that a heterogenous population of non-selective cation channels accounts for CCE in osteoblasts [Baldi et al., 2002] and human epithelial breast cells [Baldi et al., 2003].

In summary, the present results indicate that the endogenous osteoblastic TRPC3-like protein is involved in both receptor-dependent and receptor-independent activation of CCE, and represent the first evidence involving a TRPC related protein in $1\alpha,25(OH)_2D_3$ signaling for regulation of calcium entry in a steroid target cell. We propose TRPC3 as a potential candidate for mediating store-operated, non-selective cation entry into osteoblastic cells.

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