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Evidence that TRPC3 is a molecular component of the 1α,25(OH)$_2$D$_3$-activated capacitative calcium entry (CCE) in muscle and osteoblast cells☆

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Abstract

In chick skeletal muscle and in rat osteoblast-like cells (ROS 17/2.8), 1α,25-dihydroxy-Vitamin-D$_3$ [1α,25(OH)$_2$D$_3$] stimulates release of Ca^{2+} from inner stores and extracellular cation influx through both voltage-dependent and capacitative Ca^{2+} entry (CCE) channels. We investigated the involvement of TRPC proteins in CCE induced by 1α,25(OH)$_2$D$_3$. Two fragments were amplified by RT-PCR, exhibiting >85% sequence homology with human TRPC3. Northern and Western blots employing TRPC3-probes and anti-TRPC3 antibodies, respectively, confirmed endogenous expression of a TRPC3-like protein. Both cell types transfected with anti-TRPC3 antisense oligodeoxynucleotides showed reduced CCE and Mn^{2+} entry induced by either thapsigargin or 1α,25(OH)$_2$D$_3$. In muscle cells, anti-VDR antisense inhibited steroid-induced Ca^{2+} and Mn^{2+} influx and co-immunoprecipitation of TRPC3 and VDR was observed, suggesting an association between both proteins and a functional role of the receptor in 1α,25(OH)$_2$D$_3$ activation of CCE. In osteoblasts, two PCR fragments showing high homology with human INAD-like sequences were obtained. Northern blot and antisense functional assays suggested the involvement of the INAD-like protein in CCE regulation by the hormone. Therefore, we propose that an endogenous TRPC3 protein mediates 1α,25(OH)$_2$D$_3$ modulation of CCE in muscle and osteoblastic cells, which seems to implicate VDR–TRPC3 association and the participation of a INAD-like scaffold protein.

Keywords: CCE; TRPC; Muscle cells; Osteoblasts; 1α,25(OH)$_2$D$_3$; VDR; INAD

1. Introduction

It is now well documented that muscle and osteoblastic cells respond to 1α,25-dihydroxy-Vitamin D$_3$ (1α,25(OH)$_2$D$_3$) with a fast and sustained increment in $[Ca^{2+}]_i$ which depends on both IP$_3$-mediated mobilization of Ca^{2+} from the endoplasmic reticulum, and cation influx from the outside mainly through voltage-dependent Ca^{2+} channels as part of the non-genomic mechanism of action of the hormone in these cells [1,2]. However, we recently reported the existence of a store-operated, capacitative Ca^{2+} entry (CCE) route in chick skeletal muscle, and in rat osteosarcoma-derived osteoblastic-like (ROS 17/2.8) cells, which is non-genomically activated by 1α,25(OH)$_2$D$_3$ [3,4].

For *Drosophila melanogaster*, the transient receptor potential (TRP) proteins, designated as the TRP-canonical (TRPC) subfamily of the larger TRP superfamily gene products function as Ca^{2+} permeable channels mainly regulated by store depletion when expressed in heterologous systems [5]. At present, seven mammalian TRPC proteins are at least known (TRPC1–TRPC7), which are homologs of the invertebrate counterparts [5]. Overexpression of these genes into different cell lines results in CCE sharing heterogenous patterns of activation and/or modulation [6]. This work investigates the existence of TRPC proteins and their participation in the regulation of 1α,25(OH)$_2$D$_3$-activated CCE in skeletal muscle and osteoblastic cells.

2. Materials and methods

2.1. Materials

1α,25(OH)$_2$D$_3$ was kindly provided by Hoffmann-La Roche Ltd. (Basel, Switzerland). Rat monoclonal anti-VDR antibody (clone 9A7) was from Affinity Bioreagents (Golden, CO, USA). Anti-TRPC3 antibody and peroxidase-conjugated secondary antibodies were from Alomone Labs (Jerusalem, Israel) and Santa Cruz Biotechnology (Santa

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Cruz, CA, USA), and oligonucleotides from DNAgency (Malvern, PA, USA).

2.2. RNA isolation and reverse transcription (RT-PCR)

Total RNA was extracted from chick embryo skeletal muscle or ROS 17/2.8 cells as described elsewhere [7] and mRNA was isolated with a PolyATract system (Promega Corp.). The mRNA (1 μg) was transcribed and amplified by RT-PCR using a Promega Corp. Kit following manufacturer's conditions. The following primers were used F (forward) and R (reverse): F_1 5′-GAGAA-(A/G)GAGTTCAAGAATGACTA-3′, R_1 5′-GC(A/G)TGT-GCTACAAACTTCAT-3′; and R_3 5′-AGCAG(A/G/C)CCC-AGGAA(A/G)ATG(A/G)TGAA-3′, F_2 5′-AA(A/G)TTCAT-(A/T)GA(A/G)AA(C/T)AT(C/T)GG-3′ and R_2 5′-TTTTG-GACTAGGAACTAGAC-3′ for TRPC3 sequence amplification. RT-PCR fragments 443 bp (F_1–R_3), 190 bp (F_2–R_2), 390 bp (F_1–R_1) and 201 bp (F_2–R_2) were purified by electroelution from agarose gels and sequenced at the University of Chicago Cancer Research Center DNA Sequence Facility (Chicago, Ilinois, USA). The sequences have been submitted to the GenBank™ (accession numbers AY130980 and AY130981 for muscle and AF313482 and AF313481 for osteoblastic cell sequences, respectively). To perform INAD sequence amplification the following primers were used: F_3 5′-TGCTAT(A/T)GTTATCCATGAAGT-3′ R_4 5′-TAGTGTCGGGACTCCGTCTG-3′ (150 bp GenBank™ accession numbers AF313483) and F_4 5′-GATGCAGT(C/G)A(A/G)TCAGATGGC-3′ and R_5 5′-TAGTTACAGGACTCCGTCTG-3′ (500 bp).

2.3. Northern blot analysis

Isolation of mRNA was as described above. The hybridization probes were synthesized with the Prime A Gene kit (Promega Corp.) using a 446 bp partial cDNA fragment of hTRPC3 obtained by RT-PCR from human embryo kidney cell line (HEK293 cells), 201 bp (TRPC3) and 150 bp (INAD) amplified fragment from ROS 17/2.8 cells, as templates and [γ-^{32}P]dCTP. Hybridization analysis was performed as described elsewhere [8].

2.4. Cell culture and transfection of oligodeoxynucleotides (ODN)

Chick embryo skeletal muscle and ROS 17/2.8 rat osteoblastic-like cells were cultured as described [3,4] and plated onto glass coverslips imprinted with squares for localization of injected cells. Microinjection of ODNs protected with phosphorothioate linkages was performed as described previously [3]. Then, the cells were incubated for 48 h before fluorimetric measurements. The following antisense (AS) ODNs were used: AS_1, 5′-CA-TGCGTCTCAGGGATGGGCTTCCCTCCAT-3′; AS_2, 5′-TTAATCATAGCAATTAGCATGTTGAG-3′; AS_3, 5′-GTTGCTGCATCATTCACATCTCAGCATGCT-3′; AS_4, 5′-CGAGCAAACTTCCATTCTAC-3′, (against TRPC3 mRNA); AS_{13}, 5′-CAGTTTATCTGTAGCAGGATTTTCA-GGCAT-3′; AS_{14}, 5′-CTGCTGCCCCTTCTTCATAG-3′ (against starting and stop codon of mRNA of human INAD sequence) and AS_{15}, 5′-CTTCTTCGTGGCTGGAGTTC-3′ (against the 150 bp RT-PCR fragment). As control sense and scrambled (5′-ATCCTTAGAGTCGCGTACTT-3′) ODNs were used.

Transfection with phosphorothioate linkages protected ODNs using Lipofectin was performed as described previously [3]. Then, the cells were cultured in DME-0.5% FBS for 36 h before fluorimetric measurements. The following antisense-ODNs were used: AS_5, 5′-AGCAGG(A/G/C)CCAGGAA(A/G)ATGT(A/G)GAA-3′; AS_6, 5′-CTAAGGCTAGGGACGACCGT-3′; AS_7, 5′-CCATTGAAAGGAATGGCAGT-3′, and AS_8, 5′-TTTT-GGACTAGGAACTAGAC-3′ (against TRPC3 mRNA); AS_9, 5′-TGTCCTTGGTGATTTTGCAG-3′; AS_{10}, 5′-TCGATGACTTTCTGCTGCTC-3′, and AS_{11}, 5′-TCCTTC-ATCATCCCAATGTC-3′ (against regions upstream, comprising and downstream the AUG starting codon of chick-VDR mRNA, respectively), sense and scrambled (5′-ATCCTTAGAGTCGCGTACTT-3′) sequences were used as control.

2.5. Immunoblotting

Cells were lysed and immunoblot analysis was performed as previously described [3]. For immunoprecipitation, protein A sepharose (10%) was incubated overnight at 4 °C with 1:5 anti-TRPC3 antibody or donkey anti-goat (control). Then, the lysate (100 μg of protein) was added and incubated during 3 h at 4 °C. After centrifugation the pellet was washed three times with PBST and then resuspended in Laemmli sample buffer. Western blot analysis was performed using anti-TRPC3 or anti-VDR antibodies.

2.6. Intracellular calcium measurements

Changes in [Ca^{2+}] and alternatively Sr^{2+} and Mn^{2+} (Ca^{2+} surrogates) influx were monitored in Fura-2 loaded cells as previously described [3,4].

3. Results

A CCE pathway has been previously observed in chick skeletal muscle and ROS 17/2.8 rat osteoblastic cells after the stimulation of the cells with 1α,25$(OH)_2D_3$ or thapsigargin, a sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor. In these cells, CCE is a non-selective cation entry route which allows passage of either Mn^{2+}, Sr^{2+} or Ca^{2+} [3,4]. Several TRP and TRP-like (TRPL) homologues from vertebrate tissues have been cloned and characterized as

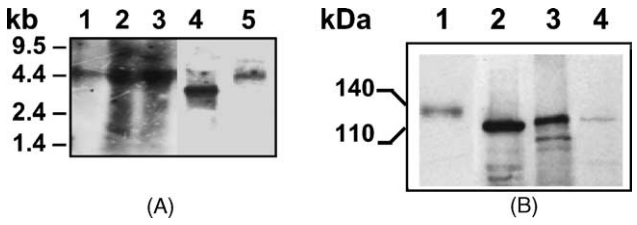

Fig. 1. Endogenous expression of TRPC3 protein in chick muscle and osteoblastic ROS 17/2.8 cells. (A) Northern blot was performed as indicated in Section 2. Lane 1: 5 μg RNA from HEK 293 cells (positive control). Lanes 2 and 3: 30 and 50 μg RNA, respectively, from muscle cells; as probe a 446 bp nucleotide sequence from HEK 293 TRPC3 was used. Lane 4: 3 μg of ROS 17/2.8 cell mRNA. Lane 5: 1.5 μg rat brain mRNA (positive control); as probe the 201 bp RT-PCR fragment obtained from ROS 17/2.8 cell mRNA was used. (B) Western blot showing the expression of endogenous TRPC3 in muscle and osteoblastic cells. Lane 1: muscle cells. Lane 2: ROS 17/2.8 cells. Lane 3: rat brain. Lane 4: ROS 17/2.8 cells using anti-TRPC3 antibody preincubated with antigen peptide. The positions of the protein size markers are indicated.

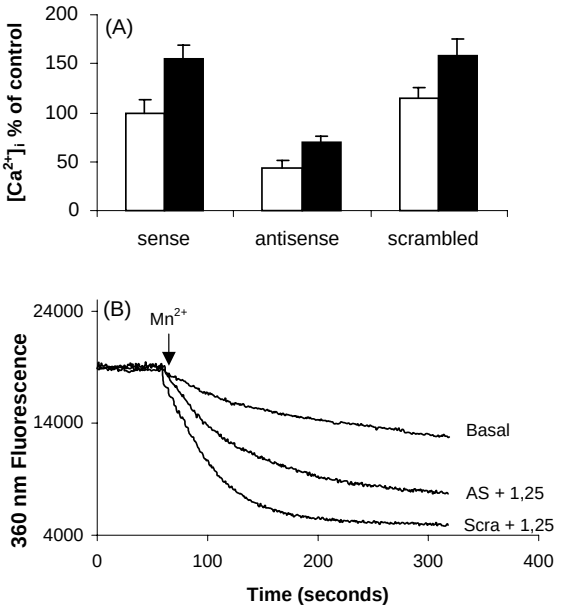

Fig. 2. Effect of antisense-ODNs against TRPC3 mRNA on $1\alpha,25(OH)_2D_3$-induced CCE. CCE was measured in fura-2-loaded cells microinjected with anti-TRP antisense, sense or scrambled oligodeoxynucleotides as indicated in Section 2. (A) CCE (measured at the peak Ca^{2+} response) after the stimulation of muscle cells with 10^{-8} M $1\alpha,25(OH)_2D_3$ (white bar) or 1 μM tapsigargin (black bar). (B) Mn^{2+} influx was recorded after addition of 100 μM Mn^{2+} to osteoblasts prestimulated with 10^{-8} M $1,25(OH)_2D_3$ (1,25). Results are representative of three independent experiment performed in triplicate.

candidates for mediating CCE in animal cells [5]. We investigated the presence of TRPC transcripts in the muscle and osteoblastic cells by RT-PCR. Two fragments of 443 and 190 bp in muscle and of 390 and 201 bp in osteoblastic cells were amplified. These fragments were found to encode continuous reading frames of 116, 63, 131, and 67 amino acids. The 116 and 63 amino acid fragments showed 71–95% of identity with human, mouse and rat TRPC3, the 131 and 67 amino acids fragments exhibited 80–100% sequence identity with TRPC3 from the same species. By Northern blot analysis a major band in muscle cells similar to that observed in HEK293 control cells corresponding to a size of ~4.4 Kb was observed (Fig. 1A, lanes 1–3). By using the 201 bp PCR fragment as hybridization probe, transcripts of about 3.5 and 4.3 kb were found in osteoblastic cells and rat brain (positive control), respectively (Fig. 1A, lanes 4, 5). As shown in Fig. 1B, by Western blot analysis using polyclonal anti-TRPC3 antibodies, a single band of about 140 and 110 kDa was detected in muscle and osteoblastic cells, respectively, and in rat brain (control). Altogether, these results indicate the endogenous expression of TRPC3 proteins in avian skeletal muscle and in rat ROS 17/2.8 cells.

Additionally, the role of a TRPC3-like protein in $1\alpha,25(OH)_2D_3$ modulation of CCE in muscle and osteoblastic cells was examined by incorporation of antisense-ODNs directed against specific regions of TRPC3 mRNA coupled to fluorimetric mesurement of CCE induced by either $1\alpha,25(OH)_2D_3$ or thapsigargin. Microinjection of AS_4, an antisense-ODN directed against EWKFAR, a highly conserved region observed in almost all TRPC proteins, significantly inhibited (56–58%) CCE induced by $1\alpha,25(OH)_2D_3$ or thapsigargin respect to control cells microinjected with sense or scrambled ODNs (Fig. 2A). Similar results were obtained by transfection of cells with a pool of ODNs (AS_5, AS_6, AS_7, AS_8) directed against regions of TRPC3–PCR products (not shown). In agreement with these data, inhibitions of 30–50% were observed when Mn^{2+} influx stimulation by $1\alpha,25(OH)_2D_3$ or thapsigargin was measured in microinjected osteoblasts with a pool of antisense-ODNs (AS_1, AS_2, AS_3) against human TRPC3 mRNA or the 201 bp TRPC3–PCR product (Fig. 2B). Scrambled or anti-TRPC3 antisense-ODN did not significantly affect neither basal (non-stimulated cells) rates of Mn^{2+} entry into cytosol nor the transient IP_3-dependent phase of Ca^{2+} release induced as a consequence of $1\alpha,25(OH)_2D_3$-stimulation of the PLC pathway (not shown).

To investigate the role of VDR in the modulation of CCE mediated by $1\alpha,25(OH)_2D_3$, we examined the existence of protein-protein interactions between TRPC3 and VDR by co-immunoprecipitation. We observed that in muscle cells VRD and TRPC3 coprecipitate suggesting the association between both proteins. In parallel, $1\alpha,25(OH)_2D_3$-dependent CCE was inhibited 45% in cells transfected with a pool of anti-VDR ODNs (AS_9, AS_{10}, AS_{11}). A similar inhibition was observed when Mn^{2+} influx stimulation by $1\alpha,25(OH)_2D_3$ was measured as the quenching of fura-2 fluorescence by Mn^{2+} upon cell transfection with the same anti-VDR ODNs (not shown).

In parallel, in osteoblastic cells, two fragments of 150 and 550 bp were amplified by RT-PCR. These fragments showed 100 and 80% homology with human INAD-like (hINADL) and multiple-PDZ-domain protein (MPDZ), respectively. Moreover, by Northern blot analysis using the ^{32}P-labelled

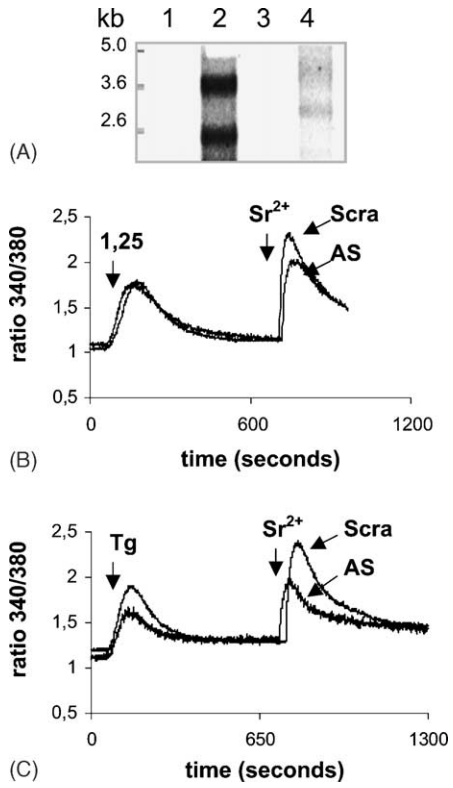

Fig. 3. Endogenous expression of a INAD-like protein in osteoblasts and its involvement in 1α,25(OH)$_2$D$_3$ CCE activation. (A) Northern blot. Lane 1: 0.3 μg; lane 2: 3 μg of osteoblast mRNA. Lane 3:0.15 μg; lane 4: 1.5 μg of rat brain mRNA. (B) CCE Sr^{2+} influx induced by10^{-8} M 1α,25(OH)$_2$D$_3$ (1,25) (C) CCE Sr^{2+} influx induced by 1 μM thapsigargin (Tg). Fura-2 loaded cells microinjected with anti-INAD antisense (AS) or scrambled (Scra) ODNs were used for (B) and (C). Results are representative of three independent experiment performed in triplicate.

150 bp RT-PCR sequence as probe, transcripts of 3.7 and 2.5 Kb were found in osteoblasts as in rat brain, positive control (Fig. 3A). In addition, microinjection of cells with anti-INAD antisense-ODNs (AS_{13} AS_{14}, AS_{15}), decreased 1α,25(OH)$_2$D$_3$ and thapsigargin-dependent Sr^{2+} influx through CCE by 22 and 40%, respectively (Fig. 3B and C).

4. Discussion

Accumulated evidence indicates that expression of certain mammalian TRP proteins results in appearance of non-selective cation channels, making them good candidates for mediating CCE [5]. This might be particularly relevant when CCE is a non-selective cation pathway, as is the case of chick skeletal muscle cells or osteoblastic cell line ROS 17/2.8 [3,4]. There is substantial evidence for store-operated non-selective cation channels in others cell types [5] and members of the TRPC3/6/7 subfamily may serve as components of channels. Overexpression of these genes into different cell lines generally results in increased capacitative Ca^{2+} entry sharing heterogenous patterns of activation and/or modulation [6]. However, none of the available evidence to date allows for direct involvement of TRP proteins as mediators of endogenous CCE. In the present work, we investigated the endogenous expression of TRP proteins and provided evidence of their participation in 1α,25(OH)$_2$D$_3$ regulation of CCE in muscle and osteoblastic cells.

Two partial nucleotide sequences highly identical to mammalian TRPC3 sequences were obtained by RT-PCR from mRNA of muscle and osteoblastic cells. Since primers used in RT-PCR were designed in order to amplify highly conserved regions in TRPC3/6/7 mammalian sequences and in view that the products obtained present low homology with other avian or mammalian proteins non-related with the TRPC family, the results obtained strongly suggest the existence of mRNA coding for TRPC proteins in chick skeletal muscle cells and in rat ROS 17/2.8 cells.

Northern blot analysis showed the presence of a transcript of about 4.4 kb in muscle cells and 3.5 kb in osteoblastic cells. The significantly smaller size of the ROS 17/2.8 cell transcript compared to that expressed in rat brain suggests that a splice variant of TRPC3 might be expressed in these cells. In fact, the existence of alternatively spliced variants of TRPC3 in rat brain and heart tissues has been recently reported [9]. However, because the full sequence of the osteoblastic TRPC3 is not known, we cannot exclude the possibility that other TRPs exist in these cells.

Additionally, using an anti-TRPC3 antibody, the expression of a protein with an apparent molecular weight similar to that expected for TRPC3 was detected in both cell types. This result further shows the expression of the TRPC3 family of proteins in avian skeletal muscle and in rat osteoblastic cells. In view that the antibody employed recognize a peptide near the carboxyl terminus of the human TRPC3 (also present in TRPC6 and TRPC7) the expression of TRPC6/7 subtypes cannot be excluded.

Moreover, the participation of TRPC3 proteins in CCE stimulated by 1α,25(OH)$_2$D$_3$ or thapsigargin in both cell types was suggested by the marked reduction observed upon transfection of antisense-ODNs directed against TRPC3 sequences. Besides the fact that the efficiency of the antisense technique 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 cells. Alternatively, non TRPC3-encoded channels may contribute to total CCE in these cells; in fact, pharmacological evidence indicating a heterogenous population of non-selective cation channels has been osbserved in the CCE in osteoblasts [4].

The results described so far strongly suggest that in muscle cells and osteoblasts, the endogenous TRPC3-like protein takes part in the capacitative Ca^{2+} entry pathway activated by 1α,25(OH)$_2$D$_3$.

TRP channels have been shown to be modulated by association of macromolecules integrating signaling supramolecular complexes. The scaffold protein INAD clusters these macromolecules through its PDZ domains [10]. The existence of a CCE pathway mediated by 1α,25(OH)$_2$D$_3$

raises the possibility that the Vitamin D receptor could interact with TRPC proteins in the modulation of this Ca^{2+} entry route by the hormone. The fact that in muscle cells, the VDR and TRPC3 coimmunoprecipitate suggests the association of both proteins. Of relevance to this observation, it has been recently reported that the VDR rapidly translocates from the nucleus to plasma membranes after short treatment of muscle cells with $1\alpha,25(OH)_2D_3$ [11]. The finding that $1\alpha,25(OH)_2D_3$ stimulation of the CCE pathway was markedly reduced in muscle cells transfected with a pool of anti-VDR antisense-ODNs, suggests that TRP and VDR participate in hormone modulation of CCE.

We speculate that a protein as INAD-like with multiple modular protein interaction domains could be well suited for recruiting different functional components of the $1\alpha,25(OH)_2D_3$ signal cascade. The observation in osteoblasts of two nucleotide sequences highly identical to human multiple PDZ domain sequences and two transcripts similar in size to that expressed in rat brain, suggest the presence of an INAD-like proteins in these cells. A functional role for INAD-like protein in hormone activation of CCE in osteoblastic cells was inferred by the reduction in $1\alpha,25(OH)_2D_3$-induced CCE Sr^{2+} influx upon cell microinjection with anti-INAD antisense-ODNs.

Altogether, the data of this paper provides for the first time molecular evidence on the existence of TRPC3 proteins in muscle and bone cells, which mediate $1\alpha,25(OH)_2D_3$ regulation of CCE through a complex integrated at least by TRPC3 and VDR, and assembled by INAD-like protein.

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