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Key Words

CaR • TRPC1 • ERK1/2 • Breast cancer cells • Cell proliferation

Abstract

The calcium-sensing receptor (CaR), is a G protein-dependent receptor that responds to increments in extracellular Ca^{2+} ($[Ca^{2+}]_o$). We previously reported that an increase in $[Ca^{2+}]_o$ induced a release of intracellular calcium and Ca^{2+} entry via store operated channels (SOCs). We also demonstrated that MCF-7 cells express Transient Receptor Potential canonical 1 (TRPC1) channels. Herein, we investigated CaR intracellular signaling pathways and examined the role of TRPC1 in CaR-induced cell proliferation, through the extracellular signal-regulated Kinases 1 & 2 (ERK1/2) pathways. Treatment by $[Ca^{2+}]_o$ increased both MCF-7 cell proliferation and TRPC1 expression. Both the $[Ca^{2+}]_o$ proliferative effect and TRPC1 protein levels were abolished by the ERK1/2 inhibitors. Moreover, $[Ca^{2+}]_o$ failed to increase cell proliferation either in the presence of CaR or TRPC1 siRNAs. Both $[Ca^{2+}]_o$ and the selective CaR activator spermine, elicited time and dose-dependent ERK1/2 phosphorylation. ERK1/2 phosphorylation was almost completely inhibited by treatment with the phospholipase C and the protein kinase C inhibitors. Treatment with 2-amino-ethoxydiphenyl borate (2-APB), and SKF-96365 or by siTRPC1 diminished both $[Ca^{2+}]_o$- and spermine-stimulated ERK1/2 phosphorylation. Moreover, down-regulation of TRPC1 by siRNA reduced the Ca^{2+} entry induced by CaR activation. We conclude that the CaR activates ERK1/2 via a PLC/PKC-dependent pathway. Moreover, TRPC1 is required for the ERK1/2 phosphorylation, Ca^{2+} entry and the CaR-proliferative effect.

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Introduction

The calcium sensing receptor (CaR), a seven-transmembrane G protein-coupled receptor capable of sensing changes in extracellular calcium concentration ($[Ca^{2+}]_o$), is expressed in human normal and cancer breast tissues [1], and in breast cancer cell lines [2]. It has also been reported to be over-expressed in some other cancer tissues [3]. The breast cancer mostly metastasizes to the bone and 70 % of these patients present osseous metastases [4]. Consequently, tumors induce bone resorption leading to the release of large quantities of Ca^{2+} into the bone microenvironment. The local Ca^{2+} level at resorption sites has been reported to rise as high as 40 mM [5]. In a early study Sanders et al., [2] reported that high $[Ca^{2+}]_o$ (5, 7.5 and 10 mM) as well as polycationic CaR agonists (neomycin and spermine) stimulate CaR inducing parathormone-related peptide (PTH-rP) secretion from MCF-7 and MDA-MB-231 cells. However, neither the involvement of the CaR nor its signaling pathways have been studied in breast cancer proliferation.

Several studies have reported that CaR-stimulation, by high $[Ca^{2+}]_o$ levels, or by its other agonists, activates the proliferation of many cell types including ovarian surface epithelial cells [6], osteoblasts and HEK-293 cells stably transfected by CaR (HEK-CaR) [7-8]. Moreover, it has also been reported that CaR-evoked proliferation is ERK1/2 dependent [9-11].

CaR-induced ERK1/2 activation implies a highly degree of signaling complexity including PLC activation [12-14]. Activation of PLC produces transient increases in intracellular calcium concentration ($[Ca^{2+}]_i$) resulting from the concomitant increase in inositol tri-phosphate (IP_3) and subsequent sustained elevations in $[Ca^{2+}]_i$ due to Ca^{2+} influx through SOC channels which, in many cases, have been identified as transient receptor potential (TRP) family members [15]. In human keratinocytes, CaR-mediated inositol 1,4,5-triphosphate (IP_3) accumulation and $[Ca^{2+}]_i$ elevation result from both release of Ca^{2+} from intracellular stores and Ca^{2+} influx through non-selective cationic channels [16]. A greater incidence of the cationic currents was suggested to be associated with cell proliferation in human astrocytoma cells U373 [17]. Moreover, many studies performed on lymphocytes and mast cells have shown that the Ca^{2+} influx, via store operated channels (SOCs), can activate ERK1/2 [18-19]. However, nothing is currently known about the link between the CaR, TRP channels, and ERK1/2 phosphorylation in breast cancer cell proliferation.

We previously demonstrated that activation of the CaR by an increase in $[Ca^{2+}]_o$ or spermine promotes an elevation in $[Ca^{2+}]_i$ through PLC cascade in MCF-7 cells [20]. We have also demonstrated that CaR-activation induces a cationic current, which is highly Ca^{2+} selective and inhibited by 2-aminoethoxydiphenyl borate (2-APB) and La^{3+}, both recognized inhibitors of the TRPC family. Moreover, we found that MCF-7 express TRPC1 [20]. Recently, Rey et al., [21] have reported the requirement of TRPC1 for the generation of transient Ca^{2+} oscillations by the CaR in HEK-293-CaR. In the present study, we first investigated the CaR-evoked ERK1/2 phosphorylation cascade and examined the potential role of TRPC1 in CaR-induced cell proliferation through the ERK1/2 pathways in MCF-7 cells. We found that CaR-stimulation induced ERK1/2 phosphorylation through a PLC/PKC cascade. Moreover, the TRPC1-mediated Ca^{2+} entry subsequent to CaR stimulation is essential to CaR-induced ERK1/2 phosphorylation and to MCF-7 proliferation.

Materials and Methods

Cell culture

The MCF-7 breast cancer cell line was obtained from the American Type Culture Collection. Cells were grown in Eagle's Minimum Essential Medium (EMEM; GIBCO) supplemented with 5% fetal bovine serum (FBS), L-glutamine (2 mM), and gentamycin (50 µg/ml) (GIBCO). The culture medium was changed every 2 days. Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere.

Cell proliferation assay

Cells were seeded in 35 mm Petri dishes at a density of $1x10^5$ cells/dish, allowed to attach overnight and then incubated with FBS free medium for a 24-h starvation period. Cells were then washed and incubated for 24 h in the same medium supplemented with different concentrations of Ca^{2+} alone or in the presence of various inhibitors. MCF-7 growth was assessed using the standard Malassez cell method. Briefly, cells were removed by trypsinisation and diluted in Trypan blue. Cell counts were performed 6 times (in a blind manner) and the results were expressed as the percentage of viable cells measured compared to those measured under control conditions.

Intracellular calcium measurement

MCF-7 cells were grown on glass coverslips for calcium imaging experiments. The cytosolic calcium concentration was measured using Fura-2-loaded cells. MCF-7 cells were loaded for 1 h at room temperature with 3.3 µM Fura-2/AM prepared in saline solution and subsequently washed three times with the same dye-free solution. The coverslip was then transferred

into a perfusion chamber of a Zeiss microscope (Thornwood, NY) equipped for fluorescence. Fluorescence was excited at 350 and 380 nm alternately, using a monochromator (Polychrome IV; TILL Photonics, Planegg, Germany), and captured by a Cool SNAP HQ camera (Princeton Instruments, Evry, France) after filtration through a long-pass filter (510 nm). Metafluor software (v. 6.2r6; Universal Imaging, West Chester, PA) was used for acquisition and analysis. All recordings were carried out at room temperature. The cells were continuously perfused with the saline solution, and chemicals were added via the perfusion system. The flow rate of the whole-chamber perfusion system was set at 1 ml/min, and the chamber volume was 500 µl.

Chemicals

The PLC inhibitor U73122, the PKC inhibitor aminoalkyl bisidolymaleimide (GF109203X), the PKC activator PMA, the selective inhibitor of MAPK kinase, U0126, the CaR agonist spermine, the Calcium entry inhibitors 2-APB, SKF96365, and Ni^{2+} were obtained from Sigma-Aldrich (La Verpilliere, France).

RNA extraction and RT-Q-PCR

Total RNA was isolated from human breast cancer MCF-7 cells by standard TRIzol extraction (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. RNA samples were treated with DNase I (Promega, France) at 37°C for 30 min. A phenol/chloroform (v/v) extraction was performed and RNA was precipitated with ethanol and dissolved in sterile distilled water. The RNA level was measured by spectrophotometry (optical density at 260 nm) and was reverse-transcribed into cDNA using an SSII kit (Invitrogen) by following the manufacturer's instructions. Complementary DNA was stored at -20°C. The PCR primers used to amplify the RT-generated TRPC1 cDNAs were designed on the basis of established GenBank sequences. The primers for TRPC1 cDNA were: (NM_003304.3) 5'-GCA CGC CAG CAA GAA AAG C-3' and 5'-GAG GTG ATG GCG CTG AAG G-3'. The amplified cDNA length is 110 bp for TRPC1. The primers for CaR cDNA were: (NM_000388) 5'-AAG AAA GTT GAG GCG TGG CAG-3' and 5'-GAG GTG CCA GTT GAT GAT GGA-3'. The amplified cDNA length is 131 bp for the CaR. The following primers were used to amplify a 226 bp of fragment β-actin cDNA 5'-CAG AGC AAG AGA GGC ATC CT-3' and 5'-ACG TAC ATG GTC GGG GTG TTGAA-3'. Primers were synthesized by Invitrogen (Fisher Bioblock, Illkrich,France). Real-time PCR was performed on a Roche LightCycler® using the Absolute™ QPCR SYBR® Green I kit (ABGene, Courtaboeuf, France) according to the manufacturer's protocol. The cDNA pool was diluted to a concentration of 5 ng/µl of which 5 µl were used, together with 0.5 µM of each specific primers in a final volume of 20 µl. The following thermal profile was used for all PCRs: 95°C for 15 min, 45 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 15 s. To verify the specificity of the amplification reaction, this PCR reaction was followed by a melting curve analysis from 65 to 95°C in 470 s, and the PCR products (10 µl) were analyzed by electrophoresis in a 1.3% agarose gel in 0.5x TBE and stained with ethidium bromide. The data obtained with the LightCycler® software 3.5 (Roche) were exported into RealQuant© software (Roche) and the results were expressed as the TRPC1/actin ratio for each sample.

Western blot analysis

Human breast cancer MCF-7 cells were cultured in T-75 flasks (3 x 10^6 cells) at 37 °C in a humidified 5% CO_2 atmosphere in 5% FBS medium for 3 days. They were then starved for 24 h in serum-free medium prior to stimulation with $[Ca^{2+}]_o$ or spermine after pre-incubation with inhibitors of various signal transduction pathways, as described in the results. At the end of the incubation period, the medium was removed; the cells were washed and unstitched using ice-cold phosphate-buffered saline (PBS) containing 0.5 mM EDTA. Afterwards, equal amounts of cells (1 million of cells/1mL) were centrifuged at 10000 *g* for 10 min at room temperature, the supernatant was removed and cells were lysed directly with 40 µl / 1 million cells of Laemmli sample buffer. The lyses were heated 3 times at 100°C for 10 minutes. The heating cycles were separated by short breaks on ice. Finally, MCF-7 cells lysates were separated on SDS-polyacrylamide gel and transferred electrophoretically onto nitrocellulose membranes (Hybond-C super membrane; Amersham Life Science) and incubated with blocking solution (10 mM Tris -HCl, pH 7.4, 150 mM NaCl, and 0.05 % Tween-20) containing 5 % dry milk for 1 h at room temperature. ERK1/2 phosphorylation was detected by overnight immunoblotting with P-ERK1/ERK2 [(Thr^{202}/Tyr^{204}) (Cell Signaling Technology)] with a 1/1000 dilution of a rabbit polyclonal and a subsequent incubation with a second, goat anti-rabbit, peroxidase-linked antiserum diluted in a blocking solution. The bands were visualized by chemiluminescence (Renaissance enhanced chemiluminescence system). Nitrocellulose membranes were stripped of antibodies and reprobed using an antiserum to ERK1/EK2 (Cell Signaling Technology) that detects this protein independently of its state of phosphorylation to confirm equal loading of ERK proteins.

For TRPC1 blotting, cells were incubated in 35 mm Petri dishes (6 x 10^4) for 2 days in 5 FBS medium. Cells were treated with an ice-cold lysis buffer containing: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM MgCl, 1 mM PMSF, 1% Nonidet P-40, and protease inhibitor cocktail from Sigma-Aldrich (La Verpilliere, France). 30 min after, the lysates were centrifuged at 15,000g at 4°C for 20 min, mixed with a sample buffer containing: 125 mM Tris-HCl pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.01% bromphenol blue, and boiled for 5 min at 95°C. Total protein samples were subjected to SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes. The membrane was blocked in a 5 % milk TNT buffer (Tris-HCl, pH 7.5, 140 mM NaCl, and 0.05 % Tween 20) overnight then probed using specific rabbit polyclonal anti TRPC1 (Alomone Labs Ltd., 1/200) and anti-β-actin antibodies (Lab Vision Co., 1/1000). The bands on the membrane were visualized by enhanced chemiluminescence (Pierce Biotechnologies Inc.). Densitometric analysis was performed using a Bio-Rad image acquisition system (Bio-Rad Laboratories).

Small interfering RNA

80 000 MCF-7 Human cells were plated in a 35 mm Petri dishes with antibiotic and 5% FCS medium. On the day of

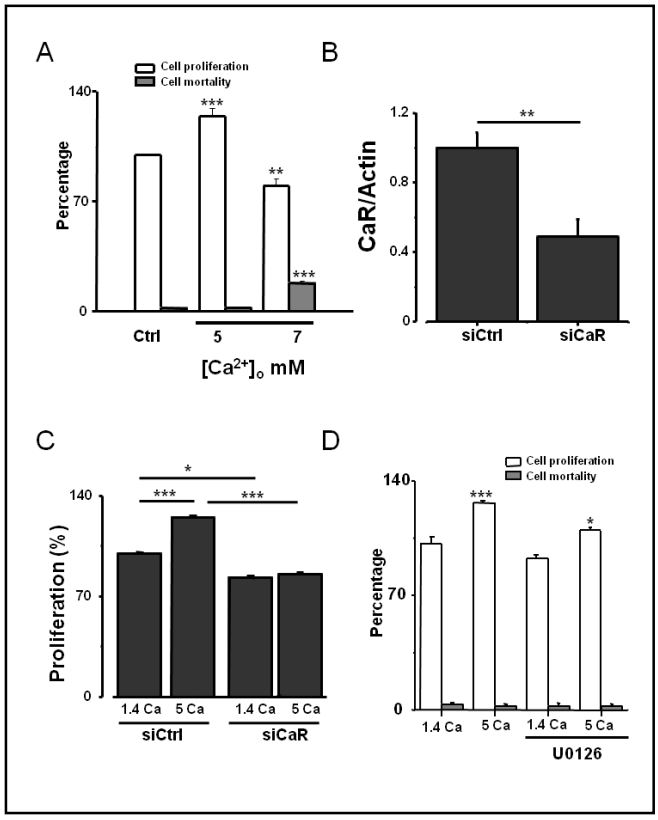

Fig. 1. $[Ca^{2+}]_o$ increased MCF-7 cell proliferation through CaR and ERK1/2 pathways. Cells were seeded at a concentration of 1×10^5 cells/petri dishes (60 mm). After a starvation period (24 h), cells were treated with 5 or 7 mM $[Ca^{2+}]_o$ for 1 day. The calcium concentration in control conditions (Ctrl) was 1.4 mM. Cells were counted after being detached with Trypsin-EDTA using the Trypan blue method. A: Effects of 5 and 7 mM $[Ca^{2+}]_o$ on cell proliferation and mortality. Experiments were repeated three times and the indicated values are mean ± SE of six dishes (** P<0.01, *** P<0.001). B: RT-Q-PCR analysis of the CaR expression 48 h after MCF-7 cell transfection with 50 nM CaR siRNA (siCaR) or control 50 nM siRNA (siCtrl) (** p<0.01). C: Cells were transfected with control siRNA (siCtrl) or siCaR and cultured overnight in EMEM medium with 5% FBS. Then, cells were serum-deprived for 6 h and incubated with $[Ca^{2+}]_o$ (1.4 mM) or (5 mM) for 24 h. After 24 h of $[Ca^{2+}]_o$ incubation, cell proliferation was determined by the Trypan blue method as described in Experimental procedures. $[Ca^{2+}]_o$ was unable to increase cell proliferation in cells transfected by the siCaR 5Ca compared with cells transfected by control siCaR 5Ca. Experiments were repeated three times and the indicated values are mean ± SE of six dishes. (* p <0.05, *** p < 0.001). D: cells were treated with normal $[Ca^{2+}]_o$ (1.4 mM) or $[Ca^{2+}]_o$ (5 mM) in the presence or absence of U0126 (5 µM) for 1 day. Cell proliferation percentage was determined by cell counting. Experiments were repeated three times and the indicated values are mean ± SE of six dishes (*p < 0.05, *** P<0.001).

transfection, cells were rinsed with fresh antibiotic and serum free medium, and transfected overnight with 50 nM siRNA-TRPC1 (siC1 H.s.308-326 (NM_003304.3) 5'-GGG UGA CUA UUA UAU GGU U(dTdT)) or 50 nM siCaR per Petri dish using "jet SI ENDO" in a final volume of 1.5 ml. Ready-to-use siRNA-TRPC1 (processing option: A4) was synthesized by Dharmacon Research Inc (Lafayette, USA). Functional non-coding siRNA#1 (Dharmacon Research Inc.) was used as a control (siCT). After transfection, cells were cultured and harvested at 48 h.

CaR siRNA was designed and synthesized in collaboration with Eurogenetec (Liege, Belgium) based on the human reference sequence NM_000388. The sequences of the siRNA will be provided by R.M. upon request. As described above, CaR siRNA transfections were carried out in Petri dishes. 50 nM of CaR siRNA was added to wells that contained the MCF-7 cells. As negative controls, parallel experiments were carried out using scrambled siRNA, which did not match the sequences of any mammalian mRNAs (Ambion, INC.). Depletion of endogenous mRNAs encoding CaR by siRNA was confirmed by RT-Q-PCR.

Immunocytochemistry and confocal imaging

Cells grown on glass coverslips were washed once with phosphate-buffered saline (PBS) and incubated in a cholera toxin subunit B Alexa Fluor® 448 conjugate (1/2000; Molecular Probes) for 15 min and then washed once with PBS and fixed in 3.5 % paraformaldehyde in PBS. PBS-glycine (30 mM) was used to quench the reaction, and the subsequent permeabilization was obtained using 0.1 % Triton X-100. The cells were washed again in PBS solution and stained with rabbit polyclonal anti-TRPC1 antibody (Alomone Labs LTD., 1/250). Alexa Fluor 546 goat anti-rabbit IgG (Moleculare probes, Cergy Pontoise, France, 1/1000) were used as secondary antibodies for TRPC1. Fluorescence was analysed on a Carl Zeiss Laser Scanning Systems LSM 510 connected to a Zeiss Axiovert 200 M with a 63 × 1.4 numerical aperture oil immersion lens at room temperature, and data were processed using Carl Zeiss LSM Image Examiner software.

Statistical analysis

Data are presented as mean ± SE. The student's t-test was used to compare treatment means with control means and to test significant differences. Differences between values were considered significant (*) when p< 0.05; very significant (**) when p< 0.01 and highly significant (***) when p< 0.001.

Results

$[Ca^{2+}]_o$ mediated cell proliferation through CaR activation on serum-deprived MCF-7 cells

We have previously reported that $[Ca^{2+}]_o$ and spermine activated the CaR in MCF-7 cells [20]. Here, we investigated whether CaR is involved in cell proliferation in serum-deprived MCF-7 cells after a 24 h incubation period. Calcium increased proliferation at 5 mM, by 24.5 ± 4.8 % (p<0.001) compared to 1.4 mM $[Ca^{2+}]_o$ (Fig. 1A). However, at 7 mM, $[Ca^{2+}]_o$ reduced

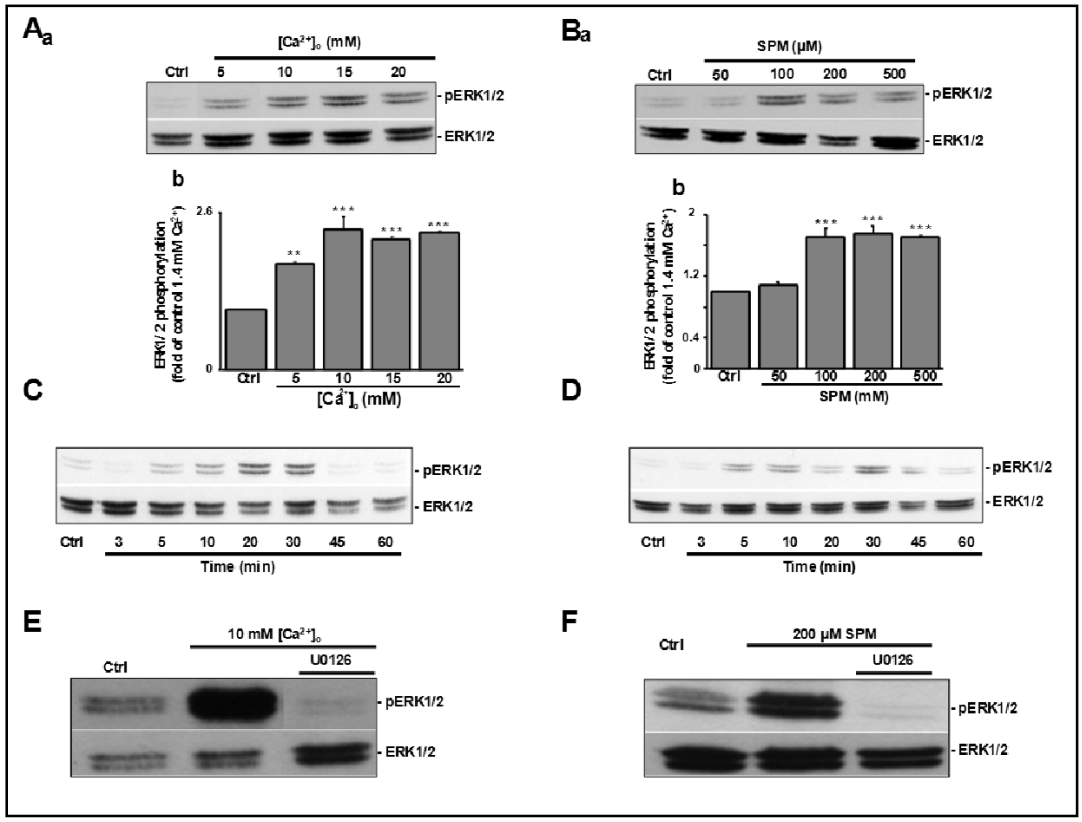

Fig. 2. CaR stimulation induced ERK1/2 phosphorylation in human breast cancer cells. Dose dependent effects of $[Ca^{2+}]_o$ (A) and spermine (SPM, B) on CaR-mediated ERK1/2 phosphorylation. Cells were stimulated with various $[Ca^{2+}]_o$ concentrations (5 - 20 mM), and spermine (50 - 500 µM) for 20 min. Cell lysates were analysed by western blotting with anti-pERK1/2 or anti-total ERK1/2. Results shown in (a) are from a representative experiment, (b) data from three independent experiments. (C-D) Time-course of ERK1/2 phosphorylation by exposure to 10 mM $[Ca^{2+}]_o$ or 200 µM spermine for different times (3 - 60 min), and cell lysates were analysed by western blotting with anti-pERK1/2 or anti-total ERK1/2. (E-F) MCF-7 cells pre-incubated for 30 min with ERK1/2 inhibitor (U0126, 5 µM) and then stimulated for 20 min with 10 mM $[Ca^{2+}]_o$ or 200 µM spermine (SPM). Cell lysates were analysed by western blotting with anti-pERK1/2 or anti-total ERK1/2.

cell proliferation (Fig. 1A). We also investigated whether the $[Ca^{2+}]_o$ induced cell mortality under our experimental conditions. The cell mortality was unchanged when cells were pre-treated with 5 mM $[Ca^{2+}]_o$ (Fig. 1A). In contrast, 7 mM $[Ca^{2+}]_o$ significantly increased the number of necrotic/apoptotic cells by 17.8 ± 1.3 % ($p<0.001$, Fig. 1A).

To further support and specify the importance of CaR in $[Ca^{2+}]_o$ response in MCF-7 cells, we used siRNA technology to down-regulate CaR expression. We transfected MCF-7 cells with either siRNA directed towards CaR (siCaR) or suitable siRNA controls (control siRNA). When compared to cells transfected with control siRNA, cells transfected with CaR showed 61 ± 4% reduction at the mRNA levels 48 h after transfection (Fig. 1B). 5 mM $[Ca^{2+}]_o$ increased cell proliferation by 25 ± 3% in cells transfected with control siRNA (Fig. 1C), in contrast, it failed to increase MCF-7 cell proliferation in cells transfected with siCaR (Fig. 1C). Taken together, these results demonstrate that $[Ca^{2+}]_o$ increased MCF-7 cell proliferation through CaR activation.

ERK1/2 mediated CaR-induced proliferation

To identify the signal transduction pathways involved in CaR-mediated of cell proliferation, we investigated the effect of ERK1/2 inhibitor, U0126 (5 µM) on 5 mM Ca^{2+}-induced proliferation. 5 mM $[Ca^{2+}]_o$ alone increased cell proliferation by 26.5 ± 1.2 % ($P<0.001$, Fig. 1D). In the presence of U0126, 5 mM Ca^{2+} induced proliferation by only 10 ± 1.6 % ($P<0.05$, Fig. 1D). Moreover, U0126 had no significant effect on basal proliferation or cell viability (Fig. 1D).

Phosphorylation of ERK1/2 in response to CaR activation by $[Ca^{2+}]_o$, and spermine in MCF-7 cells

Serum-deprived MCF-7 cells were treated with different concentrations of $[Ca^{2+}]_o$. As shown in Fig. 2A-a, $[Ca^{2+}]_o$ increased the phosphorylation of ERK1/2 in a dose-dependent manner with a maximum obtained at 10 mM (Fig. 2A-b, $p<0.001$). To prove the mediatory role of the CaR in activating ERK1/2, we used a CaR agonist, spermine. Spermine induced the phosphorylation of ERK1/

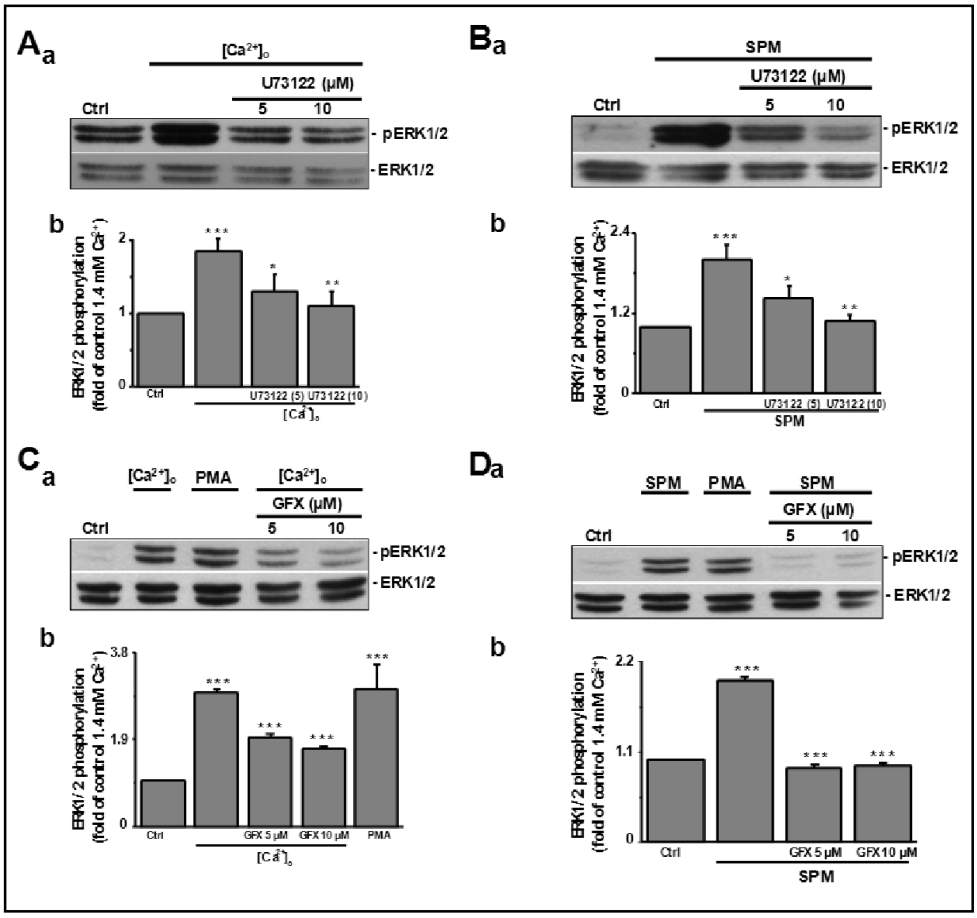

Fig. 3. Effects of PLC and PKC on CaR-evoked ERK1/2 phosphorylation in human breast cancer cells. (A-B) MCF-7 cells were treated with different concentrations of PLC inhibitor U73122 (5 - 10 μM) for 30 min before stimulation with 10 mM $[Ca^{2+}]_o$ or 200 μM spermine (SPM) for 20 min, and cell lysates were analysed by western blotting with anti-pERK1/2 or anti-total ERK1/2. Results shown in (a) are from a representative experiment, (b) data from three independent experiments. (C-D) MCF-7 cells were treated with 1μM PKC activator PMA for 10 min or pre-treated with PKC inhibitor GF109203X (GFX) with different concentrations (5 - 10 μM) for 30 min before stimulation with 10 mM $[Ca^{2+}]_o$ or 200 μM spermine (SPM) for 20 min, and cell lysates were analysed by western blotting with anti-pERK1/2 or anti-total ERK1/2. Results shown in (a) are from a representative experiment, (b) data from five independent experiments.

2 in a dose-dependent manner (Fig. 2B-a). At 50 μM, spermine failed to induce ERK1/2 phosphorylation, the maximum effect being obtained at 100 μM (Fig. 2B-a-b, $P<0.001$). Furthermore, $[Ca^{2+}]_o$ (Fig. 2C) and spermine (Fig. 2D) induced a strong ERK1/2 phosphorylation after 20 and 30 min treatments respectively. Moreover, a 30 min pre-incubation with U0126 (5 μM) totally abolished both $[Ca^{2+}]_o$ and spermine-stimulated ERK1/2 phosphorylation (Fig. 2E-F).

$[Ca^{2+}]_o$ and spermine induced ERK1/2 activation through PLC and PKC pathways in MCF-7 cells

We have previously demonstrated the recruitment of PLC in the CaR cascade in MCF-7 cells [20]. Moreover, it is well-known that PLC activation catalyzes the formation of IP_3 and DAG, an activator of protein kinase C (PKC). Thus, we investigated the hypothesis that PLC and PKC are necessary for CaR-induced ERK1/2 phosphorylation. Pre-treatment of MCF-7 cells for 30 min by 5 μM or 10 μM of a selective inhibitor of PLC (U73122) markedly reduced ERK1/2 phosphorylation induced both by $[Ca^{2+}]_o$ and spermine (Fig. 3 A-B). Nearly complete inhibition was reached at 10 μM (Fig. 3 Ab-Bb), a concentration that abolished the CaR induced intracellular calcium increase response in MCF-7 cells [20]. We further investigated the role of PKC in the downstream pathway of PLC activation. To determine whether PKC might be involved in mediating CaR-stimulated ERK1/2 activation, we evaluated the effect of an activator of PKC, PMA on ERK1/2 phosphorylation. Treatment of MCF-7 cells with PMA (1 μM) for 10 min caused a rapid phosphorylation of ERK1/2 similar to the effect obtained with both $[Ca^{2+}]_o$ and spermine (Fig. 3 C-D). To obtain further evidence of the link between PKC and ERK1/2 pathway in the CaR signaling, cells were treated for 30 min with GF109203X (5 and 10 μM), which is recognized as a highly selective and potent inhibitor of multiple PKC subtypes. Under these experimental conditions, GF109203X decreased the ERK1/2 phosphorylation induced by $[Ca^{2+}]_o$ and spermine (Fig. 3 C-D). However, GF109203X completely inhibit spermine-induced ERK1/2 phosphorylation (Fig. 3Da-b) but not that induced by $[Ca^{2+}]_o$ (Fig. 3Ca-b).

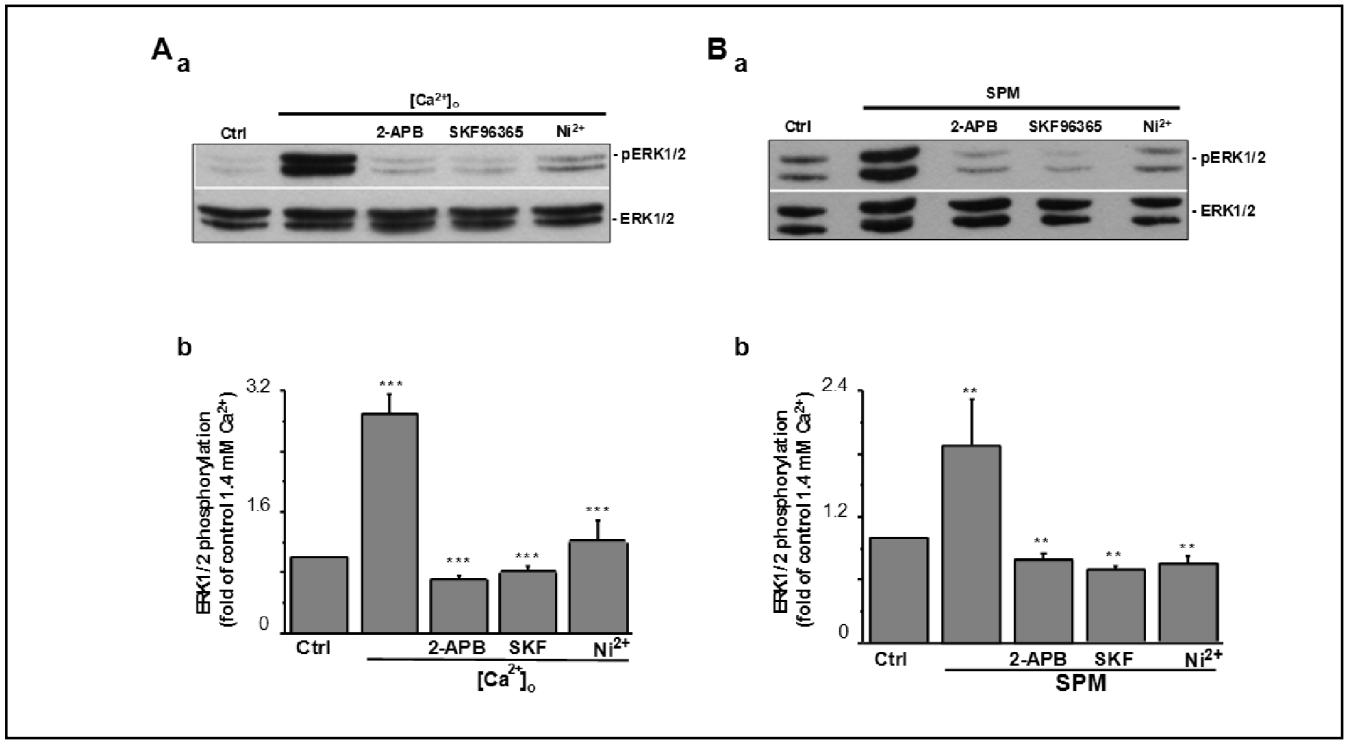

Fig. 4. Effect of Ca^{2+} entry on CaR-mediated ERK1/2 phosphorylation in human breast cancer cells. (A-B) MCF-7 cells were pre-treated for 30 min with 2-APB (50 μM), SKF96365 (20 μM) or Ni^{2+} (1 mM) before stimulation with 10 mM $[Ca^{2+}]_o$ or 200 μM spermine (SPM) for 20 min, and cell lysates were analysed by Western blotting with anti-pERK1/2 or anti-total ERK1/2. Results shown in (a) are from a representative experiment, (b) data from five independent experiments.

Role of the capacitative Ca^{2+} entry in the activation of ERK1/2

We previously demonstrated that the activation of CaR activates a Ca^{2+} entry via store operated channels [20]. Moreover, other works have documented the link between the Ca^{2+} entry and ERK1/2 phosphorylation [22]. Here, we investigated whether the Ca^{2+} entry evoked by the CaR was necessary for the activation of ERK1/2. To do this, we used organic and inorganic drugs known to inhibit the TRP channels, namely 2-APB, SKF-96365 and Ni^{2+}. As depicted in Fig. 4A, the activation of ERK1/2 by 10 mM $[Ca^{2+}]_o$ was completely prevented when cells were pre-treated by 2-APB (50 μM), SKF-96365 (20 μM), or by Ni^{2+} (1 mM) (Fig. 4Aa-b). Similar results were obtained under spermine-induced ERK1/2 activation (Fig. 4Ba-b).

We have previously demonstrated that TRPC1 is strongly expressed in MCF-7 cells and that CaR stimulation leads to the activation of selective calcium currents. We also suggested TRPC1 as a plausible candidate [20]. To determine whether the inhibition of TRPC1 channel would suppress ERK phosphorylation and Ca^{2+} entry, we used siRNA technology to down-regulate TRPC1 expression in MCF-7 cells. We first used western blot to confirm the effect of 50 nM TRPC1 siRNA on the protein expression in MCF-7 cells after 48h of transfection. Fig. 5A shows that, in siRNA-TRPC1 -transfected cells (siTRPC1), the TRPC1 protein level was significantly lower (-70 %) than in transfected control cells (siCT). Once the effect of siTRPC1 was confirmed, cells were transfected with siTRPC1 or siCT for 48 h, of which the last 24 h was for starvation period. Then, cells were stimulated with 10 mM $[Ca^{2+}]_o$ for 20 min. As shown in figure 5B-C, ERK1/2 phosphorylation was almost suppressed in MCF-7 transfected with TRPC1 siRNA when compared to MCF-7 transfected with the control siRNA suggesting the role of TRPC1 in ERK1/2 phosphorylation.

To investigate whether TRPC1 is required for Ca^{2+} entry, we carried out Ca^{2+} imaging experiments to monitor changes in $[Ca^{2+}]_i$ in cells transfected with siTRPC1 or siCT for 48 h. An increase in $[Ca^{2+}]_o$ from 1.4 to 10 mM elicited an increase in $[Ca^{2+}]_i$ both in cells transfected with siCtrl or siTRPC1 (n=143, Fig. 5D). However,

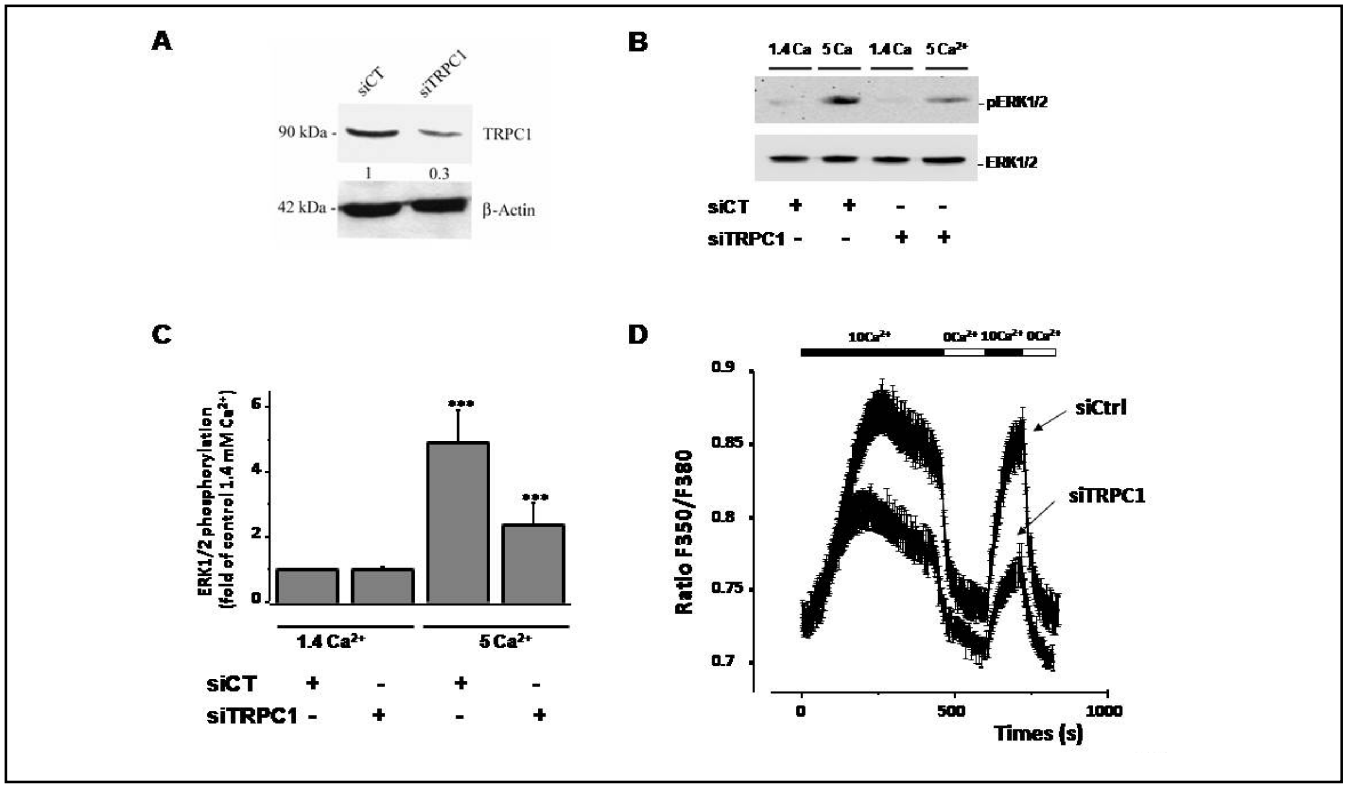

Fig. 5. Down-regulation of TRPC1 abolished both ERK1/2 phosphorylation and Ca^{2+} entry. A: Western-blot analysis of the TRPC1 expression 48 h after MCF-7 cell transfection with 50 nM TRPC1 siRNA (siTRPC1) or control 50 nM siRNA (siCT). After 48 h MCF-7 transfection with siTRPC1, cells were stimulated with 10 mM $[Ca^{2+}]_o$ for 20 min, and cell lysates were analysed by western blotting using anti-pERK1/2 or anti-total ERK1/2 antibodies. Results shown in (B) are from a representative experiment, (C) data from three independent experiments (P<0.001). D: Mean of $[Ca^{2+}]_i$ induced by 10 mM $[Ca^{2+}]_o$ (10 Ca^{2+}) recorded 48 h after transfection with siRNA TRPC1 (siTRPC1) compared to control siRNA (siCtrl, n=143, *P*<0.001).

$[Ca^{2+}]_i$ increase was significantly lower in MCF-7 transfected with TRPC1 siRNA compared with MCF-7 treated with control siRNA (n=143; *P*<0.01; Fig. 5D). Moreover, the removal of extracellular Ca^{2+} caused in both cases a sharp decline in $[Ca^{2+}]_i$ (Fig. 5D).

CaR activation induced TRPC1 overexpression through ERK1/2 dependent pathway

To further investigate the involvement of CaR in TRPC1 expression, we used real time quantitative PCR, immunoblotting and confocal imaging techniques to elucidate the relation between CaR activation and TRPC1 expression. Using real quantitative PCR we obtained a significant increase in TRPC1 mRNA in cells treated by 5 mM $[Ca^{2+}]_o$ for 24 h compared to control conditions (Fig. 6A). Moreover, in the presence of 5 µM of U0126, 5 mM $[Ca^{2+}]_o$ failed to overexpressed TRPC1 transcripts. To confirm our data, cells were immunostained with cholera toxin (green), used as plasma membrane marker and a TRPC1 polyclonal antibody (red). We observed a significant overexpression of TRPC1 in cells treated with 5 mM $[Ca^{2+}]_o$ for 24 h compared to controls (Fig. 6B). By contrast, 5 mM $[Ca^{2+}]_o$ failed to enhance expression of TRPC1 in the presence of 5 µM U0126 (Fig. 6A-B).

siRNA targeting of TRPC1 impaired the CaR-stimulated proliferation of MCF-7 cells

As TRPC1 activation and expression were correlated to CaR activation, we investigated the hypothesis of the physiological role of TRPC1 in CaR-induced cell proliferation in MCF-7 cells. To determine whether inhibition of TRPC1 would suppress MCF-7 cell proliferation induced by 5 mM $[Ca^{2+}]_o$, we used siRNA targeting to down-regulate TRPC1 expression. We pre-treated cells in the absence and in the presence of 5 mM $[Ca^{2+}]_o$ both with a transfectant solution and with siRNA control. Fig. 7A shows that both transfectant alone and siCT have no effect on MCF-7 cell proliferation and 5

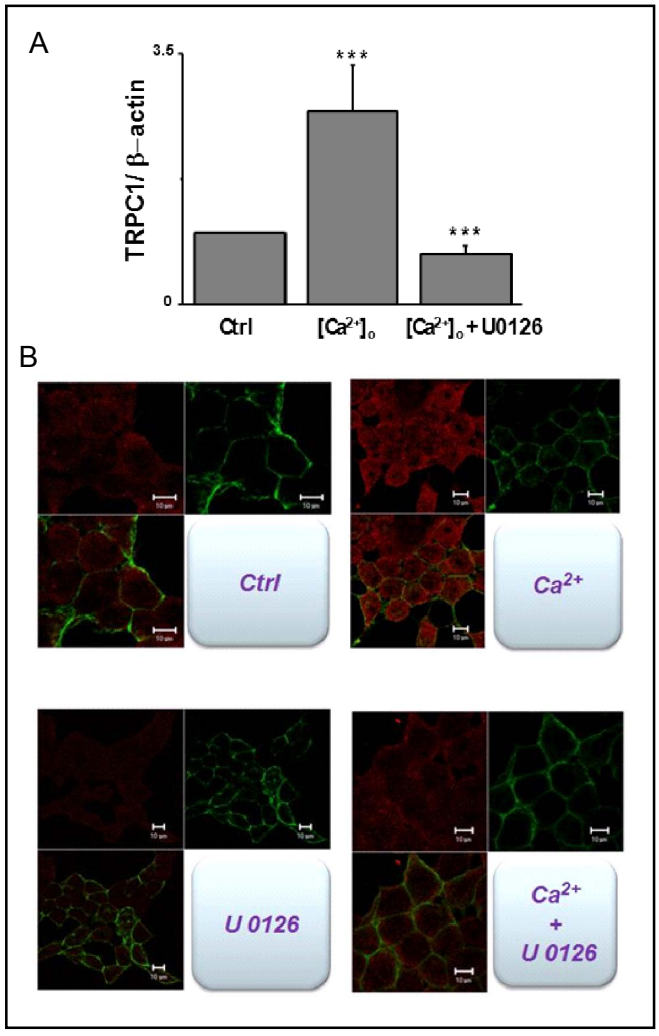

Fig. 6. ERK1/2 pathway-dependent modulation of TRPC1 expression by $[Ca^{2+}]_o$ in human breast cancer cells. A: Up-regulation of TRPC1 mRNA transcripts by high $[Ca^{2+}]_o$ treatment is demonstrated by real time-quantitative RT-PCR technique. MCF-7 cells were serum-deprived for 24h and then incubated with 5 mM $[Ca^{2+}]_o$ in the presence or in the absence of ERK1/2 inhibitor U0126 at the concentration of 5 μM. B: Up-regulation of TRPC1 protein by $[Ca^{2+}]_o$ treatment is demonstrated by a immunohistochemistry confocal technique as described in Material and Methods. MCF-7 cells were serum- deprived for 24h and then incubated for 24h with 1.4 mM (Ctrl), 5 mM $[Ca^{2+}]_o$ (Ca^{2+}), U 0126 alone (U 0126) and U 0126 with 5 mM $[Ca^{2+}]_o$ (Ca^{2+} + U 0126).

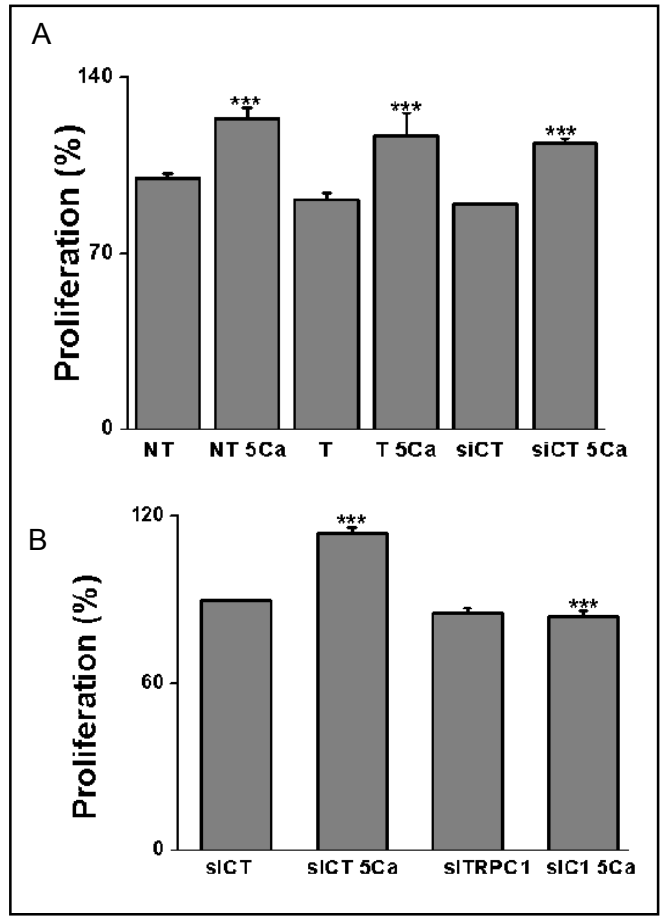

Fig. 7. Specific down-regulation of TRPC1 with siRNA inhibited the $[Ca^{2+}]_o$ proliferative effect in human breast cancer cells. A: For the proliferation assay, MCF-7 cells were seeded at a concentration of 1×10^5 cells/Petri dish (60 mm) and allowed to attach overnight. Cells were then transfected by a transfectant solution only (T), with control siRNA (siCT) or in presence of 5 mM $[Ca^{2+}]_o$ (siCT 5Ca). After, cells were cultured overnight in EMEM medium with 5% FBS. Then, cells were serum-deprived for 6 h and incubated with $[Ca^{2+}]_o$ (5 mM) for 24 h. After 24 h of $[Ca^{2+}]_o$ incubation, cell proliferation was determined by the Trypan blue method as described in Experimental procedures. $[Ca^{2+}]_o$ was unable to increase cell proliferation in cells transfected by the specific siTRPC1 5Ca (B) compared with cells transfected by control siCT 5Ca. Experiments were repeated three times and the indicated values are mean ± SE of six dishes. ***$p < 0.001$.

mM $[Ca^{2+}]_o$ increased cell proliferation under all the conditions studied (Fig. 7A). Transfection with siTRPC1 reduced cell proliferation induced by 5 mM $[Ca^{2+}]_o$ (Fig. 7B). Moreover, under each experimental condition, the mortality percentage was similar and lower than 4 % (data not shown).

Discussion

Despite the obvious significance and broad distribution of CaR in many cellular systems, including cancer cells [23-25], intracellular signaling pathways activated by the CaR in breast cancer cells are not yet

understood. This study provides evidence that $[Ca^{2+}]_o$ activates ERK1/2 thereby inducing MCF-7 cell proliferation through the CaR activation. This phosphorylation occurs through PLC and PKC-dependent pathways. Moreover, we demonstrated, for the first time, that TRPC1 is required for Ca^{2+} entry, ERK1/2 phosphorylation, and CaR-proliferative effect.

It has previously been shown that CaR enhances proliferation in astrocytoma, osteoblasts, fibroblasts, and myeloma cells [7, 17, 26, 27]. In contrast to Sanders et al., [2] who reported, on MCF-7 and MDA MB 231 cells, that incubation with CaR agonists (5, 7.5, and 10 mM $[Ca^{2+}]_o$, 300 µM neomycin and 2 mM spermine) for 6 h did not affect cell proliferation (estimated by MTT absorbance at 595 nm/655 nm), we show here that treatment of MCF-7 cells, during 24 h, by 5 mM $[Ca^{2+}]_o$ increased cell proliferation without any significant effect on the cell mortality rate. In contrast, 7 mM, $[Ca^{2+}]_o$ decreased cell proliferation and this effect could be explained by an increase in cell mortality rate.

A number of studies in several cell types, including CaR-transfected HEK-293 cells, have shown that CaR activates ERK1/2 [26, 28-32]. Consistent with these, our study reveales a significant up-regulation of phospho-ERK1/2 in MCF-7 incubated with $[Ca^{2+}]_o$, spermine or neomycin. Importantly, phospho-ERK1/2 induced by both $[Ca^{2+}]_o$ and spermine was almost completely abolished by U0126, a specific MEK1 inhibitor, indicating that their effects on ERK1/2 were mediated through the classic MEK1/ERK1/2 pathway.

Accumulating studies have shown that the activation of CaR induces cell proliferation through the activation of the ERK1/2 pathway [9, 10, 33]. Moreover, it has been reported that functional CaR is necessary for ERK1/2 activation in response to $[Ca^{2+}]_o$ [10]. In MCF-7 cells, we observed that treatment with the MEK1 inhibitor (U0126) prevented the increase in cell proliferation in response to $[Ca^{2+}]_o$, suggesting that $[Ca^{2+}]_o$-induced MCF-7 proliferation is MEK1/ERK pathway-dependent.

We previously reported that both $[Ca^{2+}]_o$ and spermine activate CaR through PLC-dependent pathways [20]. Moreover, in most cells investigated, CaR interacts with PLC, causing accumulation of IP3 [13, 30, 33, 34] and DAG, thus promoting a release of Ca^{2+} from endoplasmic reticulum and a Ca^{2+} entry [13]. To characterize the role of PLC in ERK1/2 activation in $[Ca^{2+}]_o$- and spermine, we studied the effect of the best known PLC inhibitor (U73122) on $[Ca^{2+}]_o$ and spermine activated ERK1/2 phosphorylation. U73122 inhibited both $[Ca^{2+}]_o$- and spermine–induced activation of ERK1/2,

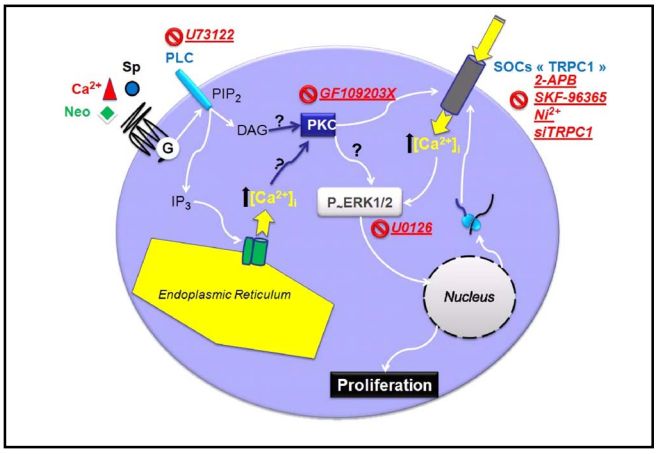

Fig. 8. Schematic diagram summarizing the principal findings on the involvement of CaR to control MCF-7 cells proliferation.

documenting the role of PLC in CaR-induced ERK1/2 activation. This phosphorylation would be due to a rise in intracellular Ca^{2+} and/or an activation of PKC by DAG. We then investigated the role of PKC in the ERK1/2 phosphorylation. The involvement of PKC in CaR-mediated ERK1/2 phosphorylation has been demonstrated in parathyroid cells and in HEK293-CaR cells based upon the effect of potent PKC activators and PKC-selective inhibitors [28, 35]. In accordance with these studies, we found that the activation of ERK1/2 by high $[Ca^{2+}]_o$ levels and spermine are reduced in the presence of GF109203X. Moreover, acute stimulation of PKC with phorbol ester PMA, under control conditions, was sufficient to induce ERK1/2 phosphorylation.

One of the novel findings in this report is the observation that the Ca^{2+} entry, presumably via TRPC1, is essential for ERK phosphorylation. Although several studies have shown that CaR activation by neomycin, spermine or elevated $[Ca^{2+}]_o$ induced a non-selective cationic current [17, 36-38], the nature of the entry channels is not presently known. In MCF-7 cells, we have previously provided direct evidence that CaR is functionally coupled to transmembrane Ca^{2+} entry via the PLC-catalyzed inositol phospholipid-breakdown signaling pathway [20]. Moreover, RT-PCR analysis showed the presence of the specific mRNA for only TRPC1 [20]. However, the relation between the Ca^{2+} entry activated by CaR and ERK phosphorylation remains unclear. Here, we show that the inhibition of the Ca^{2+} entry by the most widely used pharmacological SOCs inhibitors completely abolished ERK1/2 activation induced both by $[Ca^{2+}]_o$ and spermine. Unfortunately, specificity of SOC blockers is

largely discussed today. To overcome this problem, we used RNA interference, which represents a suitable alternative to functional blocker of channel activity. siRNA TRPC1 treatment resulted in a reduction in ERK phosphorylation induced by $[Ca^{2+}]_o$ (Fig. 5) to an extent comparable to that induced by 2-APB, SKF-96365 or Ni^{2+}. siTRPC1 treatment also reduced the Ca^{2+} entry recorded in the presence of high $[Ca^{2+}]_o$. Moreover, inhibition of PKC reduced ERK phosphorylation. The Ca^{2+} entry seems to be necessary for the rapid activation of PKCα, as was reported by Sakwe et al., [29] in HEK-CaR cells. Thus, in MCF-7 cells, we suggest that Ca^{2+} influx through TRPC1 channels triggers the translocation and stimulation of conventional Ca^{2+}-dependent PKC, perhaps the PKCα isoform, and the activated PKCα in turn activates an ERK1/2 cascade.

TRPC1 plays a critical role in sustaining both proliferation and the elevated $[Ca^{2+}]_i$ required for proliferation [39-41]. Indeed, TRPC1 expression is up-regulated during human smooth muscle cell proliferation and inhibition of its expression attenuates proliferation as well as store-depletion-induced Ca^{2+} influx [41]. Here, we found that longer applications (24 h) by $[Ca^{2+}]_o$ produced an increase in TRPC1 protein levels. Moreover, siRNA/TRPC1 treatment, which decreases TRPC1 protein levels in MCF-7 cells, reduced the percentage of proliferating cells. Importantly, TRPC1 protein levels are also markedly reduced when ERK phosphorylation was inhibited. These effects indicate that TRPC1 channels are associated with MCF-7 cell proliferation induced by the CaR activation through ERK1/2 activation. Recently Rey et al., [21] have reported, in CaR transfected HEK293 cells, that the $[Ca^{2+}]$i oscillations induced by the CaR activation were selectively abolished by TRPC1 down-regulation. However, in contrast to our results, they found these results only for the $[Ca^{2+}]$i oscillations stimulated by aromatic amino acids, yet not that induced through PLC. To summarize our results (i) the activation of CaR in MCF-7 induced only a transient $[Ca^{2+}]$i increase but (ii) not oscillations [20], (iii) CaR failed to activate cell proliferation when using siRNA against TRPC1, (iv) Ca^{2+} entry induced by the CaR activation is abolished when down-regulating TRPC1, and (v) inhibition of PKC, which is known to up-regulate TRPC1 channels [42], reduced $[Ca^{2+}]_i$ and abolished ERK phosphorylation.

We conclude (see Fig. 8), that the activation of CaR either by $[Ca^{2+}]_o$, or spermine elicits rapid, dose-dependent phosphorylation and activation of ERK1/2. This phosphorylation occurs through a PLC/PKC-dependent pathway. Moreover Ca^{2+} entry, presumably via TRPC1, is essential for ERK1/2 phosphorylation which, in turn, increases TRPC1 protein levels. Thus, the CaR proliferative effect is due to an up-regulation of TRPC1 protein through the ERK1/2 pathway. The down-regulation of TRPC1 by siRNA or inhibition of ERK1/2 phosphorylation altered the proliferative effect of CaR.

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