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Activation of the calcium-sensing receptor by high calcium induced breast cancer cell proliferation and TRPC1 cation channel over-expression potentially through EGFR pathways

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ABSTRACT

The calcium sensing receptor (CaR) is a G-protein-coupled receptor that is activated by extracellular calcium ($[Ca^{2+}]_o$). In MCF-7 human breast cancer cells, we previously reported that treatment with $[Ca^{2+}]_o$ for 24 h leads to an over-expression of the Transient Receptor Potential Canonical 1 (TRPC1) cation channel and cell proliferation. Both involve the extracellular signal-regulated Kinases 1 & 2 (ERK1/2). MCF-7 also expressed epidermal growth factor receptor (EGFR) which is involved in cell proliferation through ERK1/2. Therefore, we investigated the cross-talk between CaR and EGFR in mediating ERK1/2 phosphorylation, TRPC1 over-expression and cell proliferation. Our data show that both high $[Ca^{2+}]_o$ and EGF phosphorylate ERK1/2. Furthermore, inhibition of EGFR kinase and matrix metalloproteinases (MMPs) reduced the overall effects mediated by $[Ca^{2+}]_o$ such as activation of ERK1/2, expression of TRPC1 and cell proliferation. They indicate the important role of the CaR-EGFR-ERK axis in transmitting mitogenic signals generated by high $[Ca^{2+}]_o$ in MCF-7 cells.

Introduction

The calcium sensing receptor (CaR) is expressed in the epithelial ducts of the normal human breast [1,2]. It is localized in the latero-basal membrane and interacts with ionic transporters [1]. CaR is also expressed and functional in breast cancer cell lines and tissues [2,3]. Moreover, it was reported that the CaR is pre predominantly expressed in patients with advanced breast cancer who developed bone metastases [4]. Several studies have reported that CaR-stimulation, by high $[Ca^{2+}]_o$ levels activates the proliferation of many cell types including ovarian surface epithelial cells, osteoblast cells, aortic vascular smooth muscle cells and H-500 rat Leydig cancer cells [5–9]. When activated, the CaR stimulates several mitogen-activated protein kinases (MEK1&2, ERK 1&2, p38, and JNK) [10–13] and it has been reported that the proliferative effect of CaR is ERK1/2 dependent [6–8].

Epidermal growth factor (EGF) also has effects on breast tissue, working through its receptors (EGFR) and ErbB-2 (c-neu, HER2), both intrinsic tyrosine kinase growth factor receptors [14]. EGFR is relevant in the pathogenesis and behaviour of breast and other human cancers [15]. Moreover, high expression of EGFR in tumours correlates with poor survival and resistance to therapy [16]. Activation of the EGFR signaling pathway in cancer cells has been linked to increased cell proliferation, angiogenesis and metastasis, as well as decreased apoptosis [16]. In MCF-7 breast cancer cells, it has been reported that EGF, via EGFR, induced a growth stimulatory effect and this effect was ERK1/2 dependent [17–18].

Several data have been reported to show that G protein-coupled receptors (GPCR) will transactivate the epidermal growth factor receptor through the stimulation of matrix metalloproteinases (MMPs), which results in the extracellular release of a latent, membrane-spanning precursor of a member of the family of ligands known to activate the EGFR [19–20]. The ligand, either heparin-bound (HB)-EGF or transforming growth factor (TGF-α), will then activate the EGFR, causing stimulation of the MAP kinases [21].

We have previously reported that activation of the CaR by high extracellular Ca^{2+} leads to phosphorylation of ERK1/2 that participate in MCF-7 cell proliferation. This phosphorylation occurs through PLC and PKC-dependent pathways. Furthermore, we have also demonstrated that TRPC1 is required for Ca^{2+} entry, ERK1/2 phosphorylation, and CaR-proliferative effect [22]. Because the MCF-7 cell line expresses both the CaR and EGFR [2,3,23] and because the CaR is a G protein-coupled receptor, we hypothesized

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that the CaR activates EGFR and, in turn, ERK1/2 leading to MCF-7 cell proliferation and TRPC1 overexpression. Our results show that (i) both high $[Ca^{2+}]_o$ and EGF activated ERK1/2 inducing TRPC1 over-expression and MCF-7 cells proliferation, and (ii) the inhibition of the MMPs, known to be involved in the proteolysis of the EGF family ligands, significantly inhibited the CaR-mediated ERK1/2 phosphorylation, proliferation and TRPC1 cation channel over-expression in response to $[Ca^{2+}]_o$. Thus, in breast cancer cells, the CaR-EGFR-ERK pathways also participate in regulating 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 gentamicin (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.

RNA extraction and Q-PCR

Total RNA was isolated from human breast cancer MCF-7 cells by standard TRIzol extraction (Invitrogen, Carlsbad, CA) as 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), following the manufacture'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′-GCACGCCAGCAAGAAAAGC-3′ and 5′-GAGGTG ATGGCGCTGAAGG-3′. The amplified DNA length is 110 bp for TRPC1. The following primers were used to amplify a 226 bp of fragment β-actin cDNA 5′-CAGAGCAAGAGAGGCATCCT-3′ and 5′-A CGTACATGGTCGGGGTGTTGAA-3′. Primers were synthesized by Invitrogen (Fisher Bioblock, Ilkrich, France). Real-time PCR was performed on a Roche LightCycler® using the Absolute™ QPCR SYBR® Green I kit (ABGene, Courtaboeuf, France) according to the manufacture's protocol. The cDNA pool was diluted to a concentration of 5 ng/μl, of which 5 μl was 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 0.5× 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 (3×10^6 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 a serum-free medium prior to stimulation with $[Ca^{2+}]_o$ or EGF 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; cells were washed and unstitched using ice-cold phosphate-buffered saline (PBS) containing 0.5 mM EDTA. Afterwards, equal amounts of cells (1 million cells/1 ml) were centrifuged at 10,000*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 (10–12%) and transferred electrophoretically onto nitrocellulose membranes (Hybond-C super membrane; Amersham Life Science) and incubated with a 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 immuno-blotting 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 phosphorylation state to confirm equal loading of ERK proteins.

Cell proliferation assays

Cells were seeded in 35 mm Petri dishes at a density of 1×10^5 cells/dish, then allowed to attach overnight and subsequently 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 EGF or 5 mM $[Ca^{2+}]_o$ in the presence or absence 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.

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.

Chemicals

The EGFR inhibitor (AG1478), the MMPs inhibitor (GM6001), the MAPK kinase inhibitor (U0126) were obtained from Sigma–Aldrich (La Verpilliere, France) and were dissolved in DMSO. Final concentrations were obtained by appropriate dilution in an external control solution. The final DMSO concentration was <0.1%. EGF was dissolved in water.