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ERK binds, phosphorylates InsP3 type 1 receptor and regulates intracellular calcium dynamics in DT40 cells

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Abstract

Modulation on the duration of intracellular Ca^{2+} transients is essential for B-cell activation. We have previously shown that extracellular-signal-regulated kinase (ERK) can phosphorylate inositol 1,4,5-trisphosphate receptor type 1 (IP_3R1) at serine 436 and regulate its calcium channel activity. Here we investigate the potential physiological interaction between ERK and IP_3R1 using chicken DT40 B-cell line in which different mutants are expressed. The interaction between ERK and IP_3R1 is confirmed by co-immunoprecipitation and fluorescence resonance energy transfer (FRET) assays. This constitutive interaction is independent of either ERK kinase activation or IP_3R1 phosphorylation status. Back phosphorylation analysis further shows that type 1 IP_3R (IP_3R1) is phosphorylated by ERK in anti-IgM-activated DT40 cells. Finally, our data show that the phosphorylation of Ser 436 in the IP_3-binding domain of IP_3R1 leads to less Ca^{2+} release from endoplasmic reticulum (ER) microsomes and accelerates the declining of calcium increase in DT40 cells in response to anti-IgM stimulation.

Keywords: IP_3R1; ERK1/2; DT40; FRET; Phosphorylation

Inositol 1,4,5-trisphosphate receptors (IP_3Rs) are ubiquitously expressed intracellular calcium release channels and activated by a calcium-induced calcium release (CICR) process and sensitized by IP_3 binding and kinase phosphorylating [1–3]. IP_3Rs-mediated calcium signaling, which results from the activation of receptor tyrosine kinases and/or G protein-coupled receptors, regulates a number of cellular functions, including proliferation and apoptosis [4]. Among the three well-identified isoforms of IP_3Rs (IP_3R1, 2, and 3) in mammals, IP_3R1 dominates in T lymphocytes [5] and functions physiologically via cross-talk with other signaling pathways, including protein kinases. It has been established that IP_3R1 phosphorylation by PKA enhances Ca^{2+} release in both reconstituted lipid vesicle and intact cells. Other protein kinases involved in IP_3R1 phosphorylation include non-receptor protein tyrosine kinase Src family, protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase II, cGMP-dependent protein kinase (reviewed in Ref. [2]), Cdc2/cyclinB [6], and Akt [7].

Antigen receptors on B-cells (BCR) can elicit different maturation state-specific responses during B-cell differentiation. Many of the early signaling events are detected following BCR ligation, such as cytosolic two-phase Ca^{2+} rise or oscillations, and activation of the Ras/MAPK (mitogen-activating protein kinase) signaling cascades. The rapid IP_3-induced Ca^{2+} release from endoplasmic reticulum (ER) is followed by slow Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channel (CRAC, also called store operated calcium entry, SOCE). B-cell growth decisions in response to B-cell antigen receptor (BCR) activation are partly controlled by regulating the balance of Ca^{2+} store depletion, calcium entry through CRAC, and

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store refilling [8]. Sustained increases in $[Ca^{2+}]_i$ are required for the proliferation and differentiation of naive B-cells and failure to reach a threshold of intracellular Ca^{2+} level leads to altered cell migration and ultimately cell death [9]. Identification of the signaling molecules that regulate this balance of calcium signals in B-lineage cells therefore has important value for the understanding of both normal and abnormal immune responses. MAP kinase signaling can induce diverse biological responses in B-cells, which are dependent on the kinetics and amplitudes of activation, the intensity of the ERK signal, and the presence of additional signaling complexes [10]. Some reports have suggested that ERK/MAPK regulates Ca^{2+} signaling in different cell types [11,12]. A recent work of Jellerette et al. suggested that the metaphase-specific calcium oscillations in mouse zygotes might be attributed to the IP_3R1 phosphorylation by ERK or Cdc2 [13], which is consistent with our previous report [14].

Chicken-derived DT40 (3ko) B-cell line, in which all three types of IP_3Rs are deficient, is widely used for the research of IP_3R functions [15]. Here we transfected DT40 cells (WT and 3ko) with different mutants of IP_3R1 and/or ERK2 to investigate their potential functional interactions. Our results suggest that ERK2 binds constantly to IP_3R1 and the phosphorylation of Ser 436 in IP_3R1 by ERK2 negatively regulates IP_3R1 channel activity both *in vitro* and *in vivo*.

Materials and methods

Materials. $[^3H]IP_3$ was purchased from Perkin-Elmer (Boston), $[\gamma\text{-}^{32}P]ATP$ was from Amersham Pharmacia Biotech (Piscataway, NJ), ATP and IP_3 were from Sigma (St. Louis, MO), Recombinant mouse active MAPK1 (ERK2) was obtained from Calbiochem (San Diego, CA), and anti-ERK and anti-phospho-ERK antibodies were from Santa Cruz (CA) and Calbiochem (San Diego, CA), respectively. Anti-GFP was from BD Biosciences, anti-Chicken IgM monoclonal antibody was from Southern Biotech. Anti-IP_3R1 polyclonal antibody (T443) was a kind gift from Dr. Ilya Bezprozvanny (University of Texas Southwestern Medical Center, Dallas).

Oocyte collection. Immature fully grown GV (germinal vesicle) -stage oocytes were obtained from 4- to 6-week-old ICR mice as our previous report [16].

Immunoprecipitation and Western blotting. Triton X-100-solubilized extract from DT40 cells was prepared as described previously [17] and immunoprecipitation was carried out as described previously [14].

Back phosphorylation of the IP_3R1. Back phosphorylation of IP_3R1 immunoprecipitates was performed by a modification of the method described previously [18]. DT40 cell extracts (100 μl) were prepared by solubilization in Triton X-100-containing buffer and immunoprecipitated with T443 anti-IP_3R1 antibody. The immunoprecipitates were performed as previously reported [14], followed by 7.5% SDS–PAGE separation, transfer to PVDF membrane, and autoradiography.

Cell culture, transfection, and calcium analysis. DT40 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 1% chicken serum, 50 μM β-mercaptoethanol, penicillin, and streptomycin at 37 °C in a humidified incubator with 5% CO_2. For transient transfection, 2×10^7 cells in logarithmic phase growth were mixed with 30 μg of plasmid cDNA in 400 μl of cytomix medium [19]. Electroporation was performed in a 4-mm cuvette using Gene Pulser II apparatus (Bio-Rad) at 350 V, 975 μF. The transfected DT40 (wt) or DT40 (3ko) cells were cultured with the plasmids for a recovery period of 1–2 days in growth medium before sampling for fluorescent image or Western blotting. DT40 cells were stimulated with goat-derived anti-chicken IgM antibodies and the fluorescence was continuously monitored.

Expression constructs. CFP–IP_3R1: IP_3R1 was digested with *SacI* and *Xho*I, the ~8100 bp insert was then sub-cloned into pECFP-C1. This construct expresses ECFP as an N-terminal fusion with the IP_3R1. This fusion was chosen because its N-terminus is physically associated with the C-terminal tail of the IP_3R1 [20]. The CFP–IP_3R1 (S436A) mutant were generated as previously reported [14] and S436D mutants was generated by PCR using the Qickchange XL site-directed mutagenesis kit (Merk–Strategene). The primers used for S436D mutation were 5′-GCATTTGCC ATAGTTCCTGTTGATCCTGCTGAGGTTCGG-3′ and 5′-GGTCC CGAACCTCAGCAGGATCAACAGGAACTATGGC-3′. YFP–ERK2 (or dominant negative (dn)ERK): pEYFP was fused to the N-terminus of ERK2. The cDNA for ERK2 was achieved from mouse brain extraction using RT-PCR (ThermoScript RT, Invitrogen; Pfu DNA polymerase, Promega). PCR primers used for ERK2 were 5′-CGCGGCCGCGAAG GAGATATACATATG-3′ and 5′-ATTAGAGCTCAGTCACTACCAC TACTACTACTC-3′. The final PCR products of ERK2 were sub-cloned in-frame to pEYFP-C2 (from our Laboratory) and into the *Xho*I and *Bam*HI sites of pcDNA3.1(−)/myc-his C (Invitrogen), named YFP-ERK2. The ERK2 dominant–negative mutant (dnERK2) (Y185F) [21] was obtained using QickChange kit (Strategene). The primers used for dnERK2 mutation were 5′-TTCTTGACAGAGTTCGTAGCCA CACGTTG-3′ and 5′-TGTGGCTACGAACTCTGTCAAGAACCCT-3′. The construction of DsRedER was from our laboratory. All of above constructs were sequenced to confirm that no additional mutation was generated during PCR.

Microsome Ca^{2+} release assay. The isolation of DT40 cellular microsomes and measurement of IP_3-induced Ca^{2+} release were performed as previously reported [14].

Fluorescent imaging and FRET. The cells were imaged on a Zeiss microscope with a 20× or 40× Plan Neofluar lens (Carl Zeiss MicroImaging) equipped with a Photometrics CoolSNAP HQ cooled charge-coupled device camera (CCD, Roper Scientific, SenSys). Imaging was gathered with MetaFluor software using excitation at D480 nm and emission at 535 nm for YFP, using excitation at 568 nm and emission at 630 nm for DsRedER, and using excitation at 440 nm and emission at 480 nm for CFP. DT40 (wt) or DT40 (3ko) cells were in RPMI 1640 supplemented with 10% FCS, 1% chicken serum overlaid with mineral oil at 37 °C with a heated stage. Dual-emission ratiometric imaging was carried out with Metafluo-software. Emission filters were alternated using a computer-controlled filter wheel. Cells were excited at 440 nm and 535:480 nm ratiometric images were acquired every 10 s for 10 min. Data were pooled from at least three separate experiments for each trace.

Results

ERK2 interacts with IP_3R1 in DT40 cells

In a previous report [14], we presented the biochemical evidence showing that ERK interacted with and phosphorylated IP_3R1. In order to identify the potential physiological interaction between these two crucial signaling molecules, we transfected DT40 (3ko) cells with different mutants of ERK and/or IP_3R1. The protein expressions of these transfected recombinants were analyzed with Western blot as shown in Fig. 1. Yellow fluorescence protein (YFP)-labeled dnERK2 and cyan fluorescence protein (CFP)-labeled mouse IP_3R1 (including mutants) expressed in DT40 (3ko) cells amount to comparable levels to those of the wild type cells (Fig. 1A and B).

The binding between ERK and IP_3R1 was analyzed by co-immunoprecipitation from IP_3R1-transfected DT40