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Expression of a truncated form of inositol 1,4,5-trisphosphate receptor type III in the cytosol of DT40 triple inositol 1,4,5-trisphosphate receptor-knockout cells

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Abstract

In non-excitable cells, the inositol 1,4,5-trisphosphate receptor (IP_3R) is an intracellular Ca^{2+} channel playing a major role in Ca^{2+} signaling. Three isoforms of IP_3R have been identified and most cell types express different proportions of each isoform. The DT40 B lymphocyte cell line lacking all three IP_3R isoforms (DT40IP_3R-KO cells) represents an excellent model to re-express any recombinant IP_3R and analyze its specific properties. In the study presented here, we confirmed that DT40IP_3R-KO cells do not express any IP_3-sensitive Ca^{2+} release channel. However, with an immunoblot approach and a [^{3}H]IP_3 binding approach we demonstrated the presence of a C-terminally truncated form of IP_3R type III in the cytosolic fraction of DT40IP_3R-KO cells. We further showed that this truncated IP_3R retained the ability to couple to the Ca^{2+} entry channel TRPC6. Therefore, a word of caution is offered about the interpretation of results obtained in using DT40IP_3R-KO cells to study the cellular mechanisms of Ca^{2+} entry.

Keywords: Inositol 1,4,5-trisphosphate; Cytosol; Knockout cells

1. Introduction

Inositol 1,4,5-trisphosphate receptors (IP_3Rs) are intracellular Ca^{2+} channels that are activated by the second messenger IP_3, generated from the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C in response to a Ca^{2+}-mobilizing hormone [1,2]. IP_3Rs are located on the endoplasmic reticulum and their opening liberates stored Ca^{2+} that diffuses in the cytosol and triggers Ca^{2+}-dependent cellular responses. IP_3Rs play major roles in agonists-induced intracellular Ca^{2+} release and also in capacitative Ca^{2+} entry, a process whereby the depletion of intracellular Ca^{2+} store causes the opening of Ca^{2+} channels in the plasma membrane (for review, see [3]). Three separate isoforms of IP_3R, encoded by different genes, have been isolated, with each isoform exhibiting unique tissue-specific expression patterns [4–8]. IP_3R isoforms can assemble as homo- or hetero-tetramers to constitute functional channels [9–12]. Despite sharing considerable homology in terms of amino acid sequence, IP_3R isoforms can exhibit different binding affinities for IP_3, tissue distributions, expression levels, and potential regulation by cytosolic Ca^{2+} and phosphorylation by protein kinases [2,8,13–17]. To study their particular properties, functional assays were developed, in which specific IP_3R isoforms were transfected in mammalian cells expressing relatively low levels of endogenous IP_3Rs [18–20]. Nonetheless, in most of these studies, measurement of recombinant IP_3Rs properties has been hampered by significant contributions from endogenous IP_3Rs. The DT40 B lymphocyte cell line lacking all three IP_3R isoforms (DT40IP_3R-KO cells) has recently been developed [21]. This cell line represents an excellent model to express any recombinant IP_3R and to study its specific properties. However, it was argued that the disrupted IP_3R genes could give rise to expression of C-terminally truncated IP_3Rs in these cells [22]. Therefore, it is essential to determine whether the DT40IP_3R-KO cells are truly devoid of IP_3R translation products. In the study presented here, we provide evidence that DT40IP_3R-KO cells express a cytosolic C-terminally truncated IP_3RIII fragment that cannot form a functional Ca^{2+} channel but

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that retains the capacity to bind IP_3 and to interact with the Ca^{2+} entry channel TRPC6.

2. Materials and methods

2.1. Materials

Fura-2/AM and Fura-2 (free acid) were purchased from Calbiochem (San Diego, CA). Saponin, polyethylene glycol, Hepes, thimerosal, phosphocreatine, creatine phosphokinase, phenylmethylsulfonyl fluoride (PMSF), Isopropylβ-D-1-thiogalactopyranoside (IPTG), poly-L-lysine and soybean trypsin inhibitor were from Sigma (St. Louis, MO). The proteases inhibitor cocktail (Complete™) was from Roche Molecular Biochemicals (Laval, Qc). Immobilion-P polyvinylidene fluoride (PVDF) membranes were from Millipore (Bedford, MA). Mouse anti-IP_3RIII antibody (recognizing a N-terminal epitope) was from BD Biosciences Transduction Laboratories (Mississauga, ON). Rabbit anti-IP_3RI antibody (recognizing a N-terminal epitope) was from Affinity Bioreagent (Golden, CO). Rabbit anti-IP_3RII antibody (T2NH, recognizing a N-terminal epitope) was produced in the laboratory of. Gregory Mignery. Sheep anti-mouse IgG antibody coupled to horseradish peroxidase (SAM), Donkey anti-rabbit IgG antibody coupled to horseradish peroxidase (DAR), glutathione-sepharose 4B gel beads and ECL Plus Western blotting detection reagents were from Amersham Biosciences (Piscataway, NJ). Anti-chicken IgM (supernatant, M-4 clone) was from Southern Biotechnology Associates (Markham, ON). [^{3}H]IP_3 (23 Ci/mmol) was from Perkin Elmer (Boston, MA). IP_3 was from Alexis Biochemical (San Diego, CA). Texas red-conjugated goat antibody against mouse IgG was from Molecular Probes (Eugene, OR).

2.2. Cell culture and transfection

Wild type DT40 chicken B cells (DT40 cells) and DT40IP_3R-KO cells were cultured in RPMI 1640 (Invitrogen, Burlington, ON) supplemented with 10% heat-inactivated fetal bovine serum, 1% chicken serum, 21.4 mM $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin, 4 mM glutamine and 55 μM β-mercaptoethanol at 40 °C in a humidified atmosphere containing 5% CO_2. DT40IP_3R-KO cells were transiently transfected by electroporation in a medium containing 20 mM HEPES, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 6 mM glucose). A 25 μg of plasmid DNA encoding full-length TRPC6-Green Fluorescent Protein fusion protein (GFP-TRPC6) were added to DT40IP_3R-KO cells (10^7 cells) in a 0.8 ml cuvette (electrode gap of 0.4 cm) and electroporated at 275 mV, 975 μF, with the Gene Pulser II system (Bio-Rad). The cells were then plated in RPMI with 10% fetal bovine serum.

2.3. Cytosolic Ca^{2+} measurement

Cells (5×10^6) were washed twice by centrifugation for 4 min at 1000 × *g* with an extracellular-like medium (ECM) (120 mM NaCl, 5.3 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM dextrose and 20 mM HEPES, pH 7.4) before being incubated for 45 min at 40 °C with 5 μM Fura-2/AM in ECM. After three washes by centrifugation, cells were resuspended in 2 ml of ECM and gently stirred in a quartz cuvette maintained at 37 °C while $[Ca^{2+}]_i$ was monitored on a Hitachi F-2000 spectrofluorometer (Hialeah, FL) with alternative excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm. At the end of each recording, maximal fluorescence ratio (R_{max}) and minimal fluorescence ratio (R_{min}) were determined by adding successively 0.1% Triton X-100 and 10 mM EGTA to the cells suspension. $[Ca^{2+}]_i$ was calculated according to the equation of Grynkiewicz et al. [23].

2.4. IP_3-induced Ca^{2+} release

Cells (2.5×10^9) were washed by centrifugation for 4 min at 1000 × *g* with a cytosol-like medium (20 mM Tris–HCl at pH 7.2, 110 mM KCl, 10 mM NaCl, 5 mM KH_2PO_4 and 2 mM $MgCl_2$) supplemented with 1 mM EGTA. Cells were resuspended in 2 ml of permeabilization medium composed of the cytosol-like medium (without EGTA) supplemented with 30 μg/ml saponin, 1 μM Fura-2 acid, 20 U/ml of creatine kinase and 40 mM phosphocreatine. After 30 min at 37 °C, Ca^{2+} uptake (upon addition of ATP) and Ca^{2+} release (upon addition of IP_3) were monitored at 37 °C with a Hitachi F-2000 spectrofluorometer as previously described [24].

2.5. [^{3}H]IP_3 binding on permeabilized cells

Cells (6×10^7 cells/ binding point) were incubated in a binding medium containing (25 mM Tris–HCl pH 8.5, 100 mM KCl, 10 mM NaCl, 5 mM KH_2PO_4, 1 mM EDTA) supplemented with 30 μg/ml saponin. After 30 min at 37 °C, permeabilized cells were incubated for 15 min at 0 °C, in the presence of [^{3}H]IP_3 (15 000 cpm) and increasing concentrations of unlabeled IP_3. Non-specific binding was determined in the presence of 2 μM IP_3. Incubations were terminated by centrifugation at 5000 × *g* for 15 min at 4 °C. The pellets were solubilized and the receptor-bound radioactivity was evaluated by liquid scintillation spectrometry.

2.6. [^{3}H]IP_3 binding on cytosolic fractions

Cells (1.7×10^8 cells/binding point) were washed twice and permeabilized for 30 min at 37 °C in the binding medium supplemented with 90 μg/ml saponin. After centrifugation at 35 000 × *g* for 30 min at 4 °C, the supernatant was collected and aliquots were incubated for 5 min at 0 °C in the presence of [^{3}H]$InsP_3$ (15 000 cpm) and increasing concentrations of