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The predominant role of IP_3 type 1 receptors in activation of store-operated Ca^{2+} entry in liver cells

L. Jones [a], L. Ma [a], J. Castro [b], T. Litjens [a], G.J. Barritt [b], G.Y. Rychkov [a,*]

[a] School of Molecular and Biomedical Science, University of Adelaide, Adelaide, South Australia 5005, Australia
[b] Department of Medical Biochemistry, School of Medicine, Flinders University, Adelaide, South Australia 5001, Australia

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ABSTRACT

Physiologically, hormone induced release of Ca^{2+} from intracellular stores occurs in response to inositol 1,4,5-trisphosphate (IP_3) binding to its receptors expressed on the membranes of intracellular organelles, mainly endoplasmic reticulum. These IP_3 receptors act as channels, releasing Ca^{2+} into the cytoplasmic space where it is responsible for regulating a host of distinct cellular processes. The depletion of intracellular Ca^{2+} stores leads to activation of store-operated Ca^{2+} channels on the plasma membrane which replenishes lost Ca^{2+} and sustain Ca^{2+} signalling. There are three isoforms of IP_3 receptor, each exhibiting distinctive properties, however, little is known about the role of each isoform in the activation of store-operated Ca^{2+} entry. Recent evidence suggest that at least in some cell types the endoplasmic reticulum is not a homogeneous Ca^{2+} store, and there might be a sub-compartment specifically linked to the activation of store-operated Ca^{2+} channels, and Ca^{2+} release activated Ca^{2+} (CRAC) channel in particular. Furthermore, this sub-compartment might express only certain types of IP_3 receptor but not the others. Here we show that H4IIE liver cells express all three types of IP_3 receptor, but only type 1 and to a lesser extent type 3, but not type 2, participate in the activation of CRAC current (I_{CRAC}), while type 1 and type 2, but not type 3, participate in observed Ca^{2+} release in response to receptor stimulation. Presented results suggest that in H4IIE rat liver cells the sub-compartment of intracellular Ca^{2+} store linked to the activation of I_{CRAC} predominantly expresses type 1 IP_3 receptors.

1. Introduction

Exquisite regulation of cytoplasmic free Ca^{2+} concentration is essential for cell survival and function, with alterations controlling a variety of cell functions ranging from contraction and secretion to apoptosis [1]. Cytoplasmic Ca^{2+} concentration is altered via Ca^{2+} movement from two main sources — the extracellular space, where Ca^{2+} enters the cell via channels in the cell membrane, and intracellular Ca^{2+} stores, such as the endoplasmic reticulum (ER), Golgi, and mitochondria, which release Ca^{2+} into cytoplasmic space. In liver cells, as well as other cell types, inositol 1,4,5-trisphosphate receptors (IP_3Rs) expressed on the membranes of intracellular organelles, particularly ER, provide the main pathway for Ca^{2+} release in response to the activation of G-protein coupled receptors located on the plasma membrane [2,3]. When intracellular Ca^{2+} stores become depleted, store-operated Ca^{2+} channels on the plasma membrane are activated, allowing Ca^{2+} entry into the cell [4,5]. This mediates refilling of intracellular Ca^{2+} stores, enabling long-term calcium signalling [6].

There are three known isoforms of IP_3R — types 1, 2 and 3 [2,7]. Each has a specific affinity for IP_3, Ca^{2+} dependence, and tissue and subcellular distribution [2,8–13]. Moreover, each type of IP_3R is thought to have a specific role in generation and maintenance of Ca^{2+} oscillations in different cell types [9,14]. Recent investigations suggest that the functional specificity of different IP_3R isoforms also extends to their roles in the activation of store-operated Ca^{2+} currents across the plasma membrane, such as Ca^{2+} release activated Ca^{2+} current (I_{CRAC}). In avian B cells (DT40), which normally express all three types of the receptor, only IP_3R2 and IP_3R3 participate in the activation of I_{CRAC} through IP_3-dependent Ca^{2+} release from the intracellular stores, while IP_3R1 does not [15]. In freshly isolated rat hepatocytes, however, an IP_3R agonist with higher affinity for type 1 receptors has significantly stronger effects on Ca^{2+} release and Ca^{2+} entry than the agonists more selective for type 2 receptors, suggesting that a specialised region of the ER linked to activation of store-operated Ca^{2+} entry is enriched in IP_3R1, despite that IP_3R2 is the predominant isoform [16]. These and a wide range of other experimental results are consistent with a notion of ER Ca^{2+} store heterogeneity [17–22]. Furthermore, some dissociation between release of Ca^{2+} from intracellular stores and activation of CRAC channels on the plasma membrane lead to the hypothesis of a specialised CRAC store

Abbreviations: IP_3, inositol 1,4,5-trisphosphate; IP_3Rs, inositol 1,4,5-trisphosphate receptors; CRAC, Ca^{2+} release activated Ca^{2+}; SOCE, store-operated Ca^{2+} entry; Rn, rattus norvegicus

* Corresponding author. Tel.: +61 8 8303 3979; fax: +61 8 8303 3356.
E-mail address: Grigori.Rychkov@adelaide.edu.au (G.Y. Rychkov).

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specifically linked to activation of I_{CRAC} [15,17]. In contrast, in pancreatic acinar cells ER seem to represent a continuous Ca^{2+} store, and Ca^{2+} diffusion inside ER is virtually unrestricted [23].

In the present study we investigated the role of each type of IP_3 receptor in activation of I_{CRAC} in H4IIE rat liver cells. We have found that although all three types of IP_3 receptor are expressed in these cells in comparable amounts, only the siRNA-mediated knockdown of IP_3R1 and IP_3R3, but not IP_3R2, results in a significant reduction in the amplitude of the I_{CRAC} activated by intracellular perfusion with IP_3. At the same time, agonist-induced Ca^{2+} release from the stores was affected by the knockdown of IP_3R1 and IP_3R2, but not IP_3R3. These results lend further support for the notion of a specialised Ca^{2+} store linked to the activation of store-operated Ca^{2+} channels and suggest that the properties of this Ca^{2+} store are cell type specific.

2. Materials and methods

2.1. Cell culture

H4IIE cells were cultured at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.1% l-glutamine [24]. For some experiments cells were grown in culture medium supplemented with 100 nM insulin (Sigma) and 100 nM Dexamethasone (Sigma).

2.2. siRNA design

siRNA sequences were designed for IP_3R1, IP_3R2 and IP_3R3 according to published criteria, while control siRNAs, used to evaluate any nonspecific effects of siRNA transfection, had no homology to any known mammalian genes [25]. To aid in the identification of siRNA transfected cells a fluorescently labelled control siRNA was added to all transfections. As longer siRNA has been reported to enhance knockdown and ensure maximal specificity, IP_3R2 and its corresponding control siRNA were extended to 27 base pairs in length compared to 21 base pairs for IP_3R1 and IP_3R3 and their corresponding control (Table 1) [26]. Based on preliminary experiments, all transfections were incubated for 72 and 96 h using 80 nM siRNA. siRNAs were made by Qiagen, Germany.

2.3. Immunofluorescence and western blotting

Immunofluorescence and Western blotting were performed at 72 and 96 h post transfection as previously described [24]. Cells grown on glass coverslips were fixed with cold methanol at −20 °C for 5 min and then blocked for 15 min at room temperature with 20% FBS (v/v) in phosphate buffered saline (PBS). The coverslips were then incubated for 4 h, at room temperature, with the specific anti-IP_3R primary antibody (IP_3R1: polyclonal rabbit diluted 1:200, Affinity Bioreagents; IP_3R2: polyclonal goat diluted 1:200, Santa Cruz; IP_3R3 polyclonal mouse diluted 1:50, BD Biosciences). This was followed with either one-hour incubation with fluorescein isothiocyanate-labelled goat anti-rabbit IgG secondary antibody diluted 1:500 (Abcam) for IP_3R1, or with Texas red-labelled bovine anti-goat IgG secondary antibody diluted 1:400 (Santa Cruz) for IP_3R2 and Cy3 donkey anti-mouse diluted 1:200 (Rockland) for IP_3R3. Coverslips were mounted using a solidifying cell-mounting medium (Prolong gold, Molecular Probes), supplemented with DAPI (200 nM) to enable cell identification in the absence of IP_3 receptor labelling.

Protein extraction and Western blot techniques were performed as previously published by Su et al. [27]; however, protein samples were quantified using spectrophotometry (ShimadzuGene UV-1601) at 562 nm, with a Bicinchoninic acid protein assay kit (Sigma). For optimal protein separation a 7.5% SDS-page separating gel was used, in conjunction with a 4% SDS-PAGE stacking gel. Identification of IP_3R1, IP_3R2 and IP_3R3 protein utilised anti-IP_3R1 (diluted 1:500, Affinity Bioreagents), anti-IP3R2 (diluted 1:200, Santa Cruz) and anti-IP3R3 (diluted 1:200, BD Biosciences) primary antibodies. The secondary antibodies used included goat anti-rabbit IgG-HRP-conjugate, diluted 1:2000 (Abcam) for IP_3R1, donkey anti-goat IgG-HRP-conjugate, diluted 1:4000 (Santa Cruz) for IP_3R2 and donkey anti-mouse IgG-HRP-conjugate, diluted 1:1000 (Abcam) for IP_3R3. These combinations of primary and secondary antibodies produced western blots with prominent single bands above 240 kDa, corresponding to the predicted molecular weight of IP_3Rs.

As a control, membranes were stripped (100 mM beta-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl pH 6.8) for 30 minutes at 50 °C, and then re-probed with anti-GAPDH as per the foregoing protocol, using anti-GAPDH diluted 1:500 (rabbit polyclonal IgG, Santa Cruz Biotechnology).

To analyse the western blot, optical density measurements of IP_3Rs bands from control and IP_3Rs knockdown cells were taken using Scion Image software (Scion Corp., USA). These results were normalised to the respective GAPDH measurements, accounting for any loading differences between lanes.

2.4. Electrophysiology

Whole-cell patch clamping of H4IIE cells was performed using a computer-based patch-clamp amplifier and PULSE software (EPC-9, Heka electronics, Germany) as previously described [24]. The bath solution contained (mM): 140 NaCl, 4 CsCl, 10 $CaCl_2$, 2 $MgCl_2$, 10 glucose and 10 HEPES; adjusted to pH 7.4 with NaOH. Two different internal pipette solutions were used, depending on whether 20 μM IP_3 or 2 μM thapsigargin (Tg), a sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor, was used to promote store depletion. For use with IP_3, the internal pipette solution contained (mM): 120 Cs glutamate, 4 $CaCl_2$, 5 $MgCl_2$, 1 MgATP, 10 EGTA and 10 HEPES; adjusted to pH 7.2 with NaOH. For use with Tg the internal pipette solution contained (mM): 125 Cs glutamate, 5 $MgCl_2$, 10 EGTA, and 10 HEPES; adjusted to pH 7.2 with NaOH. Patch pipettes were pulled from borosilicate glass and fire-polished; pipette resistance ranged between 2 and 4 MΩ. Series resistance, for which no compensation was made, did not exceed 10 MΩ. In order to monitor the development of I_{SOC}, voltage ramps between −120 and +120 mV were applied every 2 s, starting immediately after achieving the whole-cell configuration. Acquired currents were filtered at 2.7 kHz and sampled at 10 kHz. All averaged data shown on the graphs are presented as a mean ± SEM. Unpaired t test has been used to compare the data sets obtained under different conditions. All voltages shown are nominal voltages not corrected for the liquid junction potential between the bath and electrode solutions. The holding potential was 0 mV throughout. Cell capacitance was compensated automatically by the EPC9 amplifier.

2.5. Measurements of $[Ca^{2+}]_{cyt}$ using Fura-2

To estimate $[Ca^{2+}]_{cyt}$ H4IIE cells grown on glass coverslips were loaded at room temperature for 30 min with 5 μM Fura-2 AM in Krebs-Ringer-HEPES buffer (KRH) containing 0.02% (v/v) pluronic acid. The KRH solution contained (mM): NaCl, 136; KCl, 4.7; $CaCl_2$, 1.3; $MgCl_2$, 1.25; glucose, 10; and HEPES, 10; adjusted to pH 7.4 with NaOH. After 30 min of de-esterification period, the fluorescence emission of Fura-2 was imaged at 340 and 380 nm by selecting regions of interest (ROI) under 40× objective of a Nikon TE300

Table 1
siRNA sequences. siRNAs designed for types 1, 2 and 3 IP_3Rs, and their respective controls.

siRNA	Sense sequence
IP_3R1	5′-AGCAGACACGAUAGUGAAAdTdT-3′
IP_3R3	5′-CAAGCAGUCUGUAUUUGGUdTdT-3′
$IP_3R1/3$ control	5′-UUCUCCGAACGUGUCACGUdTdT-3′
IP_3R2	5′-CCGUAUCUCUUACAUGCUGUCCAUAdTdT-3′
IP_3R2 control	5′-UUCUCCGAACGUGUCACGUGAAAUUdTdT-3′

epifluorescence microscope in conjunction with a Sutter DG-4/OF wavelength switcher, Omega XF04 filter set for fura-2, Photonic Science ISIS-3 ICCD camera and UIC Metafluor software, as described previously [18,24]. Values of fluorescence ratio were converted to values of $[Ca^{2+}]_{cyt}$ using an *in situ* calibration method and a K_d value of 224 for the Ca^{2+}-Fura-2 complex [28]. Paired t test has been used to compare the data obtained from cells used for measurements on the same day.

2.6. qRT-PCR

Total RNA was extracted from H4IIE cells using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Contaminating DNA was removed from total RNA extraction using TURBO DNA-free™ kit (Ambion) according to the manufacturer's instructions. RNA integrity was electrophoretically verified by ethidium bromide staining showing clear 28S and 18S bands with a ratio of about 2:1 and by OD_{260}/OD_{280} nm absorption ratio >2.0 (measured with Eppendorf BioPhotometer 6131). The same amounts of total RNA (3 μg) from different samples was then reverse transcribed with 200 U SuperScript™ III reverse transcriptase (Invitrogen) using 400 ng of oligo (dT) primers (Qiagen) according to the manufacturer's instructions. The real-time PCRs were run on Rotor-Gene 3000 (Corbett). The amplification efficiency (E) was determined by Rotor-Gene 6.0.19 software and the threshold was set manually above the baseline within the exponential growth region of the amplification curve. The relative expression ratio (R) of a target gene (*Rn_Itpr1, Rn_Itpr2, or Rn_Itpr3*) was calculated using amplification efficiency (E) and threshold cycle (C_t) of each target gene relative to that of the reference gene (*Rn_Gapdh*) in the same sample, using the following equation [29].

$$R = \frac{\left(E_{\text{target}}\right)^{C_{t\,target}}}{\left(E_{\text{ref}}\right)^{C_{t_{\text{ref}}}}}$$

3. Results

To ascertain the role of each type of IP_3 receptor in activation of the I_{SOC} in liver cells we used siRNAs to specifically knockdown each isoform of the receptor, immunofluorescence and Western blotting to confirm the effectiveness of the knockdown, and patch clamping and fura-2 Ca^{2+} imaging to investigate the functional consequences. First, the initial immunofluorescence experiments using IP_3R isoform-specific antibodies showed that non-transfected H4IIE cells or cells transfected with a control siRNA, express all three types of the IP_3 receptor (Fig. 1A). Subsequent Western blotting analysis confirmed that all three types of IP_3 receptor are expressed in H4IIE cells in clearly detectable quantities (Fig. 1B). H4IIE cells transfected with siRNA targeted against IP_3R1, IP_3R2 or IP_3R3 showed a substantial decrease in specific immunofluorescence at 72 h post transfection with maximum reduction at 96 h post transfection (Fig. 1A). The reduction in the protein levels of different IP_3Rs in transfected cells was confirmed by Western blotting (Fig. 1B). Densitometry analysis showed 60% to 80% knockdown of each IP_3R at 96 h post transfection. On average, the amounts of IP_3R1, IP_3R2 and IP_3R3 proteins were reduced by $63 \pm 7\%$ (n = 4), $60 \pm 4\%$ (n = 3), and $79 \pm 4\%$ (n = 3), respectively. IP_3R2 was the most difficult to knockdown. Several 21-base pair siRNAs designed against IP_3R2 failed to produce a significant effect, with the knockdown not exceeding 30%. Difficulties in knocking down expression of IP_3R2 have been reported previously by others [14]. Therefore, we used a 27-base pair siRNA against IP_3R2, which allowed levels of knockdown above 50%.

As we could not achieve a 100% transfection rate and a complete knockdown of IP_3Rs, a negative control siRNA labelled with Alexa Fluor-546 was used in patch clamping experiments to ascertain the level of transfection with siRNA [24]. In H4IIE cells transfected with a 21-base pair negative control siRNA, intracellular perfusion with 20 μM IP_3 activated I_{SOC} with an average maximal amplitude of -3.53 ± 0.40 pA/pF at −100 mV (n = 14) (Fig. 2). The amplitude and the time course of development of I_{SOC} in cells transfected with control siRNA were the same as those in non-transfected cells and cells treated only with the transfection reagent (not shown) [24]. Since the magnitude and time course of development of I_{SOC} recorded from cells transfected with control siRNA for 72 and 96 h were the same, these results were pooled and used as a control for both time points. Only cells containing fluorescently labelled siRNA were used for patch clamping. Transfection with siRNA against IP_3R1 reduced the average maximal amplitude of I_{SOC} measured at −100 mV to -1.17 ± 0.30 pA/pF (n = 13) and -0.60 ± 0.19 pA/pF (n = 13) at 72 h and 96 h post transfection, respectively (Fig. 2A). However, when thapsigargin was used to activate I_{SOC}, the average maximal amplitude of the current in cells transfected for 96 h with siRNA targeted against IP_3R1 (-2.43 ± 0.41 pA/pF (n = 9)) was not significantly different from that in cells transfected with control siRNA (-2.42 ± 0.36 pA/pF (n = 9)) (Fig. 2B).

Transfection with siRNA against IP_3R3 also resulted in a reduction of the I_{SOC} amplitude to -2.71 ± 0.45 pA/pF (n = 9) and -1.65 ± 0.3 pA/pF (n = 9) at 72 h and 96 h post transfection, respectively, when compared to 21-base pairs control siRNA (-3.53 ± 0.40 pA/pF (n = 14)) (Fig. 3A). However, knockdown of IP_3R3 had a substantially smaller effect on I_{CRAC} amplitude than the knockdown of IP_3R1. Increasing time post transfection to 5 days did not have any additional effect (not shown). As with IP_3R1, the knockdown of IP_3R3 only had an effect on I_{CRAC} amplitude when IP_3 was used to activate the current. When thapsigargin was used to activate I_{SOC}, the average maximal amplitude of the current in cells transfected for 96 h with siRNA targeted against IP_3R3 (-2.11 ± 0.19 pA/pF (n = 11)) was not significantly different from that in cells transfected with control siRNA (-2.42 ± 0.36 pA/pF (n = 9)) (Fig. 3B). In contrast, knockdown of IP_3R2 by a specific siRNA had no effect on the I_{CRAC} activated by IP_3 (Fig. 4). Using double transfections 24 h apart did not change the result (not shown).

To ascertain what effect the knockdown of each type of IP_3Rs has on emptying of intracellular Ca^{2+} stores, we investigated agonist-induced Ca^{2+} release using Fura-2. Physiologically, in hepatocytes IP_3-mediated empting of Ca^{2+} stores occurs in response to agonists of G-protein coupled receptors [30,31]. However, H4IIE liver cells grown in normal cell culture medium do not respond to Ca^{2+} mobilising hormones (authors unpublished). Due to their small size, these cells are not amenable to intracellular injections that we previously used to introduce IP_3 in rat hepatocytes [16], nor did we have much success with membrane permeable IP_3 analogues. However, we found that when cell culture medium is supplemented with 100 nM dexamethasone and 100 nM insulin for several days, H4IIE cells become responsive to extracellular application of the P2Y-receptor agonist ATP (20–100 μM) and exhibit Ca^{2+} release from intracellular stores and Ca^{2+} entry through CRAC channels (Supplemental Fig). Therefore, we used H4IIE cells treated with dexamethasone and insulin to determine whether the knockdown of IP_3Rs resulted in a reduction of Ca^{2+} release from the intracellular stores. Specific knockdown of IP_3Rs using corresponding siRNAs resulted in a significant reduction of the amount of Ca^{2+}released by 50 μM ATP in cells treated with siRNA against IP_3R1 and IP_3R2 ($p < 0.02$, n = 6, paired t test) but not IP_3R3 (Fig. 5). Knockdown of IP_3R1 caused about 60% reduction in the peak of Ca^{2+} release, while knockdown of IP_3R2 reduced it by about 30% (Fig. 5B).

The foregoing results obtained using Western blotting and immunofluorescence indicate that all three isoforms of IP_3 receptor are present in H4IIE cells. However, they could not give definitive information about the relative expression levels of these receptors. To estimate relative abundance of each isoform of the IP_3 receptor we used quantitative real-time PCR. In H4IIE cells grown in normal DMEM, relative to the housekeeping gene GAPDH, mRNAs for IP_3R2

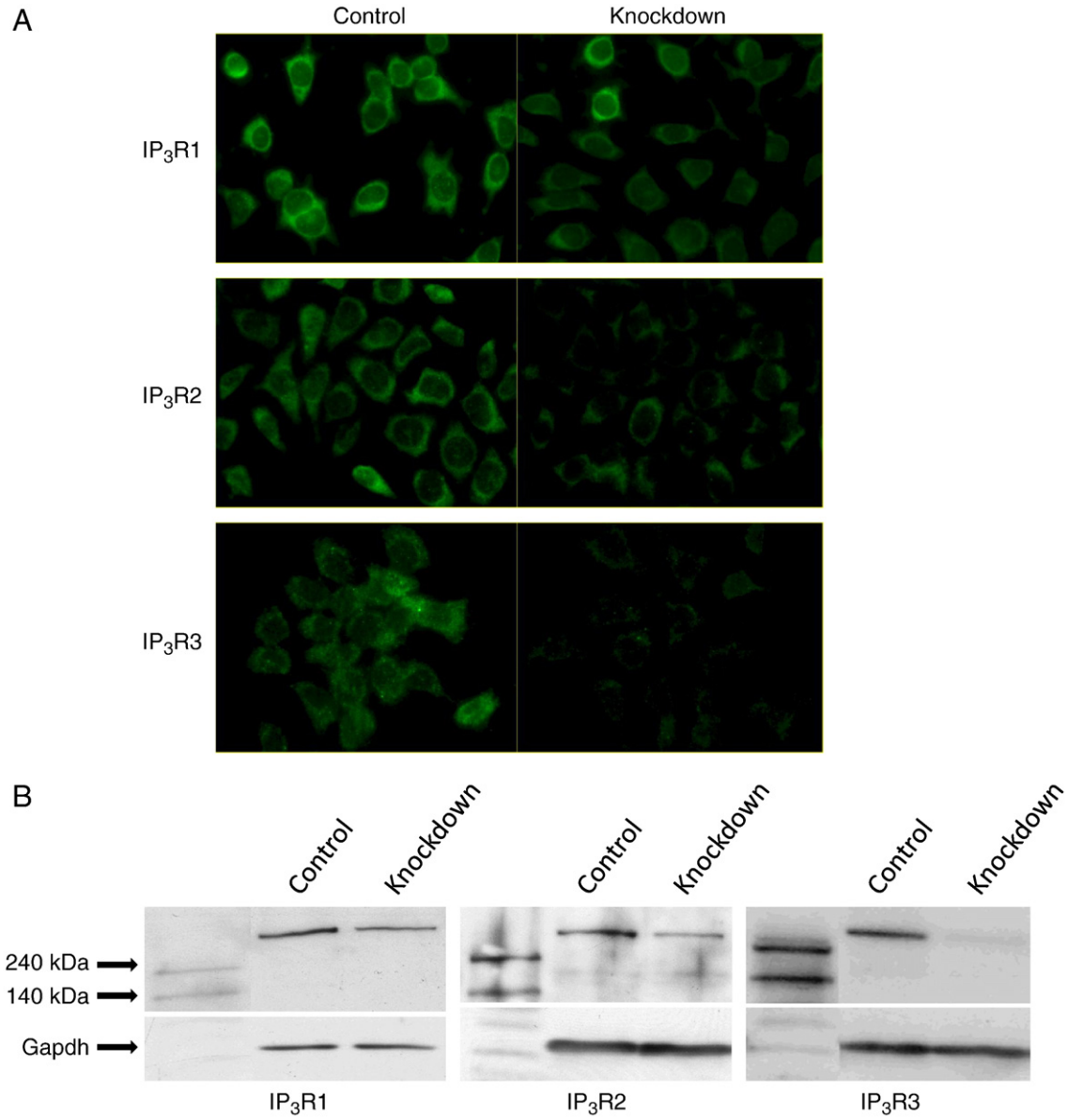

Fig. 1. Assessment of siRNA-mediated knockdown of each type of IP_3 R in H4IIE cells. A. Immunofluorescence microscopy of types 1, 2 and 3 IP_3Rs (green) in cells transfected for 96 h with negative control siRNAs (first column) and corresponding IP_3Rs siRNAs (second column). Results are representative of 4 individual experiments. B. Reduction of the types1, 2 and 3 IP_3R proteins as determined by Western blotting. Expression of GAPDH served as a loading control. Molecular weight markers are indicated on the left. Results are representative of 4 individual experiments.

and IP_3R3 were present in equal quantities, while IP_3R1 mRNA was significantly less abundant (Fig. 6). In these cells IP_3R1 mRNA amounted to about $16 \pm 0.5\%$ ($n = 4$) of the total IP_3Rs message, while IP_3R2 and IP_3R3 accounted for $42 \pm 1\%$ ($n = 4$) and $42 \pm 1.4\%$ ($n = 4$) correspondingly. Supplementation of the DMEM with dexamethasone and insulin had little effect on the mRNA levels of IP_3R2 and IP_3R3 relative to GAPDH. However, these agents increased the amount of IP_3R1 mRNA by a factor of 2 (Fig. 6). In cells treated with dexamethasone and insulin, IP_3R1 mRNA amounted for about $29 \pm 1\%$ ($n = 4$) of the total IP_3Rs message, while IP_3R2 and IP_3R3 accounted for $32 \pm 0.9\%$ ($n = 4$) and $39 \pm 1.4\%$ ($n = 4$) correspondingly.

4. Discussion

In this work we show that types 1, 2 and 3 isoforms of IP_3R do not contribute equally to activation of endogenous CRAC channels present in H4IIE liver cells. SiRNA-mediated knockdown of the least abundant IP_3R, type 1, resulted in attenuation of P2Y-receptor mediated Ca^{2+} release by approximately 60% and a virtually complete suppression of IP_3-mediated activation of I_{CRAC}. Knockdown of IP_3R2 reduced receptor mediated Ca^{2+} release by approximately 35%, but had no effect on the activation of I_{CRAC}. Knockdown of IP_3R3 had no observable effect on Ca^{2+} release at all, but resulted in a significant, but not complete reduction (~60%, 96 h post transfection) of I_{CRAC} amplitude activated by IP_3.

IP_3 receptors expressed on the membranes of intracellular Ca^{2+} stores provide the major pathway for Ca^{2+} release into the cytoplasmic space and play an integral part in physiological activation of store-operated Ca^{2+} channels [2,3]. Specific roles of different types of IP_3 receptor in generation and maintenance of cytoplasmic Ca^{2+} oscillations have been investigated in different cell types in some detail [2,10,14,32,33]. By contrast, the role of each isoform of IP_3 receptor in activation of SOCE is much less well understood.

In hepatocytes, Ca^{2+} oscillations and Ca^{2+} waves generated in response to IP_3 regulate glucose metabolism, bile acid transport and bile secretion, protein synthesis, propulsion of bile along the

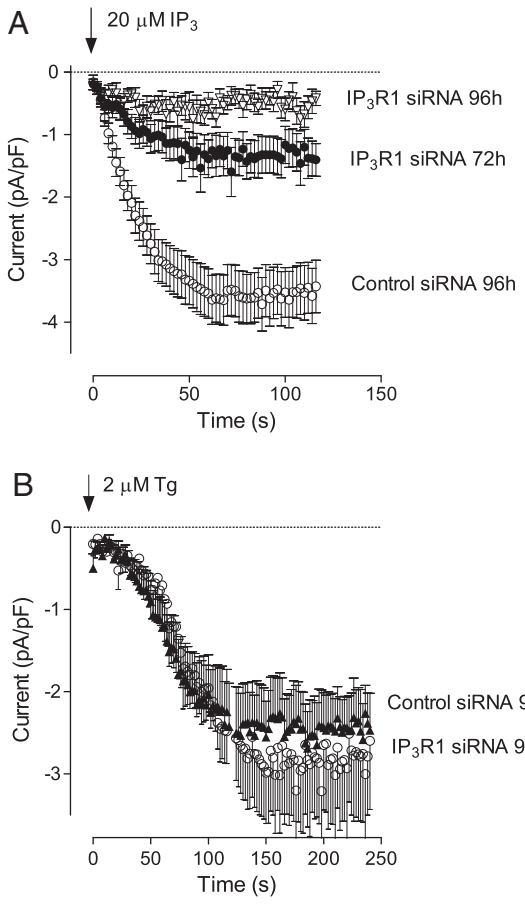

Fig. 2. Effect of IP_3R1 knockdown on I_{CRAC} in H4IIE cells. A. Development of I_{CRAC} in response to intracellular IP_3 in H4IIE cells treated with either control siRNA (n = 14) or IP_3R1 siRNA for 72 and 96 h (n = 13 for each condition). Unpaired t test showed significant difference between control group and 72 and 96 h treatments ($p < 0.0001$). B. Activation of I_{CRAC} by intracellular application of thapsigargin in H4IIE cells treated with either control siRNA (n = 9) or IP_3R1 siRNA for 96 h (n = 9). Each point represents the amplitude of I_{CRAC} at −100 mV taken from voltage ramps from −120 to 120 mV, applied every 2 seconds.

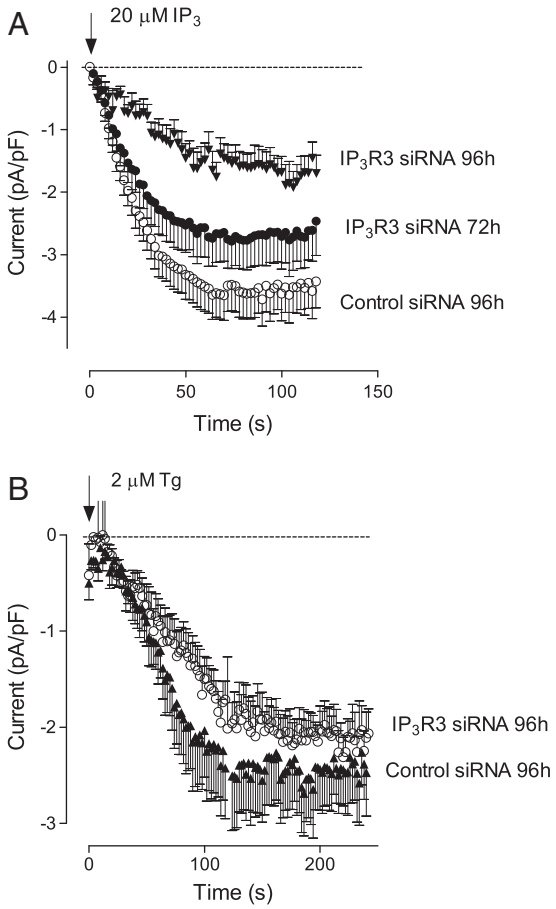

Fig. 3. Effect of IP_3R3 knockdown on I_{CRAC} in H4IIE cells. A. Development of I_{CRAC} in response to intracellular IP_3 in H4IIE cells treated with either control siRNA (n = 14) or IP_3R3 siRNA for 72 and 96 h (n = 9 for each condition). Unpaired t test showed significant difference between control group and 96 h treatment ($p = 0.003$). B. Activation of I_{CRAC} by intracellular application of thapsigargin in H4IIE cells treated with either control siRNA (n = 11) or IP_3R3 siRNA for 96 h (n = 9).

canaliculus, and many other functions [34]. Primary hepatocytes express two types of IP_3 receptors, IP_3R1 and IP_3R2, with IP_3R2 being the predominant isoform. *In situ*, IP_3R2 are mainly expressed at the canalicular membrane, while IP_3R1 are expressed elsewhere [33]. It is thought that IP_3R2 are required for the initiation of Ca^{2+} oscillations, since in hepatocytes the hormone-induced Ca^{2+} wave starts at the bile canaliculus and propagates to the basolateral region [33]. However, it has been suggested that the activation of SOCE by IP_3 in primary hepatocytes depends mainly on IP_3R1 [16]. Results of the present investigation show that in H4IIE liver cells IP_3R1 are indispensible for the activation of I_{CRAC}. Taken together these results suggest that the locations of the initial Ca^{2+} release and subsequent store-operated Ca^{2+} entry are spatially separated. It is interesting to note, that in polarised pancreatic acinar cells Ca^{2+} waves also start at the apical pole, where most of the IP_3 receptors are expressed, and propagate to basolateral region [35]. However, translocation of stromal interaction molecule 1 (STIM1), the endoplasmic Ca^{2+} sensor that interacts with Orai1, a pore-forming subunit of the CRAC channel on the plasma membrane, occurs at the basolateral region distant from the point of Ca^{2+} wave origination [35,36]. It has been suggested that such spatial separation of Ca^{2+} release and Ca^{2+} entry may contribute to the vectorial transport of Ca^{2+} in secretory epithelia [35].

The present results also confirm that IP_3Rs are not directly involved in the activation of I_{CRAC}. Thus before the discovery of STIM1 and Orai1, the molecular components of CRAC channels, IP_3 receptors were investigated as a potential direct link between Ca^{2+} stores and store-operated channels [37,38]. Later it became apparent that IP_3 receptors are likely not directly involved in the steps required for activation of I_{CRAC} following ER Ca^{2+} depletion. Thus, triple IP_3 receptor knockout DT40 chicken B-cells developed normal I_{CRAC} when

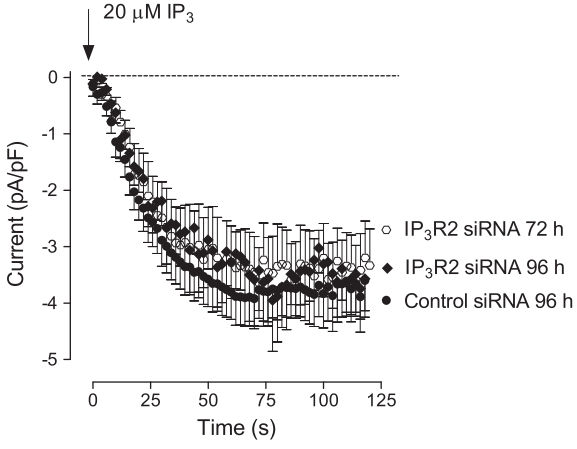

Fig. 4. Knockdown of IP_3R2 has no effect on the development of I_{CRAC}. Development of I_{CRAC} in response to intracellular IP_3 in H4IIE cells treated with either control siRNA (n = 10) or IP_3R2 siRNA for 72 and 96 h (n = 6). Each point represents the amplitude of I_{CRAC} at −100 mV taken from voltage ramps from −120 to 120 mV, applied every 2 s.

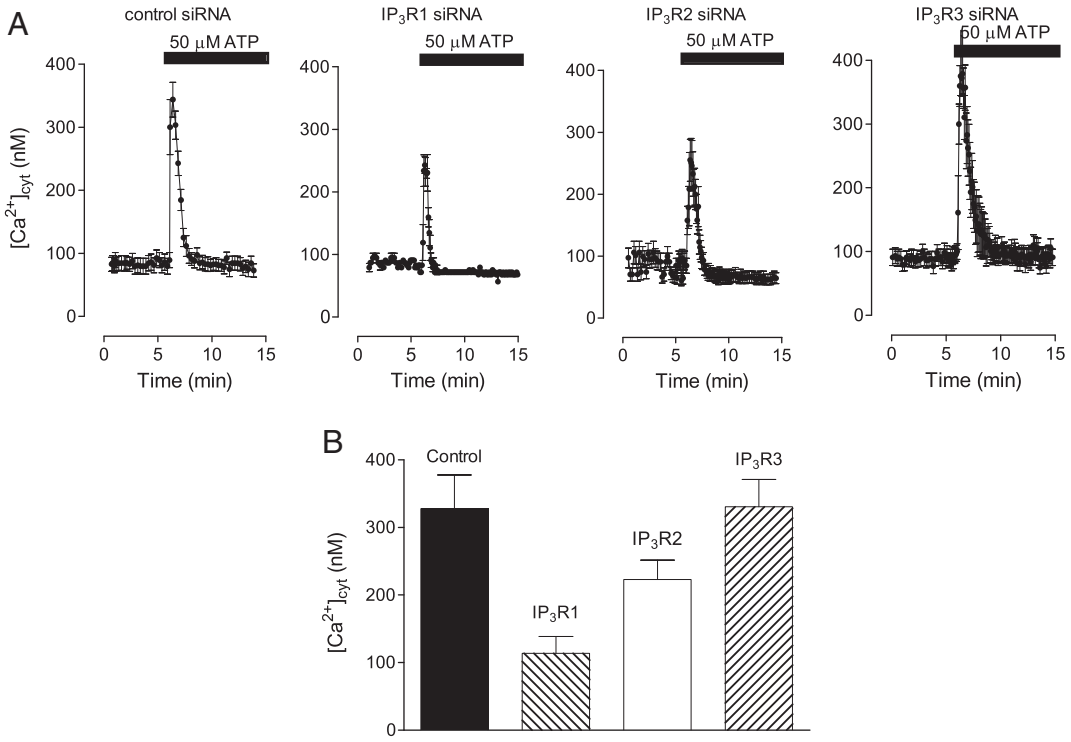

Fig. 5. Effect of the knockdown of different isoforms of IP_3 receptor on ATP-mediated Ca^{2+} release. A. Ca^{2+} release from intracellular stores initiated by application of 50 μM ATP to the bath in the absence of extracellular Ca^{2+} in H4IIE cells treated with either control siRNA, or siRNAs against different isoforms of IP_3Rs. H4IIE cells were grown in the cell culture medium supplemented with 100 nM dexamethasone and 100 nM insulin. Each trace represents averaged data ± SEM from 20 to 30 cells on the same coverslip. B. Peak Ca^{2+} release in H4IIE cells treated with control siRNA, or siRNAs against different isoforms of IP_3Rs. Data was averaged from three separate experiments. Paired t test confirmed significance of the reduction of Ca^{2+} release in cells treated with siRNA against IP_3R1 and IP_3R2 ($p<0.02$; $n=3–6$), but not IP_3R3.

stores were depleted by the inhibitors of SERCA pump [39]. Consistent with these studies, knockdown of any isoform of IP_3 receptor in H4IIE cells had no effect on the I_{CRAC} activation by thapsigargin.

Recent investigations of double IP_3 receptor knockout DT40 cell lines, each expressing only one type of IP_3 receptor, showed that cells possessing IP_3R3 alone developed normal I_{CRAC}. Cells expressing only IP_3R2 showed reduced I_{CRAC} amplitude, while cells expressing only IP_3R1 did not develop any I_{CRAC} in response to intracellular IP_3 [15]. It was concluded that the putative CRAC store in DT40 cells specifically expresses IP_3R3 and excludes IP_3R1, while IP_3R2 is partially expressed in that store. In H4IIE cells the results are opposite, it is the type 1 IP_3Rs that are fully accountable for the development of I_{CRAC}, and

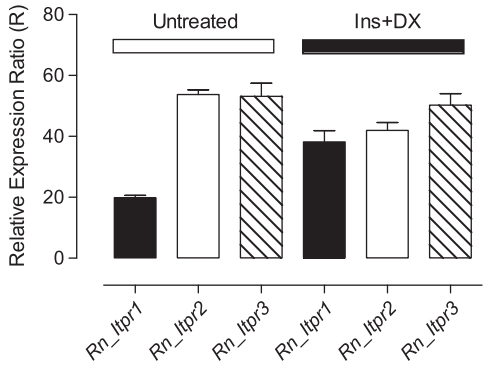

Fig. 6. Relative expression levels of each type of IP_3 receptor in H4IIE cells as determined by quantitative RT-PCR. Expression of types 1, 2 and 3 IP_3Rs relative to the expression of housekeeping gene (GAPDH) in H4IIE cells grown in the absence and presence of 100 nM dexamethasone and 100 nM insulin. Expression of IP_3R1 in dexamethasone and insulin treated cells was significantly weaker than expression of IP_3R1 in untreated cells (unpaired t test, $n=4$, $p=0.003$) and expression of any other type of IP_3R ($n=4$, $p<0.0001$).

presence of both IP_3R2 and IP_3R3 is insufficient for the development of a significant current in response to IP_3. Knockdown of IP_3R3, however, did decrease the amplitude of $I_{CRAC.}$ This suggests that IP_3R3 contributes to the activation of I_{CRAC} only when co-expressed with IP_3R1. Alternatively, knockdown of one isoform of the receptor changes the composition of heterotetramers formed by the remaining two IP_3R isoforms and leading to a change in their intracellular localization [40,41]. It is possible that, in the absence of IP_3R3, there is a higher probability of IP_3R1 and IP_3R2 forming heterotetramers but these are excluded from the putative CRAC store. The notion that I_{CRAC} dependence on IP_3R3 in H4IIE cells might be indirect is supported by the fact that the receptor-mediated Ca^{2+} release was unaffected by IP_3R3 knockdown. Interestingly, a complete lack of the effect of IP_3R3 knockdown on peak Ca^{2+} release shown here has been previously reported in HeLa cells and Cos-7 cells where IP_3R3 is the predominant isoform [14]. One possible explanation is that, of the three isoforms, IP_3R3 has the lowest affinity to IP_3 and might not be activated by the amounts of IP_3 produced by the receptor stimulation in these particular cell types, and might have a specific role involving activation in certain circumstances [14,42,43]. In patch clamp experiments, however, where saturating concentrations of IP_3 are used, IP_3R3 still can contribute to depletion of CRAC store and development of I_{CRAC}.

There are also other possible factors to be considered in comparing the effects of IP_3Rs knockdown on receptor-activated Ca^{2+} release and the amplitude of I_{CRAC}. In intact cells, receptor-mediated Ca^{2+} release will depend not only on the amount of IP_3 released but also on the spatial separation between a given subtype of IP_3 receptors and the G-protein coupled receptors on the plasma membrane. In patch clamping this effect is also likely negated by using a saturating dose of IP_3. Nevertheless, a significant reduction of the receptor-mediated Ca^{2+} release by the knockdown of IP_3R1 or IP_3R2, and a complete lack of the effect on the I_{CRAC} of the knockdown of IP_3R2 with virtually complete inhibition of I_{CRAC} by

the knockdown of IP_3R1 supports the notion of some dissociation between store release and activation of store-operated channels, and confirms that for any given cell type, one or more types of IP_3 receptor plays a specific role in activation of SOCE [15,16,44]. It seems, however, that the role that each type of IP_3 receptor plays in SOCE is not ascribed to the receptor type per se but depends on the cell type and probably the spatial location and specific properties of the ER.

In conclusion, we show here that in liver cells, among all three isoforms of IP_3Rs, the least abundant type 1 IP_3R has the biggest role in agonist-induced Ca^{2+} release and is indispensible for activation of I_{CRAC}. These results provide further evidence of heterogeneity of intracellular Ca^{2+} stores and specificity of each IP_3R isoform function.

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