The Transcription Factors NFAT and CREB Have Different Susceptibilities to the Reduced Ca^{2+} Responses Caused by the Knock Down of Inositol Trisphosphate Receptor in HEK 293A Cells

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Key Words

Inositol trisphosphate receptor • NFAT • CREB • Calcium signaling

Abstract

Background/Aims: The inositol 1,4,5-trisphosphate receptor (IP_3R), a ligand-gated Ca^{2+} channel, plays an important role in the control of intracellular Ca^{2+} . Three isoforms of IP_3R have been identified and most cell types express different proportions of these isoforms. The purpose of this study was to investigate how IP_3R signalling is involved in the activation of the Ca^{2+} -sensitive transcription factors NFAT and CREB. **Methods:** Each IP_3R isoform expressed in HEK 293A cells was knocked down using selective siRNA. Free intracellular Ca^{2+} was monitored spectrofluometrically. NFAT and CREB activities were measured with luciferase reporter constructs. **Results:** IP_3R -2-knocked down HEK 293A cells showed a deficient CCh-induced Ca^{2+} response that could be rescued by co-stimulation with VIP, a cAMP increasing agonist. NFAT transcriptional activity, but not CREB transcriptional activity, was significantly reduced in IP_3R -2-knocked down HEK 293A cells. Overexpression of IP_3R -1 could fully compensate for

IP_3R -2 knock down to mobilize Ca^{2+} and to activate NFAT. **Conclusion:** Our results show that the knock down of IP_3R -2 significantly reduced the intracellular Ca^{2+} response of HEK 293 cells. This reduced Ca^{2+} response did not affect the activation of CREB but significantly decreased the activation of NFAT, suggesting that the Ca^{2+} signals required for the activation of NFAT are stronger than those required for the activation of CREB.

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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 Ca^{2+} -mobilizing hormones [1-4]. IP_3Rs are located on the endoplasmic reticulum, and their opening liberates stored Ca^{2+} that diffuses into the cytosol and triggers Ca^{2+} -dependent cell responses. IP_3Rs play major roles in agonist-induced intracellular Ca^{2+} release and in store operated Ca^{2+} entry, a process whereby the depletion of the intracellular Ca^{2+} store causes the opening of Ca^{2+} channels in the plasma membrane [5]. The

elevation of the cytosolic free Ca^{2+} concentration is a dynamic signal regulating a variety of important cellular functions such as secretion, contraction, metabolism and gene transcription [6]. In mammalian cells, three IP_3R isoforms ($\text{IP}_3\text{R-1}$, $\text{IP}_3\text{R-2}$ and $\text{IP}_3\text{R-3}$) derived from three distinct genes have been identified, but the actual role of each isoform in physiological Ca^{2+} signaling remains to be established [7]. IP_3R isoforms are highly homologous and have the same overall structure, including an N-terminal ligand-binding domain, a C-terminal channel domain and an intervening coupling or regulatory domain [2, 8]. IP_3Rs associate in tetrameric structures that form functional Ca^{2+} channels. Individual cell types can express more than one IP_3R isoform, which may form different populations of homo- and hetero-tetrameric channels [9-12]. Although they share considerable homology in terms of amino acid sequence, IP_3R isoforms exhibit different IP_3 binding affinities, expression levels, tissue distributions and sensitivities to potential regulatory mechanisms such as Ca^{2+} , ATP and protein kinases [3, 4]. These differences are likely to confer specific pharmacological and functional properties on each IP_3R isoform, and the intracellular Ca^{2+} response may thus vary according to the IP_3R isoform(s) a cell expresses [7, 13]. Two transcription factors are known to be regulated by Ca^{2+} . Nuclear Factor of Activated T cells (NFAT) is directly activated by Ca^{2+} through the Ca^{2+} /calmodulin/calcineurin pathway, whereas cAMP Response Element Binding (CREB) is positively modulated by the Ca^{2+} /calmodulin/CamKII pathway [14-18]. Using a selective siRNA knock down approach, we evaluated the involvement of IP_3R signalling in the activation of NFAT and CREB in HEK 293A cells.

Materials and Methods

Material

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin-glutamine, Opti-MEM and HiPerfect transfection reagent were obtained from Gibco Life Technologies (Gaithersburg, MD). HEK 293A cells were from Qbiogene (Carlsbad, CA). Anti- $\text{IP}_3\text{R-1}$ antibody was obtained from Affinity Bioreagent (Golden, CO). Anti- $\text{IP}_3\text{R-3}$ antibody was obtained from BD Biosciences Transduction Laboratories (Mississauga, ON). Anti- $\text{IP}_3\text{R-2}$ antibody (epitope: NPDYRDAQNEGKNV) was customized by Genscript Corporation (Piscataway, NJ). Pan anti- IP_3R antibody (recognizing an epitope common to all three IP_3R isoforms) was from SantaCruz Biotechnology (Santa Cruz, CA). Lipofectamin 2000 transfection reagent, TRIzol®, Reverse Transcriptase Superscript II, oligonucleotides and siRNAs against human $\text{IP}_3\text{R-1}$, $\text{IP}_3\text{R-2}$ and $\text{IP}_3\text{R-3}$ (ITRPs Stealth™ select)

were obtained from Invitrogen (Burlington, ON). ATP, Poly-L-lysine hydrobromide, Bapta/AM, calmidazolium, EGF, FK 506, forskolin and VIP were from Sigma-Aldrich (Oakville, ON). Carbachol (CCh), fura2/AM, bisindolylmaleimide I (GF 1), cyclosporine A (CSA), KN-92, KN-93 were from Calbiochem (San Diego, CA). Dual Luciferase Reporter 1000 Assay System and Random Primers were from Promega (Madison, WI). Proteases inhibitors cocktail (Complete™) was from Roche Molecular Biochemicals (Laval, QC). BioMax ML film was from Eastman Kodak (Rochester, NY). iQSYBR Green Supermix was from Bio-Rad, (Mississauga, ON). The NFAT-luciferase reporter construct, in which the expression of luciferase is driven by multiple copies of the NFAT DNA-binding element, was a gift from J.D. Molken (Cincinnati Children's Hospital Medical Center, OH). The CREB-luciferase reporter construct, in which the expression of luciferase is driven by of cAMP response element, was a gift from T.E. Gunter (University of Rochester School of Medicine, NY). The cDNA encoding rat $\text{IP}_3\text{R-1}$ construct was a gift from Thomas Sudhof (Stanford University, CA).

Cell Culture

The HEK 293A cell line was cultured in complete DMEM (supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin) at 37°C in a humidified atmosphere containing 5% CO_2 . When 80% confluent, cells were passaged (1:8 dilution) every 3.5 days, using 0.25% trypsin and 1 mM EDTA.

Dynamic Video Imaging of Cytosolic Ca^{2+}

Fluorescence from fura2-loaded cells was monitored as previously described [19]. Briefly, siRNA-transfected HEK 293A cells grown on glass coverslips coated with poly-L-lysine were washed with HEPES-buffered physiological saline solution (HBSS: 20 mM HEPES pH 7.4, 120 mM NaCl, 5.3 mM KCl, 0.8 mM MgSO_4 , 1.8 mM CaCl_2 and 11.1 mM dextrose) and loaded with 0.1 µM fura2/AM for 20 min at room temperature in the dark. Cells were then washed and bathed in fresh HBSS for 20 min to ensure complete hydrolysis of the fura2/AM before inserting the coverslip into a circular open-bottom chamber that was mounted on the stage of a Zeiss Axiovert microscope fitted with an Attotfluor Digital Imaging and Photometry System (Attotfluor Inc., Rockville, MD). The system allows simultaneous data acquisition from up to 99 user-defined variably sized regions of interest per field of view. Fluorescence from isolated fura2-loaded cells was monitored by videomicroscopy using alternative excitatory wavelengths of 334 nm and 380 nm and recording emitted fluorescence at 510 nm. All experiments were done at room temperature and the data expressed as intracellular free Ca^{2+} concentration (nM) calculated from 334/380 fluorescence ratio according to Grynkiewicz et al. 1985[20]. Data acquisition was typically at 3 s intervals and lasted for 300 s.

Electrophoresis and Immunoblotting

HEK 293A cells (10^7 cells/ml) were solubilized for 1 h at 4°C in solubilization buffer (50 mM Tris/HCl at pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and the proteases

Targeted Gene	Oligonucleotide	Human siRNA sequence
IP ₃ R-1	1-1	5'-gag aau uuc cuu gua gac auc ugc a-3'
	1-2	5'-ggc gca gau aga cau ugu cag auu a-3'
	1-3	5'-ggc cug aga guu acg ugg cag aaa u-3'
IP ₃ R-2	2-1	5'-gag aag gcu cga ugc uga gac uug a-3'
	2-2	5'-gag cgg cuu cuu uaa uuc ucc cuu u-3'
	2-3	5'-ggg ccu gcu ugg gau uac aga aau a-3'
IP ₃ R-3	3-1	5'-ccg aga uga caa gaa gaa caa guu u-3'
	3-2	5'-cga gug guc uau gac cuc cug uuc u-3'
	3-3	5'-uca uca uca uug ugc uga acc uca u-3'

Table 1. Human siRNA sequences targeting IP₃Rs.

Gene	NCBI Accession Number	Forward Primer	Reverse Primer	Product Size (bp)
HPRT1	NM_013556	5-gcaaaccttgccttccctgg-3	5-acttcgagaggtcctttcacc-3	85
IP ₃ R-1	NM_002222	5-tgttacaagcccccattcaca-3	5-ttctgcatgaagccaaactg-3	184
IP ₃ R-2	NM_002223	5-accagagaactggtggatg-3	5-tgcagtgtggtggcataat-3	235
IP ₃ R-3	NM_002224	5-gtgagaactcggaggtgagc-3	5-atctgggacgtgtgttctc-3	256

Table 2. Primer sets used for quantitative PCR analyses. Accession number for HPRT1 (hypoxanthine phosphoribosyltransferase 1 gene) and IP₃Rs are the identifier assigned to the entire sequence recorded to RefSeq database available in NCBI Pubmed.

inhibitors cocktail Complete 1X). Insoluble material was precipitated by centrifugation at 35,000 xg for 30 min at 4°C. The supernatant was mixed with 2x Laemmli's buffer (60 mM Tris/HCl at pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 125 mM dithiothreitol, and 0.3% bromophenol blue) and heated for 5 min at 95°C. Samples were loaded onto a 5% polyacrylamide gel that was subjected to a constant current of 15 mA for 2 h. Proteins were electrotransferred to a nitrocellulose membrane at a constant voltage of 150 V for 2 h at 4°C. Blotted membranes were incubated for 1 h at room temperature in PBS-T (PBS containing 0.1% Tween-20) supplemented with 5% nonfat dried milk. Blots were then incubated overnight at 4°C with anti-IP₃R-1 (1:1000), anti-IP₃R-2 (1 µg/ml), anti-IP₃R-3 (1:5000) or pan anti-IP₃R (1:250) antibodies. After extensive washing with PBS-T, the blots were incubated for 1 h at room temperature with a peroxidase-conjugated secondary antibody, and after extensive washing with PBS-T, the immunostained bands were revealed with ECL Plus, according to the manufacturer's instruction and submitted to BioMax ML film. Integrated peak areas were determined using the gel analysis Quantity One software.

Selective IP₃R knock down

HEK 293A cells (250 000 cells/well) were seeded without antibiotic in a 6-well tissue culture dish 24 h prior to transient transfection with 10 µl of HiPerfect transfection reagent and 20 nM of appropriate siRNA (see Table 1 for sequences) or control scrambled siRNA mixed in 300 µl of Opti-MEM medium. SiRNA complexes were added dropwise to cells that were incubated

under normal cell culture conditions for 48 h before use. For measurement of transcriptional activities, cells were transfected with luciferase reporter constructs 24 h after siRNA transfection.

Quantitative PCR

Total RNA was extracted from HEK 293A cells, transfected or not with appropriate siRNA oligonucleotide, using TRIzol®. RNA (1 µg) was reverse-transcribed using Reverse Transcriptase Superscript II. The qPCR assays were performed using 50 ng of template cDNA. The conditions for all the reactions were as follows: an initial 5 min denaturation step at 95°C, followed by forty 40 sec cycles at 95°C, 56°C and 72°C. The qPCR assays were performed using a Rotor-Gene 6000 (Corbett Robotics, Australia) and iQSYBR Green Supermix. Results were calculated using the 2^{-ΔΔCT} relative quantification method normalized to hypoxanthine phosphoribosyltransferase 1 gene (HPRT1). The primer sets used are shown in Table 2.

Measurement of transcription factors activities

HEK 293A cells (2.5x10⁵ cells/well) were transiently cotransfected with an NFAT-luciferase reporter plasmid (pNFAT-luc, 1 µg) or a CREB-luciferase reporter plasmid (pCRE-luc, 1 µg) along with 30 ng of control *Renilla* luciferase plasmid (pRL-CMV) and 2 µl of lipofectamin Transfection Reagent in 300 µl of Opti-MEM medium. 16 h later, the cells were incubated for 5 h with different agonists or vehicle. Cells were then lysed and assayed for luciferase activity using the Dual Luciferase Reporter 1000 Assay System from Promega.

Fig. 1. Role of Ca^{2+} in the activation of NFAT and CREB. HEK 293A cells (5×10^5) were co-transfected with the NFAT-luciferase reporter vector pNFAT-Luc (A and B) or the CREB-luciferase reporter vector pCREB-Luc (C and D) along with the *Renilla* luciferase reporter vector pRL-CMV. Panel A: Cells were stimulated for 5 h with 100 μM CCh, 100 μM ATP or 100 ng/ml EGF. Cells were then lysed and their luciferase activity was evaluated as described in the methods. Panel B: Cells were stimulated for 5 h with 100 μM CCh, in the presence of the protein kinase C inhibitor bisindolylmaleimide I (GF1, 100 nM), or the intracellular Ca^{2+} chelator Bapta/AM (10 μM), or the calcineurin inhibitors FK506 (10 μM) or Cyclosporine A (CSA, 100 nM), or the calmodulin inhibitor calmidazolium (CdZ, 5 μM) or CamKII inhibitor KN93 (2 μM) or its negative control KN92 (2 μM). After lysis, their luciferase activity was evaluated as in panel A. Panel C: Cells were stimulated for 5 h with agonists as in A), with or without forskolin (F, 10 μM , inset) or VIP (300 nM). After lysis, their luciferase activity was evaluated as in panel A. Panel D: Cells were stimulated for 5 h with a combination of 100 μM CCh and 300 nM VIP, in the presence or absence of different inhibitors (GF1, BAPTA/AM, CSA, CdZ, the CamKII inhibitor KN93 (5 μM) or its negative control KN92 (5 μM). After lysis, their luciferase activity was evaluated as in panel A. The results are expressed as relative luciferase activity (fold over basal activity obtained in the absence of agonist) and are the average $\pm$ SD of at least three independent experiments. (* $p < 0.05$ compared to basal condition for A and B, and compared to CCh or CCh+VIP for C and D; † $p < 0.05$ compared to VIP; †† $p < 0.05$ compared to forskolin).

Overexpression of $\text{IP}_3\text{R-1}$

HEK 293A cells (5×10^5 cells/well) were treated with SiRNA against $\text{IP}_3\text{R-2}$ and were transiently transfected with a mixture containing 4 μl of lipofectamin transfection reagent and 2 μg of mixed DNA including pNFAT-luc (1 μg), pRL-CMV(30 ng), $\text{IP}_3\text{R-1}$ plasmid (10-50 ng) completed with pcDNA3.1 vector, in 300 μl of Opti-MEM medium. 12 h later, the cells were splitted on coverslips for Ca^{2+} assays or in 6-wells plates for measurement of NFAT activity or for immunoblotting.

Results

Ca^{2+} is involved in NFAT and CREB activation in HEK 293A cells

Carbachol (CCh) and ATP are Ca^{2+} -mobilizing hormones that activate G_q -coupled receptors. EGF is also a weak Ca^{2+} -mobilizing hormone that activates a tyrosine kinase receptor. Figure 1A shows that, in HEK 293A cells, CCh caused a marked increase in NFAT-dependent luciferase activity (1.98 ± 0.02 -fold over basal activity), whereas ATP barely increased luciferase activity (1.12 ± 0.06). These results could mean that muscarinic receptors

are more abundant than purinergic receptors on HEK 293A cells. EGF significantly decreased luciferase activity (0.79 ± 0.08). The activation of NFAT by CCh was abolished after a pre-incubation of the cells with BAPTA/AM (Fig. 1B), emphasizing the crucial role of Ca^{2+} in NFAT activation. The protein kinase C inhibitor GF1 (100 nM), the calmodulin kinase II inhibitor KN93 (2 μM) and its negative control KN-92 (2 μM) had no effect, whereas the calmodulin inhibitor calmidazolium (CdZ, 5 μM) and the calcineurin inhibitors cyclosporine A (CSA, 100 nM) and FK506 (10 μM) strongly inhibited CCh-induced NFAT activation. These results revealed that, in HEK 293A cells, CCh activates NFAT via a Ca^{2+} /calmodulin/calcineurin pathway that does not require the participation of protein kinase C and calmodulin kinase II.

CREB is essentially activated by the G_s -coupled receptor/cAMP/protein kinase A pathway, but Ca^{2+} is known to potentiate its activation. Figure 1C shows that CCh barely increased CREB activity (1.12 ± 0.61 -fold over basal activity) whereas the G_s -coupled receptor agonist VIP (300 nM) weakly activated CREB-dependent luciferase activity (1.18 ± 0.29 -fold over basal

Fig. 2. Dose-dependent CCh-induced Ca^{2+} responses in HEK 293A. Attached subconfluent HEK 293A cells were loaded with fura2/AM (0.1 μM) for 20 min at room temperature in HBSS, washed for 20 min at room temperature in HBSS, and mounted onto a video microscopy system as described in methods. Fura2 fluorescence in single cells was monitored under basal conditions for an initial period of 30 s before adding CCh (1 μM to 1 mM).

Panel A: Typical Ca^{2+} response obtained with 30 μM CCh, integrating simultaneous Ca^{2+} variation within a population of 50 cells. Panel B: Typical single Ca^{2+} spike obtained within a cell responding to 30 μM CCh. Panels C and D: Typical Ca^{2+} oscillations obtained within single cells responding to 30 μM CCh. Panel E: Typical strong Ca^{2+} transient observed within a single cell responding to 30 μM CCh. Panel F: Relative proportion of cells that did not respond (NR), or that produced a single Ca^{2+} spike, or Ca^{2+} oscillations, or a strong Ca^{2+} transient in response to the different concentrations of CCh (1 μM to 1 mM). Similar results were obtained with at least 450 cells from three different cells preparations.

activity) in HEK 293A cells. Interestingly, however, the simultaneous administration of VIP and CCh caused a synergic activation of CREB (3.94 ± 0.12 -fold over basal activity). The synergic effect of Ca^{2+} -mobilizing hormones was also observed with the weak agonists ATP (3.34 ± 0.43 -fold over basal activity) and EGF (2.04 ± 0.13 -fold over basal activity). CCh even potentiated the activation of CREB by the very potent adenylyl cyclase activator forskolin (inset of Fig. 1C). These results clearly indicated that, in HEK 293A cells, the synergic effect of agonists that activate the cAMP and the Ca^{2+} pathways causes a robust activation of CREB. Figure 1D shows that the synergic effect of VIP and CCh on CREB activation was not affected by the protein kinase C inhibitor GF1 but was strongly inhibited by BAPTA/AM, the calmodulin inhibitor Cd₂, the calmodulin kinase inhibitor II KN-93 and the calcineurin inhibitors FK506 and CSA. These results suggested that the synergic effect of Ca^{2+} on CREB activation occurs via the Ca^{2+} /calmodulin/CamKII and Ca^{2+} /calmodulin/calcineurin pathways, and does not require the participation of protein kinase C. These results demonstrated that CREB and NFAT are differently regulated by Ca^{2+} .

CCh-induced Ca^{2+} responses in HEK 293A cells

CCh is a potent Ca^{2+} -mobilizing hormone. Figure 2A shows the integrated Ca^{2+} response of 50 pre-selected HEK 293A cells stimulated with 30 μM CCh. At this

relatively high concentration, CCh caused a rapid and major increase in intracellular Ca^{2+} , with a peak level approximately 7-fold above the basal level, followed by a slow decline to a level that remained above the basal level for at least 5 min. Our dynamic video imaging system makes it possible to record individual cell responses. At the single cell level, different types of Ca^{2+} responses are elicited when HEK 293A cells are activated with 30 μM CCh (Figs. 2B, 2C, 2D and 2E). The HEK 293A cells displayed a graded response. They either responded with only a single brief spike (Fig. 2B), with Ca^{2+} oscillations that lasted for a few minutes (Figs. 2C and 2D) or with a strong Ca^{2+} transient that slowly declined toward the basal level (Fig. 2E). Figure 2F shows the relative proportion of cells that did not respond, or that produced a single Ca^{2+} spike, Ca^{2+} oscillations or a strong Ca^{2+} transient in response to the different concentrations of CCh (1 μM to 1 mM). At a low concentration of CCh (1 μM), 80% of the cells did not respond, while the remainder responded with either a single Ca^{2+} spike or Ca^{2+} oscillations. At 10 μM CCh, most cells (73%) responded with Ca^{2+} oscillations whereas only a few cells did not respond or responded with a single Ca^{2+} spike. At a high concentration of CCh (1 mM), cells responded either with Ca^{2+} oscillations (60%) or a strong Ca^{2+} transient (34%). These results illustrate the graded Ca^{2+} responses obtained in HEK 293A cells stimulated with increasing concentrations of CCh.

Fig.3. IP₃R expression and knock down in HEK 293A cells. HEK 293A cells (2.5x10⁵) were transfected with selective siRNAs against the different IP₃R isoforms. 48 h after transfection, cells were lysed and their IP₃R protein (panel A and B) or mRNA (panel C) levels were evaluated by Western blot and QPCR respectively. Panel A: Western blots showing the expression of the different IP₃R isoforms (successively reprobing the same membrane) in cells transfected with siRNAs against IP₃R-1 (1-1, 1-2, 1-3), IP₃R-2 (2-1, 2-2, 2-3), IP₃R-3 (3-1, 3-2, 3-3) or transfected with a scrambled oligonucleotide (Sc). Panel B: Densitometric analysis of IP₃R-1, IP₃R-2, IP₃R-3 and total IP₃R bands shown in Panel A. Results are expressed as means ± SD of relative units of integrated peaks (ratio of IP₃R/HSP90 densities). Panel C: Relative levels of mRNAs encoding IP₃Rs in cells transfected with selective siRNAs against the different IP₃R isoforms (white bars) or transfected with a scrambled oligonucleotide (black bars). The results are expressed as % of total mRNAs (IP₃R-1 + IP₃R-2 + IP₃R-3) measured in cells transfected with a scrambled oligonucleotide. These results are representative of at least three independent experiments. (* p<0.05 compared to control cells).

IP₃R expression and knock down in HEK 293A cells

To evaluate the susceptibility of NFAT and CREB transcriptional activity to IP₃R signalling, we used sequence-specific siRNAs (see Table 1) to selectively knock down the expression of IP₃R-1, IP₃R-2 or IP₃R-3 in HEK 293A cells. The Western blots shown in Figure 3A and 3B demonstrate that under our experimental conditions, IP₃R-1, IP₃R-2 and IP₃R-3 were efficiently and selectively silenced by their respective siRNAs. Moreover total IP₃R amounts (revealed with the pan antibody) were importantly reduced after silencing IP₃R-2. Not targeting siRNA (Sc) did not alter IP₃R expression.

QPCR analyses suggested that HEK 293A cells contain 11.9 ± 0.5% IP₃R-1, 63.5 ± 1.2% IP₃R-2 and 24.5 ± 0.6% IP₃R-3 (Fig. 3C; black bars). Similar proportions were previously reported by Wojcikiewicz [12]. QPCR analyses also revealed that siRNAs can efficiently knock down their respective IP₃R mRNAs (Fig. 3C, white bars). The level of IP₃R-1 mRNA fell to 7.8 ± 0.6% after transfection with siRNA 1-1, 4.8 ± 0.5% with siRNA 1-2, and 5.2 ± 0.3% with siRNA 1-3. The level of IP₃R-2 mRNA fell to 8.4 ± 0.4% after transfection with siRNA 2-1, 6.0 ± 1.7% with siRNA 2-2, and 9.8 ± 0.7% with siRNA 2-3. The level of IP₃R-3 mRNA fell to 1.8 ± 0.5% after transfection with siRNA 3-1, 2.0 ± 0.6% with siRNA 3-2, and 4.0 ± 0.4% with siRNA 3-3. These results showed that siRNAs efficiently knocked down IP₃Rs at the mRNA and protein levels.

Deficient Ca²⁺ responses in IP₃R-2- knocked down HEK 293A cells

To study the functional consequence of selective IP₃R knock down, we analyzed the Ca²⁺ response of HEK 293A cells stimulated with a low concentration of CCh (3 μM). Figures 4A and 4B show that 3 μM CCh increased intracellular Ca²⁺, with a peak amplitude of 78.6 ± 19.3 nM in control cells that had not been transfected with siRNA. The Ca²⁺ response was not significantly different in cells transfected with siRNAs against IP₃R-1 (70.5 ± 11.2 nM) or IP₃R-3 (64.4 ± 13.2 nM), but was significantly lower in IP₃R-2-knocked down cells (25.4 ± 7.3 nM). At the single cell level, Figure 4C shows that a large proportion (79.4 ± 5.4%) of IP₃R-2-knocked down cells did not respond to 3 μM CCh, whereas 17.2 ± 4.7% of cells responded with a single Ca²⁺ spike and only 3.4 ± 1.7% of cells responded with Ca²⁺ oscillations. This response profile was weaker than that of the control cells, which had a lower proportion of non-responding cells (53.7

$\pm 10.7\%$) and higher proportions of cells responding with a single Ca^{2+} spike ($32.9 \pm 6.3\%$) and with Ca^{2+} oscillations ($13.5 \pm 5.0\%$), when stimulated with $3 \mu\text{M}$ CCh. The response profiles of $\text{IP}_3\text{R-1}$ -knocked down cells ($49.4 \pm 5.3\%$ non-responders, $30.5 \pm 2.5\%$ with a single Ca^{2+} spike and $20.1 \pm 3.3\%$ with Ca^{2+} oscillations) and $\text{IP}_3\text{R-3}$ -knocked down cells ($53.4 \pm 3.7\%$ non-responders, $30.9 \pm 3.9\%$ with a single Ca^{2+} spike and $15.8 \pm 2.1\%$ with Ca^{2+} oscillations) were not significantly different from that of control cells.

We previously demonstrated that protein kinase A increases the Ca^{2+} release activity of $\text{IP}_3\text{R-2}$ and $\text{IP}_3\text{R-3}$ [21, 22]. A potentiating effect of protein kinase A was

clearly observed when HEK 293A cells were co-stimulated with CCh and VIP, a cAMP increasing agonist and a PKA activator. Figures 4D and 4E show that $3 \mu\text{M}$ CCh combined with 300 nM VIP caused a strong increase in intracellular Ca^{2+} with a peak amplitude of $184.5 \pm 31.9 \text{ nM}$ in control cells that had not been transfected with siRNA. This Ca^{2+} response was not significantly different in cells transfected with siRNAs against $\text{IP}_3\text{R-1}$ ($179.9 \pm 39.6 \text{ nM}$) or $\text{IP}_3\text{R-3}$ ($175.7 \pm 14.5 \text{ nM}$) but was significantly lower in $\text{IP}_3\text{R-2}$ -knocked down cells ($92.8 \pm 23.2 \text{ nM}$). At the single cell level, Figure 4F shows that a large proportion ($45.2 \pm 6.5\%$) of $\text{IP}_3\text{R-2}$ -knocked down cells did not respond to $3 \mu\text{M}$ CCh combined with

Fig. 6. Reduced NFAT activation in IP₃R-2-knocked down HEK 293A cells. siRNA-treated HEK 293A cells (5×10⁵) were co-transfected with the NFAT firefly luciferase reporter vector pNFAT-Luc (A) or the CREB firefly luciferase pCREB-Luc (B) and with the *Renilla* luciferase reporter vector pRL-CMV. Cells were then stimulated for 5 h, with increasing concentrations of CCh (0.1 μM–1 mM) (panels A and B), in the presence of 300 nM VIP (panel B). Cells were then lysed and their luciferase activity was measured as described in the methods. These results are the means ± SD of at least four independent experiments done in triplicate. (* P<0.05 compared to control).

300 nM VIP, whereas $24.7 \pm 2.3\%$ responded with a single Ca²⁺ spike and $30.1 \pm 7.8\%$ responded with Ca²⁺ oscillations. This response profile was weaker than that of control cells, which had a lower proportion of non-responders ($11.1 \pm 3.9\%$) and of cells with a single Ca²⁺ spike ($16.7 \pm 3.0\%$) but a much larger proportion of cells with Ca²⁺ oscillations ($72.2 \pm 2.6\%$). The response profiles of IP₃R-1-knocked down cells ($15.6 \pm 4.9\%$ non-responders, $25.4 \pm 2.1\%$ with a single Ca²⁺ spike and $59.0 \pm 5.6\%$ with Ca²⁺ oscillations) and IP₃R-3-knocked down cells ($13.8 \pm 1.3\%$ non-responders, $21.5 \pm 5.2\%$ with a single Ca²⁺ spike and $64.7 \pm 4.8\%$ with Ca²⁺ oscillations) were relatively similar to that of the control cells. These results suggested that IP₃R-2 makes an important contribution to CCh-induced and CCh/VIP-induced Ca²⁺ responses in HEK 293A cells.

We also studied the functional consequences of selective IP₃R knock down on the Ca²⁺ response of HEK 293A cells stimulated with a maximal concentration of CCh (1 mM). Figures 5A and 5B show that 1 mM CCh increased intracellular Ca²⁺, with peak amplitude of 729.3 ± 60.6 nM in control cells that had not been transfected with siRNA. The Ca²⁺ response was not significantly different in cells transfected with siRNAs against IP₃R-1 (737.5 ± 37.05 nM) or IP₃R-3 (745.5 ± 34.9 nM) but was significantly lower in IP₃R-2-knocked down cells (595.9 ± 22.7 nM). At the single cell level, all the cells responded to the high concentration of CCh (1 mM). Figure 5C shows that a significant proportion ($24.4 \pm 8.6\%$) of IP₃R-2-knocked down cells responded with a single Ca²⁺ spike whereas $53.0 \pm 2.9\%$ responded with Ca²⁺ oscillations, and $22.7 \pm 11.4\%$ responded with a strong Ca²⁺ transient. This response profile was weaker than that of control cells, which had a lower proportion of single Ca²⁺ spikes ($6.1 \pm 4.6\%$) and higher proportions of Ca²⁺ oscillations ($65.9 \pm 2.9\%$) and strong Ca²⁺ transients (28.0

$\pm 5.0\%$). The response profiles of IP₃R-1-knocked down cells and IP₃R-3-knocked down cells stimulated with 1 mM CCh were not significantly different from that of control cells. Figures 5D and 5E show that 1 mM CCh combined with 300 nM VIP caused a large increase in intracellular Ca²⁺, with peak amplitude of 974.2 ± 23.6 nM in control cells that had not been transfected with siRNA. This Ca²⁺ response was not significantly different in cells transfected with siRNAs against IP₃R-1 (955.1 ± 80.1 nM), IP₃R-2 (907.4 ± 63.5 nM) or IP₃R-3 (1034 ± 33.6 nM). Figure 5F also shows that, at the single cell level, the response profiles of IP₃R-1-, IP₃R-2-, and IP₃R-3-knocked down cells stimulated with 1 mM CCh and 300 nM VIP were not significantly different from that of control cells. These results demonstrated that IP₃R-2-knocked down HEK 293A cells have deficient CCh-induced Ca²⁺ responses (even at maximal CCh concentration), but that the potentiating effect of VIP can compensate for the lack of IP₃R-2.

Deficient NFAT activation in IP₃R-2-knocked down HEK 293A cells

The dose-response curves for CCh-induced NFAT activation (Fig. 6A) indicated that the response of IP₃R-2-knocked down HEK 293A cells (EC_{50} : 27.0 ± 4.6 μM; maximal amplitude: 1.40 ± 0.02 -fold over basal activity) was significantly reduced compared to the response of control HEK 293A cells (EC_{50} : 14.3 ± 1.7 μM; maximal amplitude: 2.47 ± 0.06 -fold over basal activity). The responses of IP₃R-1-knocked down cells (EC_{50} : 10.1 ± 1.4 μM; maximal amplitude: 2.60 ± 0.07 -fold over basal activity) and IP₃R-3-knocked down cells (EC_{50} : 12.3 ± 2.6 μM; maximal amplitude: 2.61 ± 0.11 -fold over basal activity) were not significantly different from that of control cells. In Figure 6B, the dose-response curves for CCh-induced CREB activation (co-stimulation with 300

Fig. 7. Overexpression of IP₃R-1 rescues CCh-induced Ca²⁺ activity and NFAT activation in IP₃R-2-knocked down HEK 293A cells. siRNA-treated cells (5x10⁵) were transfected with increasing amounts of IP₃R-1 DNA (ng), with NFAT firefly luciferase reporter vector (pNFAT-Luc), and with *Renilla* luciferase reporter vector (pRL-CMV), as described in methods. 12 h later, cells were analyzed for their IP₃Rs content by Western blot (panel A), for their CCh-induced NFAT activity (panels E and F) and for their CCh-induced Ca²⁺ responses (panels B, C, and D) as described in methods. Panel A: Western blot showing total IP₃R protein level (using pan antibody, upper panel), IP₃R-1 protein level (using anti-IP₃R-1 antibody, middle panel) and HSP90 protein level (lower panel), in cells transfected with siRNAs against IP₃R-2 and with increasing amounts of IP₃R-1 DNA (ng). Panel B: IP₃R-2-knocked down HEK 293A cells were transfected with increasing amounts of IP₃R-1 DNA, loaded with fura2/AM (0.1 μM), and stimulated with 3 μM CCh. Panel C: Average Ca²⁺ responses (maximal amplitude) measured in the experiment shown in panel B. Panel D: Relative proportion of cells that did not respond (NR), or that responded with a single Ca²⁺ spike, or with Ca²⁺ oscillations, in the experiment depicted in panel B. Panels E and F: IP₃R-2-knocked down HEK 293A cells transfected with increasing amounts of IP₃R-1 DNA were stimulated for 5h with 3 μM CCh (panel E) and 1 mM CCh (panel F). Cells were then lysed and their luciferase activity was measured as described in the methods. These results are the means ± SD of data obtained in three independent experiments (* P<0.05 compared to control).

nM VIP) indicate that the responses of IP₃R-1-knocked down cells (EC₅₀: 6.8 ± 3.1 μM; maximal amplitude: 4.74 ± 0.45-fold over basal activity), IP₃R-2-knocked down cells (EC₅₀: 7.3 ± 3.1 μM; maximal amplitude: 4.34 ± 0.33-fold over basal activity) and IP₃R-3-knocked down cells (EC₅₀: 5.4 ± 1.3 μM; maximal amplitude: 4.99 ± 0.57-fold over basal activity) were not significantly different from that of control cells (EC₅₀: 6.7 ± 5.2 μM; maximal amplitude: 4.88 ± 837-fold over basal activity) (Fig. 6B). These results suggested that IP₃R-2 plays a key role in NFAT transcriptional activity, but not in CREB transcriptional activity.

IP₃R-1 can compensate for IP₃R-2 in NFAT activation

Since IP₃R-2 is the most abundant IP₃R isoform expressed in HEK293A cells, its knock down is susceptible to affect cellular activities, like NFAT activation, that require robust Ca²⁺ responses. To verify whether the decreased NFAT activity observed in IP₃R-2-knocked down cells is selectively due to the IP₃R-2 isoform, we verified whether the overexpression of IP₃R-1 could compensate for the lack IP₃R-2. IP₃R-2-knocked down cells that were transfected with 50 ng/well of DNA coding for IP₃R-1, showed a similar level of expression

of total IP₃R as control cells (Fig. 7A, upper panel). Reprobing the blot with a selective anti-IP₃R-1 antibody (Fig. 7A, middle panel) confirmed that IP₃R-1 was efficiently expressed under these conditions. HSP90 immunodetection (Fig. 7A, lower panel) served as internal control for the amounts of loaded protein.

Figures 7B and 7C show that 3 μ M CCh increased intracellular Ca²⁺, with a peak amplitude of 71.5 ± 23.1 nM, in control cells that had not been transfected with IP₃R-2 siRNA nor IP₃R-1 DNA. As previously observed, the Ca²⁺ response was significantly decreased in IP₃R-2-knocked down cells (peak amplitude of 31.0 ± 18.5 nM). Ca²⁺ responses were progressively increased in IP₃R-2-knocked down cells that were transfected with increasing amounts of IP₃R-1 DNA (peak amplitude of 22.3 ± 14.2 nM in cells transfected with 10 ng IP₃R-1 DNA; peak amplitude of 50.1 ± 25.2 nM in cells transfected with 20 ng IP₃R-1 DNA; peak amplitude of 82.9 ± 37.5 nM in cells transfected with 50 ng IP₃R-1 DNA). The CCh-induced Ca²⁺ response in cells transfected with 50 ng IP₃R-1 DNA was similar to that of control cells. At the single cell level, Figure 7D shows that a large proportion ($81.4 \pm 1.5\%$) of IP₃R-2-knocked down cells did not respond to 3 μ M CCh, whereas $16.9 \pm 1.2\%$ of cells responded with a single Ca²⁺ spike and only $1.7 \pm 0.8\%$ of cells responded with Ca²⁺ oscillations. This response profile was weaker than that of control cells, which responded to 3 μ M CCh with a lower proportion of non-responding cells ($45.2 \pm 6.8\%$) and higher proportions of cells showing a single Ca²⁺ spike ($38.9 \pm 3.8\%$) or Ca²⁺ oscillations ($15.9 \pm 3.6\%$). After transfection with 50 ng of IP₃R-1 DNA, cells responded to 3 μ M CCh similarly as control cells, with $54.3 \pm 3.8\%$ of cells that did not respond $34.7 \pm 4.2\%$ of cells that responded with a single Ca²⁺ spike, and $11.8 \pm 0.6\%$ of cells that responded with Ca²⁺ oscillations.

Since the substitution of IP₃R-1 can compensate for the lack of IP₃R-2 in CCh-induced Ca²⁺ responses (Figs. 7C and 7D), we verified whether IP₃R-1 could compensate for the lack of IP₃R-2 in CCh-induced NFAT activation. Figures 7E and 7F show that IP₃R-2 knocked down cells that were transfected with increasing amounts of IP₃R-1 DNA (10 ng/well, 20 ng/well, or 50 ng/well) progressively increased their capacity to activate NFAT at low (3 μ M) and maximal (1 mM) CCh concentrations. In cells transfected with 50 ng IP₃R-1 DNA, both concentrations of CCh activated NFAT similarly as in control cells. These results indicated that IP₃R-1 can functionally substitute for IP₃R-2 in CCh-induced Ca²⁺ mobilization and NFAT activation in HEK 293A cells.

Discussion

IP₃Rs play major roles in agonist-induced intracellular Ca²⁺ release and therefore they are involved in the regulation of many Ca²⁺-dependent cellular activities. In the present study, we selectively knocked down each IP₃R isoform expressed in HEK 293A cells to evaluate the contribution of IP₃R signalling to the activation of the transcription factors NFAT and CREB.

We showed that the activation of endogenous muscarinic receptors by CCh increases intracellular Ca²⁺ and turns on NFAT transcriptional activity. We also showed that the simultaneous activation of muscarinic and VIP receptors produces a synergistic effect on CREB transcriptional activity. Pharmacological inhibitors of important components of the Ca²⁺ signaling pathway revealed that CCh-induced NFAT activation depends on a Ca²⁺/calmodulin/calcineurin pathway whereas VIP/CCh-induced CREB activation depends on a Ca²⁺/calmodulin/CamKII pathway that is also affected by calcineurin. It is well documented that calcineurin is involved in the activation of NFAT. The dephosphorylation of NFAT by calcineurin is the most important step leading to its activation and its translocation to the nucleus, where it induces the transcription of NFAT-dependent genes [14, 15, 23, 24]. It is also well documented that Ca²⁺ exerts a positive regulatory effect on CREB activation. The effect of Ca²⁺ on CREB activity is mediated by CamKII or CamKIV [25, 26]. Recent studies also reported a positive effect of calcineurin on CREB activation. Calcineurin activity was required to maintain CREB-dependent transcription in cortical neurons [27]. The inhibition of calcineurin blocked CREB-dependent gene expression in pancreatic islets, and chromaffin cells [28, 29]. All these data point to a positive involvement of calcineurin in CREB activation via cross-talk between the cAMP and Ca²⁺ signaling pathways.

We showed that the knock down of IP₃R-2 had a significant impact on CCh-induced Ca²⁺ responses, whereas the knock down of IP₃R-1 and IP₃R-3 did not significantly affect intracellular Ca²⁺ mobilization. Co-stimulation with VIP could functionally compensate for the lack of IP₃R-2 in HEK 293A cells stimulated with CCh. We and others have shown that phosphorylation of IP₃Rs by PKA increases their Ca²⁺ release activity [21, 22, 30-33]. The potentiating effect of PKA on IP₃R activity could explain the potentiating effect of VIP on CCh-induced Ca²⁺ responses.

We evaluated the impact of selective IP₃R knock down on NFAT and CREB activation. As observed for

the Ca^{2+} responses, we showed that only the knock down of the $\text{IP}_3\text{R-2}$ isoform affects the activation of NFAT by CCh. The knock down of $\text{IP}_3\text{R-1}$ or $\text{IP}_3\text{R-3}$ did not affect the activation of NFAT by CCh. The activation of CREB was not significantly affected by the knock down of any of the IP_3R isoforms. These results suggested that the activation of NFAT is more dependent on the optimal efficiency of the Ca^{2+} release machinery than the activation of CREB. It must be noted, however, that under the conditions required to activate CREB, cAMP exerts a potentiating effect on IP_3 -induced Ca^{2+} release. It was also recently reported that a family of modulators (TORCs: transducers of regulated CREB activity) that are activated by a cooperative effect of Ca^{2+} and cAMP can directly activate CREB [34]. TORCs constitute another pathway through which Ca^{2+} and cAMP cooperate in the activation of CREB. The cooperative effects of Ca^{2+} and cAMP on the activation of CREB likely compensate for the lack of $\text{IP}_3\text{R-2}$.

Since $\text{IP}_3\text{R-2}$ is the most abundant IP_3R isoform expressed in HEK293A cells, its knock down is susceptible to affect several cellular activities, like NFAT, that require robust Ca^{2+} responses. In pancreatic acinar cells, the simultaneous knock out of $\text{IP}_3\text{R-2}$ and $\text{IP}_3\text{R-3}$, the principal isoforms expressed in these cells, considerably reduced their Ca^{2+} mobilization capacity and their amylase secretion [13]. Also in atrial myocytes deficient in $\text{IP}_3\text{R-2}$, their principal isoform, the positive inotropic effect of endothelin was abolished and these cells were protected from arrhythmogenic conditions [35]. These studies revealed the consequences of abolishing the main IP_3R isoform on Ca^{2+} -dependent cellular activities. Hence it is plausible that the important role of

$\text{IP}_3\text{R-2}$ in the activation of NFAT in HEK 293A cells is not related to a selective effect of $\text{IP}_3\text{R-2}$ but rather to its abundance. We showed that, when expressed at a similar level, $\text{IP}_3\text{R-1}$ could fully compensate for $\text{IP}_3\text{R-2}$ knock down to mobilize Ca^{2+} and to activate NFAT in HEK 293A cells. These results show that IP_3Rs play an important role to provide the Ca^{2+} required for the activation of NFAT, though any IP_3R isoform appears to be apt to play that role.

In conclusion, we showed that the knock down of $\text{IP}_3\text{R-2}$ significantly reduced the intracellular Ca^{2+} response of HEK 293 cells. This reduced Ca^{2+} response did not affect the activation of CREB but significantly decreased the activation of NFAT. These results suggest that the Ca^{2+} signals required to activate NFAT are stronger than those required to activate CREB.

Abbreviations

CCh (carbachol); IP_3 (inositol 1, 4, 5-trisphosphate); IP_3R (inositol 1,4,5-trisphosphate receptor); PBS (phosphate buffered saline); NFAT (Nuclear Factor of Activated T cells); CREB (cAMP Response Element Binding); EGF (epidermal growth factor).

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