

## PUBLISHER'S NOTE

# Expression of Concern: RyR1 and RyR3 isoforms provide distinct intracellular Ca<sup>2+</sup> signals in HEK 293 cells

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There was an error published in Fig. 6 in *J. Cell Sci.* (2002) **115**, 2497-2504 (doi:10.1242/jcs.115.12.2497).

There is a duplication of a segment of signal in Fig. 6A. According to the authors, this was due to an error in the process of assembling the trace. They made every effort to find the original data, but, as the work dates back more than twenty years, they were unable to recover the original files.

The authors state that this error does not significantly impact the interpretation of the article, the data reliability or the reader's understanding of the paper and that the scholarly integrity of the article remains intact. "Data reported in Fig. 6A and B aimed to illustrate variability of spontaneous Ca<sup>2+</sup> signals in two RyR3-expressing cells; spontaneous Ca<sup>2+</sup> release events are also reported in Fig. 5 and duplication of signal in Fig. 6Ab does not impact on the main interpretation of the article which was that HEK293 cells expressing RyR3 display spontaneous Ca<sup>2+</sup> release events and those expressing RyR1 do not. This suggested that, at least in these cells, isoform-specific functional properties may contribute to generation of intracellular Ca<sup>2+</sup> signals."

Without the original data, it is difficult to determine how the issues arose, so the journal is publishing this note to alert readers to our concerns, with the agreement of the authors.

# RyR1 and RyR3 isoforms provide distinct intracellular Ca<sup>2+</sup> signals in HEK 293 cells

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## Summary

Ryanodine receptors (RyRs) are expressed on the endoplasmic reticulum of many cells, where they form intracellular Ca<sup>2+</sup>-release channels that participate in the generation of intracellular Ca<sup>2+</sup> signals. Here we report studies on the intracellular localisation and functional properties of transfected RyR1 or RyR3 channels in HEK 293 cells. Immunofluorescence studies indicated that both RyR1 and RyR3 did not form clusters but were homogeneously distributed throughout the endoplasmic reticulum. Ca<sup>2+</sup> release experiments showed that transfected RyR1 and RyR3 channels responded to caffeine, although with different sensitivity, generating a global release of Ca<sup>2+</sup> from the entire endoplasmic reticulum. However, video imaging and confocal microscopy analysis revealed that, in RyR3-expressing

cells, local spontaneous Ca<sup>2+</sup> release events were observed. No such spontaneous activity was observed in RyR1-expressing cells or in control cells. Interestingly, the spontaneous release events observed in RyR3-expressing cells were restricted to one or two regions of the endoplasmic reticulum, suggesting the formation of a further subcellular organisation of RyR3 in Ca<sup>2+</sup> release units. These results demonstrate that different RyR isoforms can engage in the generation of distinct intracellular Ca<sup>2+</sup> signals in HEK 293 cells.

Key words: Ryanodine receptor channels, Calcium signalling, Calcium stores, Endoplasmic sarcoplasmic reticulum, Calcium release

## Introduction

The endoplasmic reticulum of most eukaryotic cells contains relatively high levels of Ca<sup>2+</sup> and represent an important source for Ca<sup>2+</sup> release into cytosol in response to stimulation of cells by a variety of agonists (Berridge et al., 2000). There are many components involved in the organisation of intracellular Ca<sup>2+</sup> stores. Ca<sup>2+</sup> pumps of the sarcoplasmic endoplasmic reticulum Ca<sup>2+</sup> ATPases (SERCA) family accumulate Ca<sup>2+</sup> into the lumen of the endoplasmic reticulum, where specific Ca<sup>2+</sup>-binding proteins (such as calsequestrin and calreticulin) provide an efficient mechanisms of storage, mediated by their high capacity and low affinity for Ca<sup>2+</sup> (Pozzan et al., 1994; Sorrentino and Rizzuto, 2001). Ca<sup>2+</sup> accumulated in the intracellular stores of the endoplasmic reticulum is released into the cytosol through Ca<sup>2+</sup>-release-specific channels in connection with intracellular signalling mechanisms. Two distinct families of intracellular Ca<sup>2+</sup>-release channels, ryanodine receptors (RyRs) and inositol (1,4,5)-trisphosphate receptors [Ins(1,4,5)P<sub>3</sub>Rs] are known (Sorrentino et al., 2000; Patel et al., 1999). In cells, like neurons and muscle cells, where Ca<sup>2+</sup>-mediated signalling is highly developed, these Ca<sup>2+</sup>-release proteins are selectively localised to specific regions of the cells (Franzini-Armstrong and Protasi, 1997; Golovina and Blaustein, 1997; Petersen et al., 2001). The sarcoplasmic reticulum of skeletal muscle cells is a highly organised network of tubules that is well characterised with respect to the redistribution of proteins involved in Ca<sup>2+</sup>

homeostasis. In this context, RyRs are selectively localised in the region of the junctional membranes of the terminal cisternae (i.e. the part of the sarcoplasmic reticulum facing the t-tubule). RyRs appear to be part of a large multi-protein complex, where calsequestrin, triadin, junctin are the best-characterised components. By contrast, SERCAs are mainly excluded from junctional membranes and are found mostly in the longitudinal SR (Franzini-Armstrong and Protasi, 1997). Selective redistribution of intracellular Ca<sup>2+</sup>-release channels within the endoplasmic reticulum of neurons has been reported (Berridge, 1998). In pancreatic acinar cells, co-localisation of SERCA pumps and intracellular Ca<sup>2+</sup>-release channels seems to be discontinuous in most of the endoplasmic reticulum, with evidence for clustering in some regions (Petersen et al., 2001). Obviously, the subcellular localisation of intracellular Ca<sup>2+</sup>-release channels may significantly affect their contribution to Ca<sup>2+</sup> signalling (Berridge et al., 1998; Berridge et al., 2000). However, in many other cells, intracellular Ca<sup>2+</sup>-release channels are less evidently restricted in their localisation within the endoplasmic reticulum.

We report here on experiments with human HEK 293 cells transfected with either the RyR type 1 (RYR1) or the type 3 (RyR3) isoforms. Since epithelial HEK 293 are not a cell type specialised for RyR-mediated Ca<sup>2+</sup> release, we reasoned that they might represent a good model to study the localisation and the activity of RyR channels in a non-specialised endoplasmic reticulum (i.e. in contrast to the sarcoplasmic reticulum). In

addition, HEK 293 cells should provide a simpler environment to compare functional properties and dynamics of Ca<sup>2+</sup> release of different isoforms of RyRs expressed under the same cellular context, independently of the variable number of accessory proteins.

We have found that heterologous RyR1 and RyR3 channels are homogeneously distributed in the endoplasmic reticulum of HEK 293 cells, at least at the level of resolution of confocal microscopy. Functionally, the transfected channels, when stimulated with caffeine, are able to release Ca<sup>2+</sup> with a global response involving the entire endoplasmic reticulum. In contrast, expression of recombinant RyR3 channels, but not of RyR1 channels, results in the appearance of spontaneous and localised Ca<sup>2+</sup>-release activity in HEK 293 cells. At the same time, spontaneous Ca<sup>2+</sup>-release activity in RyR3-expressing HEK 293 cells is restricted to one or two sites, indicating that preferential domains for generating localised Ca<sup>2+</sup> release events are present in these cells.

## Materials and Methods

### Cloning of the mink RyR3 cDNA and construction of the RyR3 expression vector

Cloning of the mink RyR3 cDNA has been described previously (Marziali et al., 1996). To construct the RyR3 expression vector, the twelve clones isolated by Marziali et al. were joined by either PCR or ligation of DNA fragments previously digested with suitable restriction enzymes. The full length RyR3 cDNA was finally cloned into the *EcoRV/NotI* sites of the expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands). A GCCGCC Kozac consensus sequence was inserted by PCR just upstream of the first ATG codon. The 15 kb RyR3 cDNA used for the construction of the expression vector does not contain the 15 and 18 bp insertions described by Marziali et al. In addition, the sequence corresponding to exon A, as defined by Marziali et al., is present. The RyR1 cDNA (Nakai et al., 1997) was subcloned into the *EcoRV* site of pcDNA3 to obtain pcDNA3-RyR1.

### Cell culture and transfection

Human embryonic kidney (HEK 293) cells were maintained in  $\alpha$ -MEM medium supplemented with 2 mM glutamine (Bio-Wittaker, Walkersville, MD), 100  $\mu$ g/ml streptomycin, 100 U/ml penicillin (Bio-Wittaker), 1 mM sodium pyruvate (Bio-Wittaker), 10% heat-inactivated fetal calf serum (FCS) (Bio-Wittaker) at 37°C under 5% CO<sub>2</sub>. DNA transfection of the RyR3 expression vector was carried out using the calcium phosphate method. 8 $\times$ 10<sup>5</sup> cells were plated on a 100 mm tissue culture dish 24 hours before transfection. One hour before DNA addition, the medium was changed with fresh medium. 10  $\mu$ g of RyR3 expression vector were mixed in a solution containing HBS (5 g/l Hepes, 8 g/l NaCl, pH 7.1), 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.7 mM NaH<sub>2</sub>PO<sub>4</sub>, 120 mM CaCl<sub>2</sub> and incubated for 30 minutes. Calcium phosphate precipitates were added to cells and incubated for 6 hours. Transfections of the pcDNA3-RyR1 clones were performed with the GenePORTER™ method (Gene Therapy Systems, San Diego, CA), following the manufacturer's instructions. For stable transfections, Geneticin sulphate G418 (Life Technology, Groningen, The Netherlands) was added 48 hours after transfection, at a final concentration of 800  $\mu$ g/ml. Single colonies were transferred to a 96-well multiplate, expanded and tested for RyR expression.

### Immunofluorescence staining

Cells were fixed with 3% paraformaldehyde/2% sucrose in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>) for 5

minutes at room temperature, washed, and incubated for 15 minutes in 2% BSA or 5% goat serum in PBS. After washing, cells were permeabilised with Hepes Triton Buffer (20 mM Hepes pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl<sub>2</sub>, 0.5% Triton X-100). For RyRs detection, cells were alternatively incubated overnight at 4°C with a rabbit polyclonal antibody specific for the RyR1 or the RyR3 isoform at a 1:1000 dilution (Giannini et al., 1995), or for 1 hour at room temperature with a mouse monoclonal antibody recognising RyR1 and RyR3 isoforms at a 1:1000 dilution (Alexis Biochemicals, San Diego, CA). Polyclonal antibodies against calreticulin were kindly provided by M. Michalak (University of Alberta, Canada) and were used at a 1:20 dilution for 1 hour at room temperature. Polyclonal antibodies against Ins(1,4,5)P<sub>3</sub>R1 were kindly provided by P. De Camilli (Boyer Centre for Molecular Medicine, Yale University School of Medicine, New Haven, CT) and was used at a 1:1000 dilution for 1 hour at room temperature. FITC- and TRITC-conjugated secondary antibodies were from Sigma. AlexaFluor®-conjugated secondary antibodies were from Molecular Probes (Eugene, OR). Cy<sub>3</sub>-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). All secondary antibodies were used accordingly to the manufacturer's instructions. The expression vector containing the Green Fluorescent Protein (GFP) cDNA fused to a Golgi targeting signal was a gift of R. Rizzuto (University of Ferrara, Italy). Images were collected with an epifluorescence Axioplan 2 imaging Microscope (Zeiss, Thornwood, NY) equipped with a MicroMAX digital CCD camera (Princeton Instruments, Trenton, NY), and digitised, stored and subsequently processed with a Meta Imaging Serie 4.5 software (Universal Imaging Corporation®, West Chester, PA).

### Microsomal proteins preparation

Cells were harvested by a rubber scraper, pelleted in 50 ml tubes and homogenised in ice cold buffer A (320 mM sucrose, 5 mM Na-Hepes pH 7.4 and 0.1 mM PMSF) using a Teflon potter. Homogenates were centrifuged at 7000 *g* for 5 minutes at 4°C. The supernatant obtained was centrifuged at 100,000 *g* for 1 hour at 4°C. The microsomes were resuspended in buffer A and stored at -80°C. Protein concentration of the microsomal fractions was quantified using the Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA).

### Western blot analysis

Microsomal proteins were separated by SDS-PAGE, as described (Conti et al., 1996). Proteins were then transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) using a transfer buffer containing 192 mM glycine, 25 mM Tris, 0.01% SDS and 10% methanol for 5 hours at 350 mA at 4°C. Filters were blocked for 3 hours in a buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 7.4, 0.2% Tween-20, 5% nonfat milk, and incubated overnight at room temperature with specific antibodies. Rabbit polyclonal antibodies able to distinguish the three RyRs were used as described (Giannini et al., 1995).

### Intracellular Ca<sup>2+</sup> measurements

Untransfected and transfected HEK 293 cells were loaded with 5  $\mu$ M Fura 2-AM (Calbiochem® La Jolla, CA) in Krebs-Ringer-Hepes medium (125 mM NaCl, 5 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 2 mM CaCl<sub>2</sub>, 6 mM glucose and 25 mM Hepes, adjusted to pH 7.4 with NaOH) for 30 minutes at room temperature in the dark. The Fura 2 fluorescence was recorded on an inverted stage microscope (Nikon) using a 40 $\times$  objective. Fura 2 was excited alternatively at 340 and 380 nm using dual monochromators. Images were acquired with a digital CCD camera (Princeton Instruments) and Ca<sup>2+</sup> signalling was analysed using computer software (Metafluor, Universal Imaging Corporation®, West Chester, PA).

Some of the cytosolic Ca<sup>2+</sup> measurements were performed as follows: the culture medium was replaced with an extracellular medium containing: 121 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl<sub>2</sub>, 1.8 mM CaCl<sub>2</sub>, 6 mM NaHCO<sub>3</sub>, 5.5 mM glucose, 25 mM Hepes pH 7.3. Cells were loaded with 1 μM Fura 2-AM or Fluo 3-AM for 45 minutes followed by a 30 minute de-esterification period. For video imaging, the coverslips bearing the Fura-2-loaded cells were mounted on a Nikon Diaphot inverted microscope and alternately excited with 340 and 380 nm light from twin monochromators; the excitation wavelengths being switched with a rotating mirror chopper (Glen Creston Instruments, Stanmore, UK). Emitted light was filtered at 510 nm and collected with an intensified CCD camera (Photonic Science, Tunbridge Wells, UK). The video signal was digitised, stored and subsequently processed off-line with an Imagine image-processing unit (Synoptics, Cambridge, UK). Ratio images were acquired at 2 second intervals and [Ca<sup>2+</sup>]<sub>cyt</sub> was generated from a modified formula (Grynkiwicz et al., 1985), which accounted for signal artefacts (Bootman and Berridge, 1996).

Confocal Ca<sup>2+</sup> measurements with Fluo-3-loaded cells were performed as described (Bootman et al., 1997a), with the modification that images were captured (256×240 pixels) at an effective rate of 15 frames/second (after averaging two consecutive images taken at 30 frames/second) using a Noran Oz laser scanning confocal microscope. For the line scans, a single pixel-wide line across the image was repetitively scanned at 250 lines/second. The confocal slit was set to result in a z-section thickness of ~1 μm. All experiments were performed at room temperature (20–22°C). Fluo 3 was excited using the 488 nm laser line, and the emitted fluorescence was collected at wavelengths >505 nm. Offline analysis of the confocal data was performed using a modified version of NIH Image. Absolute values for Ca<sup>2+</sup><sub>i</sub> were calculated according to the equation:

$$[Ca^{2+}]_i = K_d [(f - f_{min}) / (f_{max} - f)]$$

f<sub>min</sub> and f<sub>max</sub> were determined by permeabilising the cells with A23187 in the presence of 10 mM EGTA or 10 mM CaCl<sub>2</sub> respectively. The K<sub>d</sub> of Fluo-3 was assumed to be 810 nM (Thomas et al., 2000).

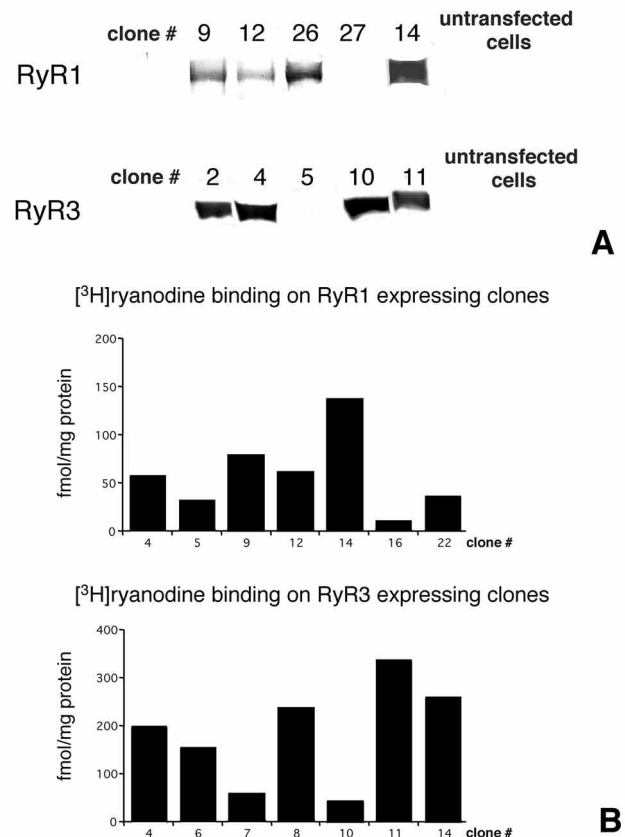
### [<sup>3</sup>H]ryanodine-binding assay

Microsomal preparations (30 μg) from control HEK 293, RyR1 and RyR3-expressing cells were incubated for 1.5 hours at 36°C with 20 nM [<sup>3</sup>H]ryanodine in 200 μl of a solution containing 0.2 M KCl, 10 mM Hepes pH 7.4, 10 μM Ca<sup>2+</sup> and a mixture of protease inhibitors: aprotinin (76.8 nM), benzamidin (0.83 mM), iodoacetamide (1 mM), leupeptin (1.1 mM), pepstatin (0.7 mM) and PMSF (0.1 mM). The bound [<sup>3</sup>H]ryanodine was separated from free ligand by filtering through Whatman GF/B glass fiber microfilters. The filters were washed with 3×5 ml of ice-cold buffer, as described above, and 2×5 ml 10% EtOH. Radioactivity remaining in the filters was measured by liquid scintillation counting. Specific binding was calculated as the difference between total and nonspecific binding measured in parallel assays in the presence of 20 μM unlabeled ryanodine. All experiments were performed in duplicate.

## Results

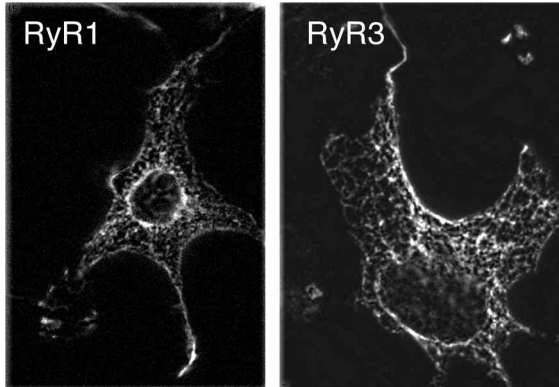
### Expression of RyRs in HEK 293 cells

Expression vectors for RyR1 or RyR3 were transfected into HEK 293 cells and different stable clones were obtained following G418 selection. Expression of RyRs was verified in all selected clones by western blot analysis using isoform specific antibodies and by [<sup>3</sup>H]ryanodine binding. Fig. 1A shows western blot analysis performed on microsomes prepared from representative RyR1 and RyR3-expressing clones in which a band specific for RyR1 or RyR3 was clearly



**Fig. 1.** Western blot analysis and [<sup>3</sup>H]ryanodine-binding assay on RyR1- and RyR3-expressing clones. (A) 50 μg of microsomal proteins obtained from RyR1- or RyR3-expressing clones were separated on 5% SDS-PAGE and analysed by western blot with anti-RyR1 or anti-RyR3 polyclonal antibodies. (B) 50 μg of RyR3 microsomes from RyR1- or RyR3-expressing clones were incubated for 1.5 hours at 36°C with 20 nM [<sup>3</sup>H]ryanodine in a solution containing 0.2 M KCl, 10 mM Hepes pH 7.4, 10 μM Ca<sup>2+</sup>. Nonspecific binding was determined by the addition of a 1000-fold excess of unlabeled ryanodine (20 μM). The amount of [<sup>3</sup>H]ryanodine bound was measured by membrane filtration on Whatman GF-B filters.

detected. Although low levels of endogenous RyR2 have been found in HEK 293 cells (Querfurth et al., 1998), we did not detect any evidence of endogenous RyR by western blot analysis in our untransfected HEK 293 cells, similar to previous results (Tong et al., 1999). In addition, overexpression of RyR1 or RyR3 did not induce expression of any other RyR isoforms in the selected clones (not shown). Subfractionation of the microsomal fraction of RyR-transfected cells by sucrose gradient centrifugation showed that the recombinant RyR protein formed a tetramer with sedimentation properties similar to those of the native channels (not shown). A more quantitative measurement of the RyR channel content was obtained by performing [<sup>3</sup>H]ryanodine-binding experiments on microsomal preparations from transfected cells. Fig. 1B shows the intracellular content of RyRs in different clones of transfected cells, ranging from 30.5 to 136.5 fmol/mg of protein for RyR1-expressing clones and from 41 to 335 fmol/mg of protein for RyR3-expressing clones. No significant



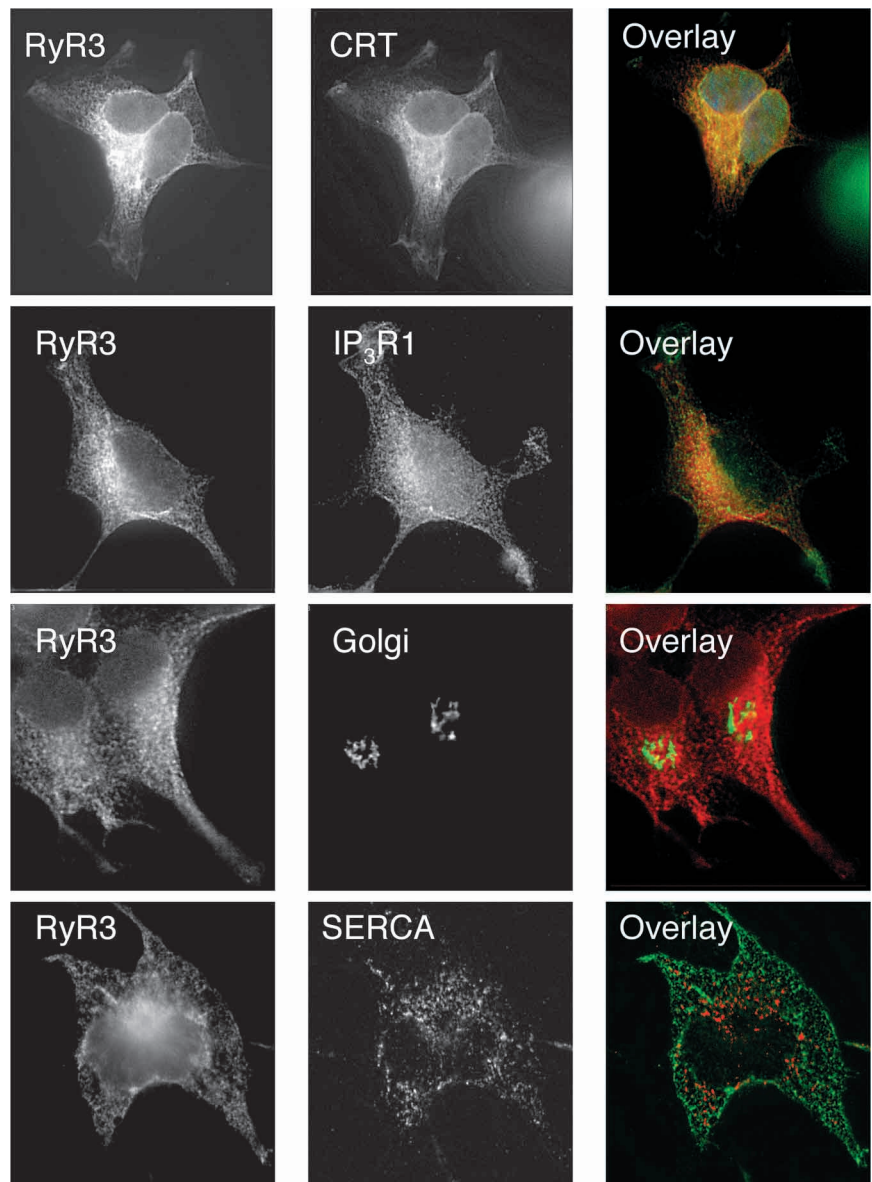
**Fig. 2.** Subcellular distribution of RyR1 and RyR3 in transfected cells. Cells decorated with monoclonal antibodies against RyR1 and RyR3 were analysed by epifluorescence microscopy.

levels of [<sup>3</sup>H]ryanodine binding were detected in HEK 293 untransfected cells.

#### Intracellular localisation of RyRs in transfected cells

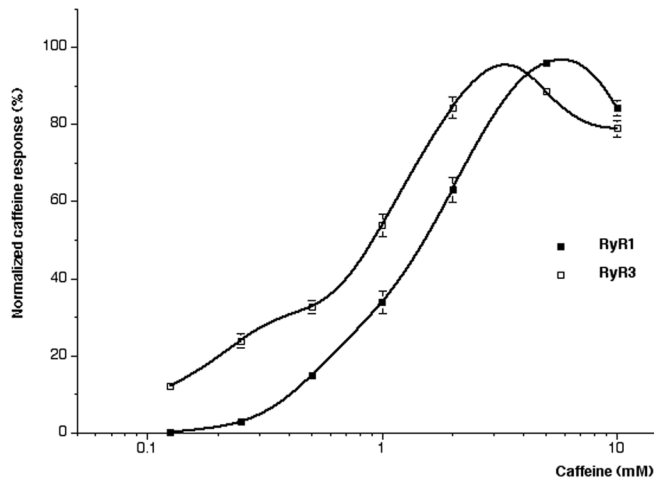
The subcellular distribution of RyR1 and RyR3 in HEK-293-transfected cells is shown in Fig. 2. Sequential scanning over an interval ranging from 0.25 to 1  $\mu$ m showed that RyR1- and RyR3-specific signals could be detected throughout the endoplasmic reticulum of transfected cells and that RyRs were distributed as a thin network spreading as far as the cell periphery. No major differences could be detected between the distribution of RyR1 and RyR3 proteins in transfected cells.

To further investigate the subcellular distribution of RyRs, co-localisation experiments were performed using different markers of the endoplasmic reticulum. In particular, Fig. 3 shows selected planes displaying the intracellular localisation of the



**Fig. 3.** Colocalization experiments of RyR3 with endoplasmic reticulum markers. RyR3-expressing cells were decorated with polyclonal antibodies against calreticulin or Ins(1,4,5) $P_3$ R1 and with monoclonal antibodies against SERCA pump. Calreticulin was detected by a FITC conjugated anti-goat antibody. Ins(1,4,5) $P_3$ R1 was detected by an Alexa-conjugated anti-rabbit antibody. SERCA pump was detected by a Cy3-conjugated anti-mouse antibody. The Golgi apparatus was stained by transfecting the RyR3-expressing cells with the cDNA for a Green Fluorescent Protein containing a Golgi targeting signal. Cells were counterstained with monoclonal antibodies against RyR3 and TRITC-, Cy3- or Alexa-conjugated anti-mouse secondary antibodies. Panels show representative sections from top to bottom of the cells at about 1  $\mu$ m intervals.

Ca<sup>2+</sup>-binding protein calreticulin, the Ins(1,4,5) $P_3$ Rs, the SERCA pumps and the Golgi apparatus compared with the RyR3 distribution. Experiments performed on RyR1-expressing cells produced results comparable with those obtained with cells expressing RyR3 (data not shown). The top panels in Fig. 3 show the intracellular distribution of RyR3 compared with that of endogenous calreticulin. In most of the cells, brighter fluorescence signals were observed in the perinuclear region of the cell, while a reticular pattern of staining was detected in the cell periphery. An overlay of representative images from sequential scanning showed that the patterns of staining obtained with anti-calreticulin and anti-RyR3 antibodies are extensively superimposable and that they are consistent with an endoplasmic reticulum distribution of the two proteins. In addition to RyRs, Ins(1,4,5) $P_3$ Rs play a role in the generation of intracellular Ca<sup>2+</sup> signalling. Western blot analysis and binding experiments with D-myo-[<sup>3</sup>H]Ins(1,4,5) $P_3$  performed on microsomes prepared from RyR-expressing cells and untransfected cells revealed that the different cell populations express equal amounts of the Ins(1,4,5) $P_3$ R type 1 isoform (data



**Fig. 4.** Caffeine response of RyR1- and RyR3-expressing cells. RyR1- or RyR3-expressing HEK 293 cells were loaded with 5  $\mu$ M Fura 2-AM for 30 minutes and analyzed for caffeine-induced Ca<sup>2+</sup> release at excitation wavelength of 340 and 380 nm. Release was normalized on the maximum Ca<sup>2+</sup> release at 5 mM caffeine. Bars indicate the mean  $\pm$  s.e.m. of the percentage of Ca<sup>2+</sup>-release increments induced by different caffeine concentrations.

not shown). Immunolocalisation experiments with antibodies against the Ins(1,4,5)P<sub>3</sub>Rs revealed that RyR3 and Ins(1,4,5)P<sub>3</sub>R were codistributed in the endoplasmic reticulum surrounding the nucleus and in the central part of cell, although some peripheral regions of the cells, negative for RyR staining, were positive for Ins(1,4,5)P<sub>3</sub>R immunofluorescence. RyR-expressing cells were also stained with a monoclonal antibody against SERCA. The SERCA staining was also compatible, as expected, with a distribution in the endoplasmic reticulum. Similarly to what was observed for Ins(1,4,5)P<sub>3</sub>R, calsequestrin and RyRs, SERCA staining was more intense in the perinuclear region of the cells, in agreement with a higher density of endoplasmic reticulum in this region of the cells. Interestingly, however, SERCA immunostaining revealed a punctuate distribution of fluorescence, as if there were discrete regions of higher density of SERCA pumps. Overlay of SERCA images with RyRs pictures revealed only a partial overlap of the two antigens within the endoplasmic reticulum. Recent work has revealed that the Golgi apparatus may function as an Ins(1,4,5)P<sub>3</sub>-operated Ca<sup>2+</sup> store functionally distinguishable from those of the endoplasmic reticulum (Pinton et al., 1998). The subcellular localisation of the Golgi apparatus in RyR-expressing cells was investigated following cell transfection with plasmids coding for a Green Fluorescent Protein (GFP) fused to a Golgi targeting signal (Pinton et al., 1998). In most of the cells, the Golgi apparatus appears as a perinuclear convoluted structure, but no apparent overlay between Golgi apparatus fluorescence signal and RyR3 staining was observed (Fig. 3), suggesting that RyRs were not enriched in the Golgi, compared with the endoplasmic reticulum membranes.

#### Intracellular Ca<sup>2+</sup> measurements in HEK 293 cells expressing RyR1 and RyR3

The functional activity of heterologous RyRs expressed in HEK 293 cells was verified by intracellular Ca<sup>2+</sup>-release analysis of

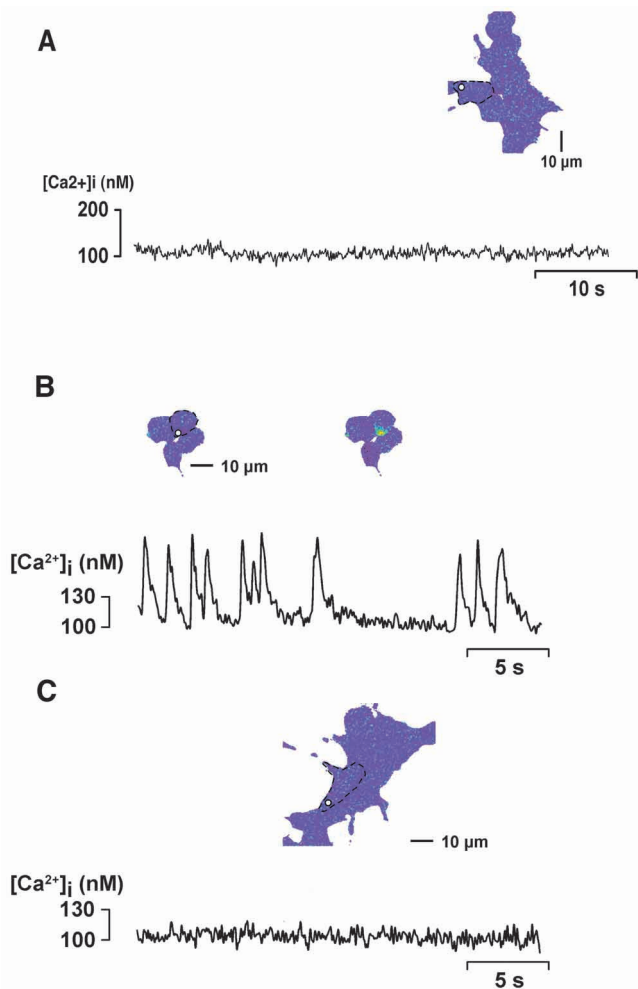
transfected cells. Cells were stimulated with caffeine, an activator of RyR Ca<sup>2+</sup>-release channels and analysed by video imaging of Fura-2-loaded cells. Caffeine solutions were applied to cells using either bulk solution changes or using a continuous gravity-driven superfusion. Neither of these methods evoked a change in Ca<sup>2+</sup> levels. This differs from data reported by Querfurth et al., who found expression of low levels of endogenous RyR2 in HEK 293 cells stimulated with caffeine, but agrees with studies by Tong et al., who did not detect endogenous RyR expression in caffeine-stimulated HEK 293 cells (Querfurth et al., 1998; Tong et al., 1999). HEK 293 cells expressing RyR1 or RyR3 recombinant proteins were stimulated with increasing concentrations of caffeine (Fig. 4). RyR3-expressing cells were found to respond to lower concentrations of caffeine than RyR1. Threshold values range between 0.125–0.250 mM for RyR1 ( $n=63$ ) and 0.0625–0.1250 mM for RyR3 ( $n=58$ ). In this study, we observed that RyR1 and RyR3 in HEK 293 cells display a different sensitivity to caffeine stimulation, with RyR3 being more sensible to lower caffeine concentrations than RyR1, as noted previously in myogenic cells (Fessenden et al., 2000). No systematic difference in the basal calcium levels between the control and transfected cells was observed (data not shown).

#### RyR3-expressing cells display spontaneous Ca<sup>2+</sup> release events

Confocal microscopy of Fluo-3-loaded cells indicated that the RyR3-expressing cells displayed spontaneous subcellular Ca<sup>2+</sup>-release events, similar to the Ca<sup>2+</sup> sparks and Ca<sup>2+</sup> puffs previously described in various cell types (Bootman et al., 1997a). In the majority of the cells (>90%,  $n=300$ ), the spontaneous localized Ca<sup>2+</sup> signals arose repetitively from a single site (Fig. 5B), more frequently occurring in a peripheral region of the cell, with the rest of the cell being quiescent. No subcellular Ca<sup>2+</sup> signals were recorded from control cells (Fig. 5C). Similar spontaneous activity was observed in five different RyR3-expressing clones, which expressed different amounts of RyR3 protein. Irrespective of the level of RyR3 expression, there was usually only a single spontaneously active site within the cells. Why such spontaneous events are restricted to 1 or 2 points in the endoplasmic reticulum, while RyR3 channels are widely distributed within the endoplasmic reticulum is not clear. It is reasonable to envision that occurrence of spontaneous Ca<sup>2+</sup> events in specific sites could be due to the presence of yet unidentified modulatory proteins at these points. Although RyR1-expressing and RyR3 cells responded similarly to caffeine stimulation, RyR1 cells did not display any spontaneous events either when unstimulated (Fig. 5A) or during applications of low (0.25–1.00 mM) caffeine concentrations (not shown). The higher caffeine sensitivity, the capability of generating spontaneous Ca<sup>2+</sup> events in RyR3-expressing cells, as well as the characteristics and frequency of these spontaneous events were not dependent on the RyR level of expression, as they could be observed in cells with a RyR3 content as low as 41 fmol/mg of protein but not in cells with a RyR1 content as high as 136.5 fmol/mg of protein.

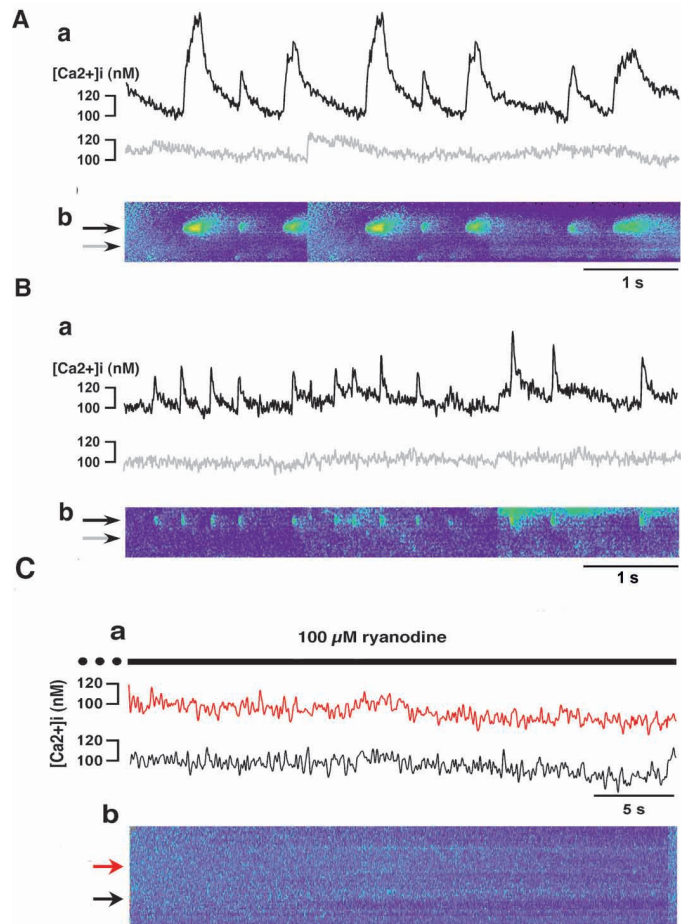
#### Characterisation of spontaneous Ca<sup>2+</sup> events in RyR3-expressing cells

The characteristics of the spontaneous and localized Ca<sup>2+</sup>



**Fig. 5.** RyR3-expressing cells display spontaneous localized Ca<sup>2+</sup> signals. (B) Typical spontaneous Ca<sup>2+</sup> signals recorded using confocal microscopy of single Fluo-3-loaded RyR3-expressing cells. The subcellular regions from which the localized Ca<sup>2+</sup> signals were recorded are depicted by white circles. The dashed lines indicate the borders of the cells. A and C illustrate the lack of spontaneous Ca<sup>2+</sup> signals in RyR1-expressing cells and control cells, respectively.

release events differed significantly among cells. Some sites produced signals that peaked after a couple of hundred milliseconds and had a total duration of up to a second (Fig. 6Aa). In other cells, the Ca<sup>2+</sup> signals were more reminiscent of the Ca<sup>2+</sup> sparks in cardiac muscle, with a rapid rising phase (time to peak  $\leq 50$  mseconds) and a recovery time of 100–200 mseconds (Fig. 6Ba). In addition to differences in the temporal properties of the localized Ca<sup>2+</sup> signals, the spatial spread of the signals was extremely variable, as shown by the line-scan plots in Fig. 6Ab, Bb. To determine whether the spontaneous Ca<sup>2+</sup> signals arose from the activation of RyRs, the cells were superfused with ryanodine at a concentration of 100 μM, which has been shown to inhibit RyR opening. Superfusion of cells with ryanodine for 5 minutes completely blocked all spontaneous Ca<sup>2+</sup>-release events (Fig. 6C). Application of caffeine (40 mM) following inhibition of RyRs with ryanodine evoked only a slight quench of the Fluo 3 fluorescence, with no obvious Ca<sup>2+</sup> release (not shown).



**Fig. 6.** Variable characteristics of spontaneous Ca<sup>2+</sup> signals in RyR3-expressing cells. A and B illustrate the considerable variability of spontaneous Ca<sup>2+</sup> signals in two RyR3-expressing cells. The traces in Aa and Ba depict the Ca<sup>2+</sup> changes in the regions along the line scan images marked by the arrows. Panel C depicts the effect of 100 μM ryanodine treatment on spontaneous Ca<sup>2+</sup>-release activity in a single RyR3-expressing cell.

## Discussion

The aim of the present study was to investigate the localisation in the endoplasmic reticulum of different isoforms of the RyR family of Ca<sup>2+</sup>-release channels and the capability of these channels to generate intracellular Ca<sup>2+</sup> signals in nonspecialised cells such as HEK 293. Following transfection of HEK 293 cells with expression plasmids encoding either RyR1 or RyR3 channels, RyRs were found to be resident proteins of the endoplasmic reticulum. Immunostaining with antibodies against RyRs revealed a specific signal compatible with a homogeneous distribution of these channels in the endoplasmic reticulum. RyR staining displayed an extensive overlap with other endoplasmic reticulum proteins, such as calreticulin and Ins(1,4,5)P<sub>3</sub>Rs, but not with the SERCA pumps. From a functional point of view, stimulation of RyR-transfected cells with caffeine, a specific agonist of these channels, gave rise to a significant increase in cytoplasmic Ca<sup>2+</sup> concentration, indicating that recombinant RyRs can localise and regulate functional intracellular Ca<sup>2+</sup> stores in HEK 293 cells. Confocal analysis of Ca<sup>2+</sup> release at the single cell level,

revealed that, following caffeine stimulation, a global Ca<sup>2+</sup> response in both RyR1 and RyR3 HEK 293 cells was observed, with Ca<sup>2+</sup> being released in an homogeneous fashion from all of the endoplasmic reticulum. Interestingly, while even low concentrations of caffeine induced a global Ca<sup>2+</sup> response in both RyR1 and RyR3 HEK 293 cells, spontaneous and localised Ca<sup>2+</sup> release events were observed in RyR3, but not in RyR1 cells. These data represent the first evidence of the generation of localised Ca<sup>2+</sup>-release events in nonmuscle cells expressing a recombinant RyR isoform.

Discrete localised increments of intracellular Ca<sup>2+</sup>, named Ca<sup>2+</sup> sparks, arising from co-operative opening of groups of few RyRs, have been reported in cardiac (Cannell and Soeller, 1999), skeletal (Tsugorka et al., 1995; Klein et al., 1996) and smooth muscles (Jaggard et al., 2000; Mironneau et al., 2001; Coussin et al., 2000), and neurons (Koizumi et al., 1999). Studies on embryonic skeletal muscle cells derived from RyR1- or RyR3-knockout mice have revealed that both RyR isoforms have the ability to produce, independently from each other, Ca<sup>2+</sup> sparks with nearly identical properties, although different from those observed in normal muscles expressing both isoforms (Shirokova et al., 1999; Conklin et al., 1999; Conklin et al., 2000). In contrast, transfection of RyR1 and RyR2 in non-muscle cells did not result in the generation of Ca<sup>2+</sup> sparks, from which it was concluded that RyRs by themselves may not be sufficient to support generation of localized Ca<sup>2+</sup> release events and that a muscle-specific environment may be required for the organisation of active Ca<sup>2+</sup> release units and generation of Ca<sup>2+</sup> sparks (Bhat et al., 1997; Bhat et al., 1999). Accordingly, expression of heterologous RyR1 or RyR3 has been shown to result in Ca<sup>2+</sup> sparks in 1B5 muscle cells, which do not express endogenous RyRs (Fessenden et al., 2000; Ward et al., 2000; Protasi et al., 2000). The ability of both RyR1 and RyR3 to generate Ca<sup>2+</sup>-release activity in 1B5 skeletal muscle cells has been confirmed recently (Ward et al., 2001). Comparison of the release events mediated by RyR1 or RyR3 channels in 1B5 cells led to the conclusion that, although both isoforms can support spontaneous release events, the spatio-temporal properties and frequency of events mediated by the two RyR isoforms differ.

In contrast to the above conclusions, results presented here clearly indicate that spontaneous Ca<sup>2+</sup>-release events can be supported by RyR3 channels in non-muscle cells, indicating that RyR3 channels themselves are sufficient to support generation of localised Ca<sup>2+</sup>-release activity. In contrast with the results obtained with HEK 293 cells expressing RyR3, HEK 293 cells transfected with RyR1 did not display any spontaneous Ca<sup>2+</sup>-release events either when unstimulated or following application of low caffeine concentrations. The observation that RyR1 and RyR3, when expressed under equivalent cellular conditions display a different behaviour in terms of spontaneous activity suggests that isoform-specific functional properties of RyR3 channels may be important for generation of specific intracellular Ca<sup>2+</sup> signals. Regulatory properties of RyR channels have been extensively studied (Meissner, 1994). Studies of RyR3 channel properties have revealed that they can participate in Ca<sup>2+</sup>-induced Ca<sup>2+</sup>-release processes, which may facilitate the generation of Ca<sup>2+</sup> sparks (Murayama and Ogawa, 1996; Murayama and Ogawa, 1997; Murayama and Ogawa, 2001; DiJulio et al., 1997; Sonleitner et al., 1998; Sorrentino and Reggiani, 1999).

A second interesting observation is the finding that, in RyR3-expressing cells, spontaneously generated Ca<sup>2+</sup>-release events were restricted to one or, at most, two sites. Localised Ca<sup>2+</sup> release events are expected to result from the activation of a variable number of intracellular Ca<sup>2+</sup>-release channels (Thorn et al., 1993; Stricker, 1999; Marchant and Parker, 2001; Bootman et al., 1997b; Koizumi et al., 1999). The mechanism leading to the formation of these sites is not clear, although clustering or organisation of channels in functional Ca<sup>2+</sup>-release units is obviously required. In frog sympathetic neurons Ca<sup>2+</sup>-release events have been found to arise in the cell periphery, a region with high density of RyRs and SERCA pumps; this suggests the presence of a functional specialisation of the endoplasmic reticulum that may promote generation of localised Ca<sup>2+</sup> release events (McDonough et al., 2000). In transfected HEK 293 cells, RyR1 and RyR3 channels seemed to be uniformly distributed throughout the endoplasmic reticulum, similarly to what has been observed for other endoplasmic reticulum proteins. It cannot be excluded, however, that either RyR channels or proteins of the endoplasmic reticulum involved in Ca<sup>2+</sup> signalling may organise at a level not detectable by the techniques used.

In conclusion, we have found that, in HEK 293 cells, RyR1 and RyR3 channels are homogeneously distributed in the endoplasmic reticulum. These channels, when stimulated with caffeine, are able to release Ca<sup>2+</sup> with a global response involving the entire endoplasmic reticulum. In contrast, expression of recombinant RyR3, but not RyR1, channels results in the appearance of Ca<sup>2+</sup> sparks. These data provide direct evidence that differential expression of RyR isoforms may be important for generating specific patterns of Ca<sup>2+</sup> signalling. In spite of initial attempts, we still have no evidence of what determines the formation of discrete Ca<sup>2+</sup>-release events in specific domains of the endoplasmic reticulum of HEK 293 cells transfected with RyR3 channels. More work is in progress to address this important point.

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