Caffeine chelates calcium in the lumen of the endoplasmic reticulum

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ABSTRACT

Cytosolic Ca^{2+} signals are often amplified by massive calcium release from the endoplasmic reticulum (ER). This calcium-induced calcium release (CICR) occurs by activation of an ER Ca^{2+} channel, the ryanodine receptor (RyR), which is facilitated both by cytosolic- and ER- Ca^{2+} levels. Caffeine sensitizes RyR to Ca^{2+} and promotes ER Ca^{2+} release at basal cytosolic Ca^{2+} levels. This outcome is frequently used as a readout for the presence of CICR. By monitoring ER luminal Ca^{2+} with the low-affinity genetic Ca^{2+} probe erGAP3, we find here that application of 50 mM caffeine rapidly reduces the Ca^{2+} content of the ER in HeLa cells by about 50%. Interestingly, this apparent ER Ca^{2+} release does not go along with the expected cytosolic Ca^{2+} increase. These results can be explained by Ca^{2+} chelation by caffeine inside the ER. Ca^{2+}-overloaded mitochondria also display a drop of the matrix Ca^{2+} concentration upon caffeine addition. By contrast, in the cytosol, with a low free Ca^{2+} concentration (10^{-7} M), no chelation is observed. Expression of RyR3 sensitizes the responses to caffeine with effects both in the ER (increase of Ca^{2+} release) and in the cytosol (increase of Ca^{2+} peak) at low caffeine concentrations (0.3-1 mM), that have no effects in control cells. Our results illustrate the fact that simultaneous monitoring of both cytosolic- and

ER-Ca^{2+} are necessary to understand the action of caffeine, and raise concerns against the use of high concentrations of caffeine as a readout of the presence of CICR.

KEYWORDS: Endoplasmic reticulum, calcium, intracellular calcium stores, caffeine, aequorin, calcium microdomains, CICR, Ryanodine Receptor

ABBREVIATION FOOTNOTE: Abbreviations used are: GFP, green fluorescent protein; GAP, GFP-Aequorin Protein; ER, endoplasmic reticulum; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; $[Ca^{2+}]_C$, cytosolic free Ca^{2+} concentration; $[Ca^{2+}]_{ER}$, Ca^{2+} concentration inside ER; $[Ca^{2+}]_M$, Ca^{2+} concentration inside mitochondria; TBH, 2,5-di-*tert*-butylhydroquinone; erGA, ER-targeted GFP-Aequorin; IP$_3$, inositol 1,4,5, trisphosphate; CCh, carbachol.

INTRODUCTION

In many excitable cells the cytosolic Ca^{2+} signals generated by Ca^{2+} entry through the plasma membrane are amplified by Ca^{2+} release from the endoplasmic reticulum (ER) via an autocatalytic process known as Ca^{2+}-induced Ca^{2+} release (CICR) ([1]). In this process Ca^{2+} itself is a potent activator of endomembrane Ca^{2+} channels, mainly the ryanodine receptors (RyRs). Mammalian tissues express three isoforms, RyR1, RyR2, and RyR3, each encoded by a different gene. RyR1 and RyR2 are predominantly expressed in the sarcoplasmic reticulum of skeletal muscle and heart, respectively, where they play an essential role to trigger muscle contraction ([2], [3]). RyR3 was originally identified in brain ([4]), but it is also expressed in many other cell types, including non-excitable ones ([5], [6]). CICR can also be triggered by ER release induced by inositol trisphosphate (1) and can also amplify [Ca^{2+}]$_C$ signals generated by Ca^{2+} release from other intracellular organelles, such as Golgi apparatus or the endo-lysosomal system ([1], [7]).

RyRs are able to sense sudden increases of cytosolic Ca^{2+} concentration ([Ca^{2+}]$_C$) and transduce them into an increase of ER Ca^{2+} permeability that produces a massive Ca^{2+} release. Increased Ca^{2+} concentration in the ER lumen ([Ca^{2+}]$_{ER}$) does also facilitate activation of RyRs and Ca^{2+} release ([8]). The second messenger cyclic ADP ribose (cADPR) increases the accumulation of Ca^{2+} into ER and facilitates Ca^{2+} release from the store by sensitization of the RyR ([7]). Caffeine sensitizes RyRs to both [Ca^{2+}]$_C$ and [Ca^{2+}]$_{ER}$ and facilitates CICR ([8]). At millimolar concentrations, caffeine produces ER Ca^{2+} release at basal [Ca^{2+}]$_C$, and this action has been used as a readout of the presence of RyRs in a given cell or tissue.

Caffeine is not a clean tool, as it may interfere with fluorescence of Ca^{2+} probes ([9]). In addition, loading the cells with Ca^{2+} probes may decrease the responses to caffeine ([10]). This has been attributed to the increase of Ca^{2+} buffering by the cytoplasm and to subtle perturbations of Ca^{2+} diffusion in the cytosol that result in dissipation of high Ca^{2+} microdomains ([10-13]). In the present paper, the role of Ca^{2+} chelation by caffeine in the ER lumen is examined.

EXPERIMENTAL PROCEDURES

Cell culture and gene transfection

The stably transfected HeLa clones expressing ER targeted GAP3 (erGAP3) or ER targeted GAP1 (IgGAP1) have been previously described ([14], [15]). Stable HeLa clones expressing mutated mitochondrially targeted (D119A) GFP-aequorin (mitmutGA) were generated by lipofectamine transfection of HeLa cells followed by 0.8 mg/ml G-418 selection. Single-cell clones were selected by limited dilution. All the HeLa clones were maintained in DMEM-GlutaMAX medium (GIBCO) supplemented with 100 μg/ml streptomycin and 100 U/ml penicillin, 10% (v/v) foetal bovine serum and 0.2 mg/ml G-418, at 37 ^{o}C under 5% CO_2. For imaging experiments, erGAP3 expressing cells were seeded on poly-L-lysine coated 12 mm-diameter coverslips at a density of 4 x 10^4 cells. For bioluminescence experiments, IgGAP1- or mitmutGA-expressing cells were seeded on poly-L-lysine coated 4-well plates at a density of 7 x 10^4 cells/well. In experiments shown in Figures 5 and 6, erGAP3 HeLa cells were transfected with 1 μg rabbit smooth muscle RyR3 cDNA cloned into pcDNA3 (kindly donated by Dr. S.R.W. Chen; University of Calgary, Canada) using lipofectamine 2000 (Invitrogen). In some experiments (Figure 6) RyR3 (1 μg) was cotransfected with the pmCherry-N3 (Clontech, 0.01 μg).

Western blot analysis

HeLa cells transfected wit RyR3- or control empty vector pcDNA were grown for 24-36 h, washed with ice-cold PBS and extracted with RIPA buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% TX-100; 0.1 % SDS and 0.5% deoxycholate) for 30 min. After centrifugation at 13,000 rpm for 5 min, the supernatant was diluted in Laemmli sample buffer and 50 μg protein samples were separated by PAGE. Membrane was first probed overnight with a mouse anti-RyR1 antibody (1:5000; ThermoFisher) followed by a horseradish peroxidase-labelled secondary antibody (1:1000; Bio-Rad) and incubated for 1h. To control for protein loading, membrane was probed with a mouse anti-tubulin antibody (1:5000; Sigma). Sample proteins were quantified by the Bradford assay.

Immunofluorescence

Stable HeLa clone expressing erGAP3 (see below) were seeded on 12 mm coverslips and transiently transfected with RyR3 cDNA. Cells were fixed for 24-36h with 4% PFA in phosphate buffered saline (PBS) for 20 min; 10% normal goat serum was added for blocking nonspecific binding sites. Expression of RyR3 was detected by incubating the mouse anti-RyR1 antibody (1:200; ThermoFisher) diluted in PBS and containing 10% goat serum, overnight at 4ºC. After washing with PBS, the secondary Alexa Fluor 568-conjugated antibody (1:200; Molecular Probes) was added and incubated for 60 min at 22ºC. Cultures were washed with PBS three times and mounted in Vectashield (Vector). GAP was detected as green fluorescence (excited at 470/40 nm and filtered at 540/50 nm) in a Zeiss Axioplan Z microscope equipped with a 63x/1.2w Korr objective and an AxioCam MR camera. The red fluorescence was excited at 560/40 and filtered at 605/50 nm). The Zeiss ApoTome^{R} system was used for optical sectioning and images were analysed with AxioVision and Image J software.

Fluorescence Ca^{2+} imaging

For simultaneous imaging $[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_C$, HeLa cells stably- expressing erGAP3 Ca^{2+} indicator were loaded with Rhod-3 by incubating with Rhod-3/AM (2 μM, Molecular Probes) for 1h at 22 ºC. Cells were imaged in a Zeiss axioplan upright microscope equipped with a water-immersion 25X objective (Plan-Neofluar, Zeiss; NA= 0.8) and sequentially excited at 402, 470 and 546 nm; fluorescence was read at >515 nm (535DF35) and >590 nm (LP590). GAP was excited using the two filters, ET402/15x and 470/35 DF, and a 505DRLP dichroic mirror. Cells were alternately epi-illuminated at 402 and 470 nm and light emitted above 505 nm was recorded using a Carl Zeiss AxioCam 12 bit camera handled by the AxioVision 4.6.3 software. Output images were background-subtracted and ratioed pixel-to-pixel using ImageJ software. The ratio F470/F402 was used as an index of $[Ca^{2+}]_{ER}$. The fluorescence excited at 546 nm (F546), was read using a FT580 dichroic mirror and a LP509 emission filter. F546 was expressed as F/F$_0$, as an index of $[Ca^{2+}]_C$. F$_0$ was the average of the fluorescence values obtained during the first 5-10 frames. The cells were under continuous perfusion (5-6 ml/min) at 22-25 ºC, with Extracellular-Like Solution (ELS) with the following composition (in mM): NaCl, 145; KCl, 5; MgCl$_2$, 1; CaCl$_2$, 1; glucose, 10; sodium-

HEPES, 10, pH 7.4. If not specified otherwise, all stimuli were diluted in ELS. Other details were as described previously ([14](), [16](), [17]()).

Aequorin Luminescence Ca^{2+} measurements

For measuring [Ca^{2+}]$_{ER}$ by bioluminescence, cells expressing the ER-targeted low-affinity Ca^{2+} probe IgGAP1 ([15]()) were incubated for 10 min at 22 °C in Ca^{2+}-free ELS containing 0.5 mM EGTA and 10 µM of the reversible sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor 2,5-di-tert-butylhydroquinone (TBH) ([18]()). GAP1 aequorin was then reconstituted by incubation with 1 µM coelenterazine *n* in the same medium during 1h, prior to measurements. Cells were then placed in a purpose-built luminometer (Cairn Research, UK) and perfused with ELS containing 1 mM CaCl$_2$, at 5 ml/min, to load the ER Ca^{2+} stores. At the end of each run, cells were lysed with 0.1 mM digitonin dissolved in 10 mM CaCl$_2$, in order to release all the residual aequorin luminescence. The total luminescence (L$_{TOTAL}$) was calculated by adding up all the L values from each time point up to the end of the experiment. Data are shown as the ratio between luminescence (L) to the total luminescence at each time point (L/LTOTAL). The [Ca^{2+}]$_{ER}$ was estimated by interpolation in the calibration curve ([15]()).

In the experiments of mitochondrial Ca^{2+} (Figure 4), HeLa cells expressing mitochondrially targeted mutated (D119A)-GFP-aequorin (mutmitGA) ([19]()) were reconstituted with coelenterazine *n* as described for the ER. The reconstitution medium also contained 10 µM of the SERCA inhibitor TBH in order to prevent refilling of the Ca^{2+} stores. Intracellular-Like Solution (ILS) was used in the permeabilized experiments. It had the following composition (in mM): KCl, 140; K$_2$HPO$_4$/KH$_2$PO$_4$, 1; MgCl$_2$, 1; Mg-ATP, 1; sodium succinate, 2; sodium-pyruvate, 1; K-HEPES, 20; pH 7.2. The cells were first permeabilized by perfusion with 50 µM digitonin in ILS containing 0.5 mM EGTA and 10 µM TBH during 1 min. The solution was then switched to ILS without digitonin containing 50 µM free-Ca^{2+}. This solution was made by blending titrated solutions of EDTA-Mg^{2+} and EDTA-Ca^{2+} in the required amounts, calculated using the program MaxChelator ([20]()). Calibration of L/L$_{TOTAL}$ into [Ca^{2+}]$_M$ was calculated according to the published constant's values previously published ([21]()). All the measurements were performed at 22 °C.

RESULTS

The activity of RyRs visualized by the cytosolic transient induced by caffeine is variable in different cell types (10). For example, chromaffin cells (22) respond with a fast and sharp $[Ca^{2+}]_C$ increase to a caffeine challenge (Figure 1A), whereas cerebellar granule neurons show a smaller response, which can be detected with Fluo-3 (Figure 1B), but not with Fura-2 (Figure 1C) (10). Even though RyRs are mainly expressed in excitable cells, some non-excitable cells are also able to express RyR (5). Figure 1D illustrates the $[Ca^{2+}]_C$ peak to caffeine in Human Embryonic Kidney cells 293 (HEK 293) cells loaded with fluo-3. By contrast, when these cells were loaded with Fura-2, they do not show an increase in $[Ca^{2+}]_C$ in response to caffeine stimulation (Figure 1E), similarly to granule neurons (compare to Figure 1C). The $[Ca^{2+}]_C$ response to the IP$_3$-producing physiological agonist ATP was larger and faster than that to caffeine. Direct measurements of the changes in $[Ca^{2+}]_{ER}$ with ER-targeted aequorin (Figure 1E) were consistent with the $[Ca^{2+}]_C$ results, demonstrating that the decrease of $[Ca^{2+}]_{ER}$ on stimulation with ATP is faster than the one induced by caffeine.

We have recently developed a new family of fluorescent genetically-encoded Ca^{2+} indicators dubbed GAP (GFP-Aequorin Protein), optimized for $[Ca^{2+}]$ measurements in organelles (23, 24). One of the main advantages of the GAP family is that it permits double-wavelength ratiometric measurements. This ability makes possible not only detecting Ca^{2+} transients but also estimations of the basal Ca^{2+} concentrations. In addition, the affinity of the GAP sensors for Ca^{2+} can be tailored for environments with different Ca^{2+} concentrations. For example, GAP3, with a Kd = 489 μM and scarcely sensitive to Mg^{2+} and pH, is optimal for measurements in high $[Ca^{2+}]$ environments such as the lumen of ER (14) or the Golgi apparatus (25).

Measurements in a HeLa cell line expressing erGAP3 stimulated with a maximal concentration of ATP are illustrated in Figure 2. The upper traces (A and B) show the time-course of the two individual fluorescence traces, at 402 and 470 nm. The fluorescence excited at the second wavelength increases with $[Ca^{2+}]$, while the first one decreases (14, 24). Note the specular behaviour of both wavelengths when ER is emptied by stimulation with the IP$_3$-producing agonist ATP (Figure 2A and 2B). The signal is calibrated by a complete discharge of the ER Ca^{2+} store, by adding a second

ATP pulse in Ca^{2+}-free medium containing the SERCA inhibitor 2,5-di-*tert*-butylhydroquinone (TBH). Trace C in Figure 2 illustrates the changes in the F_{470}/F_{402} ratio, which is proportional to the $[Ca^{2+}]_{ER}$, and has been normalized here by dividing the values by the baseline fluorescence (R/R_0, Figure 2C). Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_C$) can be simultaneously measured by loading the erGAP3 expressing cells with a red cytosolic Ca^{2+} probe such as Rhod-3 (26). The trace, normalized against the baseline fluorescence (F/F_0), is shown in Figure 2D. Simultaneous measurement of Ca^{2+} in the cytosol and in the ER allows precisely studying the temporal relationship between $[Ca^{2+}]_C$ and $[Ca^{2+}]_{ER}$ in order to draw mechanistic conclusions. We observe, for example, that the onset of the decrease of $[Ca^{2+}]_{ER}$ coincides with the increase of $[Ca^{2+}]_C$, consistent with the cytosolic Ca^{2+} being released from the ER. It is also noticeable that the $[Ca^{2+}]_C$ increase reaches its peak prior to $[Ca^{2+}]_{ER}$ reaches its minimum (Figure 2C and 2D). This result may seem surprising at first glance, but it can be easily rationalized by considering the fact that the $[Ca^{2+}]_C$ peak only reveals that Ca^{2+} clearance from the cytosol exceeds Ca^{2+} release from the stores, and this may occur as soon as the rate of release begins to decrease, even though net Ca^{2+} exit from the ER continues.

Figure 3 illustrates the effects of caffeine in HeLa cells on $[Ca^{2+}]_C$ and $[Ca^{2+}]_{ER}$. The dynamics of the individual GAP3 wavelengths, F_{402} and F_{470}, are shown in the upper panel (Figure 3A) whereas the F_{470}/F_{402} ratio (proportional to $[Ca^{2+}]_{ER}$) and the Rhod-3 fluorescence (F/F_0, proportional to $[Ca^{2+}]_C$) are shown in the lower panel (Figure 3B). The traces of the individual GAP3 fluorescences were specular, indicating that the changes are not artefactual. Moreover, bioluminescence measurements with aequorin also confirmed the $[Ca^{2+}]_{ER}$ drop induced by caffeine (Supplementary Figure S1). Surprisingly, the caffeine-induced $[Ca^{2+}]_{ER}$ decrease does not go along with changes in the $[Ca^{2+}]_C$ (Rhod-3 trace in Figure 3B). Only an overshoot in $[Ca^{2+}]_C$ due to the SERCA inhibition by TBH is detected. In contrast, stimulation with the IP_3-producing agonist ATP/carbachol triggered a decrease of GAP3 fluorescence (ER Ca^{2+} release) along with a Rhod-3 fluorescence increase ($[Ca^{2+}]_C$ increase). The simplest explanation for this discrepancy would be a chelation of Ca^{2+} inside the ER. This would not produce Ca^{2+} fluxes through the ER membrane and hence no changes in $[Ca^{2+}]_C$. Also, if caffeine chelated ER Ca^{2+} independently of activating RyRs, then the Ca^{2+} responses

should be insensitive to RyR blockers. Indeed, ryanodine (10 uM) did not block the caffeine-induced [Ca^{2+}]$_{ER}$ decrease (Supplementary Figure S2).

If changes of [Ca^{2+}]$_{ER}$ were due to chelation by caffeine, then a similar decrease in luminal [Ca^{2+}] should occur in other organelles with high Ca^{2+} content. We tested this prediction in mitochondria overloaded with Ca^{2+} by incubation of permeabilized cells with 50 μM [Ca^{2+}]$_C$ (Figure 4). Under these conditions the mitochondrial Ca^{2+} concentration ([Ca^{2+}]$_M$) reached 500-600 μM and perfusion with 50 mM caffeine decreased [Ca^{2+}]$_M$ by about 50%. This outcome indicates that caffeine is also able to chelate Ca^{2+} within the mitochondrial matrix.

We next compared the effect of caffeine in cells expressing RyRs. Figure 5 illustrates the dose-response relationship for caffeine concentrations between 0.3 and 50 mM in control or in RyR3-expressing cells. In control cells there was little or no effect on [Ca^{2+}]$_{ER}$ at low concentrations of caffeine (0.3 or 1 mM), whereas higher concentrations (10 or 50 mM) produced a graded release (Figure 5A). In no case [Ca^{2+}]$_C$ was increased, as demonstrated by the Rhod-3 trace recorded simultaneously (scale at right). The experiment was terminated with stimulation with the IP$_3$-producing agonist ATP, alone or in the presence of the SERCA inhibitor TBH, to demonstrate that ATP stimulation produced a decrease of [Ca^{2+}]$_{ER}$ concomitant with an increase of [Ca^{2+}]$_C$, as expected. The last ATP stimulation (maximum release) allows calibration of the [Ca^{2+}]$_{ER}$ signal. Note that [Ca^{2+}]$_{ER}$ decrease induced by 50 mM caffeine was about 50% of the maximum.

When the same stimulation protocol was applied to cells expressing RyR3 (Figure 5B) the action of caffeine was very much sensitized. Low caffeine concentrations (0.3 and 1 mM) produced now a clear [Ca^{2+}]$_{ER}$ release (see GAP3 trace), and the 1 mM stimulus triggered [Ca^{2+}]$_{ER}$ oscillations. In addition, the drops of [Ca^{2+}]$_{ER}$ were in all cases associated with synchronic [Ca^{2+}]$_C$ increases (see Rhod-3 trace). Moreover, the ER Ca^{2+} release was already maximum at 10 mM caffeine, as no further decrease of [Ca^{2+}]$_{ER}$ was seen at 50 mM. Finally, these high caffeine concentrations discharged ER completely, whereas the Ca^{2+} release from the ER of control cells was only 50% at 50 mM caffeine. A detailed quantification of the cytosolic- and ER-Ca^{2+} responses in both types of cells is provided in the Figure legend and in supplemental Table S1. Expression of the RyR3

protein in the transfected cells was confirmed by Western blot (Figure 5C) and by immunofluorescence (Figure 5D).

In order to assess more precise and quantitatively the effect of caffeine, we marked the RyR-expressing cells with mCherry-Fluorescent Protein (Cherry), and stimulated them with increasing caffeine concentrations (0.3 to 50 mM). Figure 6A compares the averaged responses, grouped separately for Cherry-positive or -negative cells. Note that the non-synchronic oscillatory behavior of individual cells is hindered by the averaging. The RyR3 expressing cells (red trace) responded better to caffeine at all the concentrations tested. The response was almost maximal at 10 mM caffeine, whereas in the non-expressing cells (black trace) $[Ca^{2+}]_{ER}$ was decreased by only 10-20% of the total ER Ca^{2+} (Figure 6A). Figure 6B summarizes the results. It is clear that the apparent affinity for caffeine was much higher in the RyR3-expressing cells. Cells transfected with the empty plasmid behaved as the untransfected cells (results not shown).

Finally, we tested the effect of theophylline, another methylxanthine able to sensitize CICR (8), on $[Ca^{2+}]_{ER}$, and compared it with that provoked by caffeine. Results are shown in Supplementary Figure S3. Both xanthic acid derivatives had similar effects at 20 mM although the theophylline-induced $[Ca^{2+}]_{ER}$ decrease was somewhat smaller and slower than the one induced by caffeine.

DISCUSSION

RyR-mediated CICR is important for amplifying cytosolic Ca^{2+} signals in both excitable and non-excitable cells. Detection of CICR mechanisms can be performed on the basis of immunoreactivity, ryanodine binding or functional tests measuring modifications of Ca^{2+} signaling. One of the easiest protocols assesses the increase of $[Ca^{2+}]_C$ in response to caffeine. Caffeine sensitizes RyRs to cytosolic Ca^{2+} and releases this cation from the ER at resting $[Ca^{2+}]_C$. The response is vigorous in some cell types (Figure 1A), and weaker in others (Figures 1B and 1D). The own Ca^{2+} probes, which are mobile Ca^{2+} chelators, can hinder small $[Ca^{2+}]_C$ peaks making them practically undetectable (see examples in Figures 1C and 1E). This effect has been attributed to buffering of the $[Ca^{2+}]_C$ microdomain by the Ca^{2+} probe and to an increase of Ca^{2+} diffusion velocity by mobile Ca^{2+} buffers, thus accelerating dissipation of high Ca^{2+} microdomains (10-12, 27-29).

The above interferences illustrate the problems and limitations of indirect measurements, which can be circumvented by direct measurement of $[Ca^{2+}]_{ER}$. These direct $[Ca^{2+}]_{ER}$ measurements were first made possible in intact cells with ER-targeted aequorin (30), although low affinity Ca^{2+} probes are required for quantitative measurements (22, 31). Suitable fluorescent probes have also been recently developed (14, 23, 24).

Direct measurements of $[Ca^{2+}]_{ER}$, either with luminescent (Figure 1F) or with fluorescent probes (Figure 3) in cells stimulated with caffeine revealed that this xanthine produces a rapid reduction of $[Ca^{2+}]_{ER}$, suggesting a release of Ca^{2+} from the ER to the cytosol. Unexpectedly, this $[Ca^{2+}]_{ER}$ decrease did not go along with a $[Ca^{2+}]_C$ increase (Figure 3B). This intriguing result can be explained by chelation of Ca^{2+} in the ER lumen. Caffeine is quite lipid-soluble and diffuses through cell membranes, including the ER membrane. On the other hand, it has been reported that caffeine binds Ca^{2+}, although with very low affinity (association constant, 30 M^{-1}) (32, 33). Although interaction of caffeine with Ca^{2+} at the cytosol, where $[Ca^{2+}]_C$ is about 10^{-7} M, should be negligible, the interplay inside compartments with high Ca^{2+} would increase in proportion to the calcium concentration. Thus, at 1 mM Ca^{2+} and 50 mM caffeine, near 50 % of the total calcium in the ER should be bound to caffeine. The decrease of the ER

GAP3 signal in HeLa cells on adding caffeine is consistent with this computation. Note that chelation of Ca^{2+} at the stores could also activate store-operated Ca^{2+} entry. Theophylline is also able to bind Ca^{2+} with similar affinity as caffeine (33), and this is consistent with our results (Figure S3).

As mentioned in the introduction, a decrease of [Ca^{2+}]$_{ER}$ elicited by caffeine is often used for functional detection of the presence of RyRs in cells and tissues. Our present results alert against this practice, as reduction in [Ca^{2+}]$_{ER}$ can reflect binding of Ca^{2+} by caffeine into the ER lumen rather than a real ER Ca^{2+} release. The range of caffeine concentrations used is also relevant, as binding is evident at concentrations of 10-50 mM whereas activation of CICR by binding to RyR occurs at concentrations of 1 mM or less (Figures 5 and 6). Our results evidence that measurements of either the cytosolic or the ER-Ca^{2+} concentration both have severe limitations to explain CICR, so that simultaneous monitoring of [Ca^{2+}]$_{ER}$ and [Ca^{2+}]$_C$ is necessary to draw founded conclusions.

Caffeine does also bind other divalent metals with more affinity than Ca^{2+} (32). These metals are present at trace amounts in living organisms, but are often concentrated inside the intracellular organelles of living cells. This opens up the possibility that binding of these trace elements within intracellular calcium stores such as ER, Golgi apparatus or mitochondria, might be involved in the action mechanism of caffeine.

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COMPETING INTERESTS

The Authors declare that there are no competing interests associated with the manuscript.

AUTHORS CONTRIBUTION

Jonathan Rojo-Ruiz, Macarena Rodríguez-Prados and Alba Delrío-Lorenzo did most of the experimental work. Maria Teresa Alonso and Javier-García-Sancho provided conceptual input and designed the experiments. All authors participated in analysis, discussion and interpretation of data, revised the article, and gave final approval. Javier García-Sancho put together all data and wrote the manuscript.

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FIGURE LEGENDS

Figure 1. Comparison of cytosolic Ca^{2+} ([Ca^{2+}]$_C$) increases induced by caffeine in different cell types. (A) Bovine chromaffin cells (CRF); **(B, C)** cerebellar granule neurones (GN); **(D, E, F)** HEK293T cells (HEK). Cells were stimulated with: depolarizing high K (70 mM; K); 50 mM caffeine (CAF); or 10 μM ATP (ATP), as shown. [Ca^{2+}]$_C$ was measured either with Fura-2 (**A, C, E** and **F**; loading with 4 μM Fura-2/AM), Fluo-3 (**B, D**; loading with 1 μM Fluo-3/AM) or aequorin (**F**; cells expressing the ER-targeted aequorin were reconstituted with 1 μM coelenterazine *n* in Ca^{2+}-free medium for 60-90 min. Experiment was started by refilling the ER with 1 mM Ca^{2+}). Temperature, 22 ºC. F/F$_0$: Fluo-3 fluorescence expressed as fraction of the baseline value. Reproduced with permission from ([10](#)).

Figure 2. Simultaneous fluorimetric measurements of [Ca^{2+}]$_{ER}$ and [Ca^{2+}]$_C$ in HeLa cells. Cells expressing erGAP3 and loaded with Rhod-3 were challenged first with 100 μM ATP. The same stimulus was applied at the end of the experiment in Ca^{2+}-free medium containing 10 μM of the SERCA blocker 2, 5-di-tert-butylhydroquinone (TBH) to empty completely the ER calcium stores. Traces are the average values of 39 cells present in the same field, and are representative of 10 similar experiments. **(A)** and **(B)** show the changes of the individual fluorescences excited at 402 and 470 nm, respectively. The fluorescence values have been normalized by expressing them as F/F$_0$. **(C)** Ratio of the two GAP fluorescences (F$_{470}$/F$_{402}$), which is proportional to [Ca^{2+}]$_{ER}$. These values are normalized by expressing them as fraction of the baseline value (R/R$_0$). **(D)** Rhod-3 fluorescence, normalized as F/F$_0$. This value is proportional to [Ca^{2+}]$_C$.

Figure 3. Comparison of the effects of caffeine (CAF, 50 mM) or IP$_3$-producing agonists (ATP/CCh) on [Ca^{2+}]$_{ER}$ (GAP3 fluorescence) and [Ca^{2+}]$_C$ (Rhod-3 fluorescence) in HeLa cells. (A) Individual GAP3 fluorescences. Stimulation protocol as shown: ATP/CCh stands for 100 μM ATP + 100 μM carbachol. **(B)** Comparison of the effects of caffeine and ATP on [Ca^{2+}]$_{ER}$ (GAP3) and [Ca^{2+}]$_C$ (Rhod-3). Values are the average traces of 29 cells present in the same field and are normalized to the baseline value as R/R$_0$ or F/F$_0$. Other details as in Figure 2. Results are representative of 2 independent experiments with the same protocol where a total of 66 cells were

studied. The Standard Error of the Mean (SEM) values of the GAP3 fluorescence in the different peaks ranged from 0.01 to 0.02. For the Rhod-3 fluorescence the SEM in the different peaks oscillated between 0.04 and 0.07. The peak following stimulation by ATP/CCh, expressed as delta F/F_0 was (mean±SEM, n=66) 0.50±0.07, whereas after the repeated stimulation with caffeine the delta F/F0 was only 0.08±0.04.

Figure 4. Effects of caffeine (CAF, 50 mM) on the mitochondrial Ca^{2+} content ([Ca^{2+}]$_M$) in permeabilized HeLa cells. The plasma membrane of cells expressing GFP-aequorin targeted to the mitochondrial matrix (mitGA) ([19], [34]) was permeabilized by treatment with 50 µM digitonin for 1 min. and perfused with an intracellular-like solution (ILS) containing 100 nM Ca^{2+}. The SERCA blocker TBH was added at 10 µM to avoid interferences with ER. At the time shown, perfusion of the cells was switched to ILS containing 50 µM Ca^{2+} to provoke mitochondrial calcium uptake through the mitochondrial calcium uniporter (17). At the times shown, caffeine (50 mM) was added (CAF). Results are shown as Luminescence/total Luminescence (left ordinate axis), which is proportional to [Ca^{2+}]$_M$ (approximate calibration at right ordinate axis). Experiment representative of three similar ones.

Figure 5. Comparison of the responses to caffeine in control (A) and RyR3-expressing HeLa cells (B). HeLa cells were transiently-transfected with RyR3 cDNA. ER (GAP3, scale at left) and cytosolic Ca^{2+} transients (Rhod-3, scale at right). Responses to different caffeine concentrations (CAF; 0.3, 1, 10 and 50 mM) are shown. The effects of ATP (100 µM) or the same stimulus in Ca^{2+}-free medium + TBH (to obtain maximum ER Ca^{2+} release) are included for comparison. The single-cell traces shown are representative of 25 untransfected cells **(A)** or 14 RyR3-transiently transfected cells **(B)**. The SEM values oscillated between 0.02 and 0.04. Results are representative of seven (control) and five (RyR3-transfected) independent experiments with a total number of 274 cells (control) and 70 cells (RyR3-transfected) studied. Detailed quantification is summarized in supplemental Table S1. **(C)** RyR3 expression in the transfected HeLa cells. Whole extracts obtained from control cells transfected with empty vector (control) or with RyR3 cDNA (+RyR) were subjected to Western blot with either a specific anti-RyR3 or an anti-tubulin antibody as a control of protein loading. **(D)** Inmunofluorescence of stably-expressing erGAP3-HeLa cells transiently

transfected with RyR3. Cells were fixed, permeabilized and immunostained with anti-RyR antibody; erGAP is shown in green and the RyR in red. Calibration mark, 10 µm.

Figure 6. Dose-response plots of the caffeine-induced [Ca^{2+}]$_{ER}$ decreases in HeLa cells. Cells were co-transfected with RyR3 and Cherry-Fluorescent Protein (Cherry). Unicellular ROIs selected for Cherry-positive or negative cells were averaged separately. **(A)** Average traces of 26 RyR3-transfected cells (red line) and 19 control cells (black line) in a representative experiment. Protocol includes sequential stimulation with 0.3, 1, 10 and 50 mM caffeine, followed by stimulation with ATP (100 µM), removal of external Ca (Ca0) and maximal ER-Ca^{2+} discharge with ATP+Ca0+TBH. **(B)** ER-Ca^{2+} emptying (expressed as the decrease of R/R$_0$) plotted against caffeine concentration. *Max* stands for maximal ER-Ca^{2+} discharge. The mean values in RyR3-expressing (red) or control (black) cells correspond to experiment shown in **A**. The vertical bars stand for SEM values. Similar results were obtained in 4 independent experiments with different transfections.

Fig. 1

A. CRF, Fura-2

B. GN, Fluo-3

C. GN, Fura-2

D. HEK, Fluo-3

E. HEK, Fura-2

F. HEK, Fura-2-loaded, Aequorin

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

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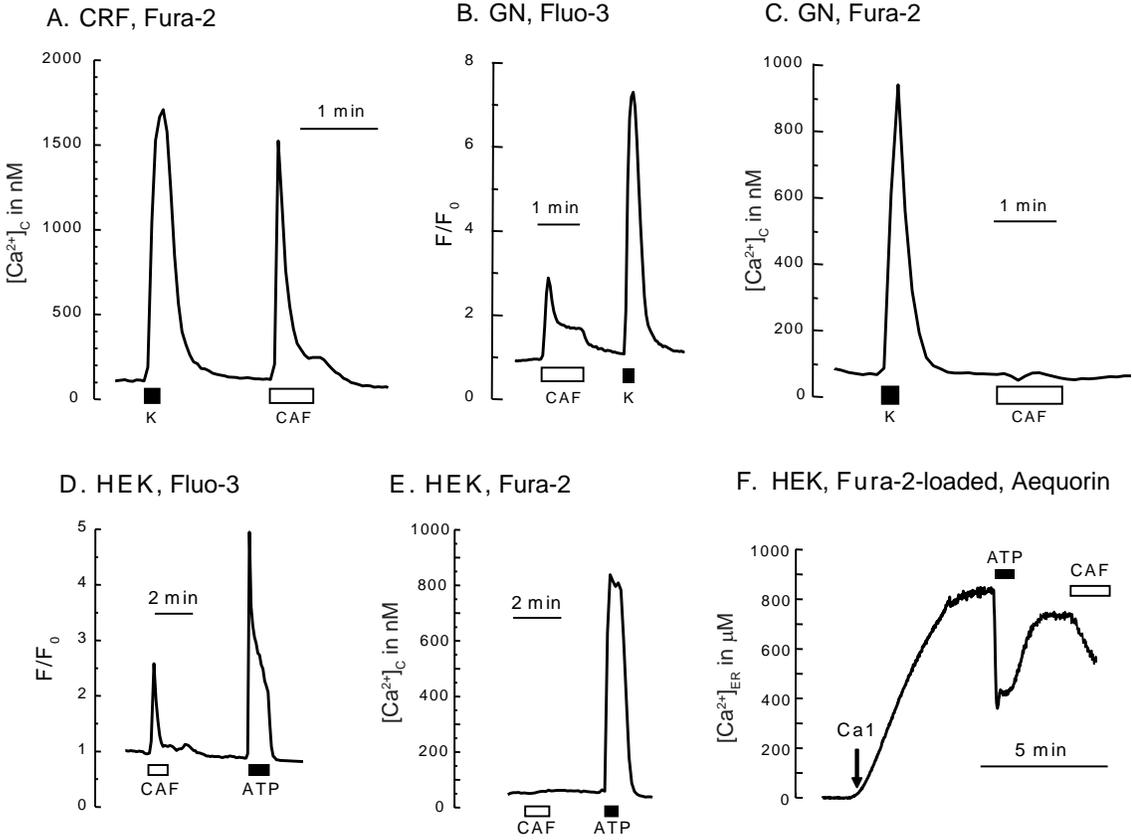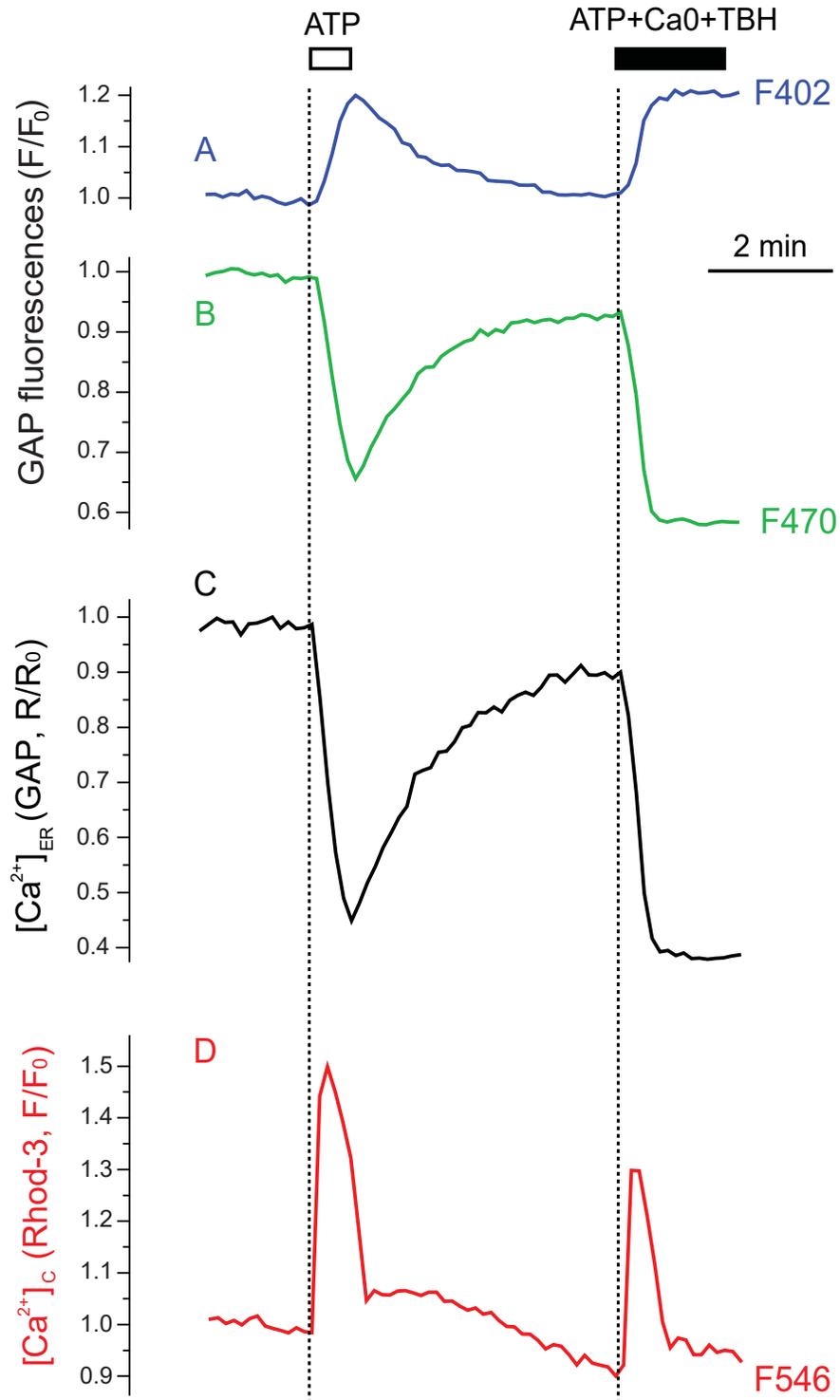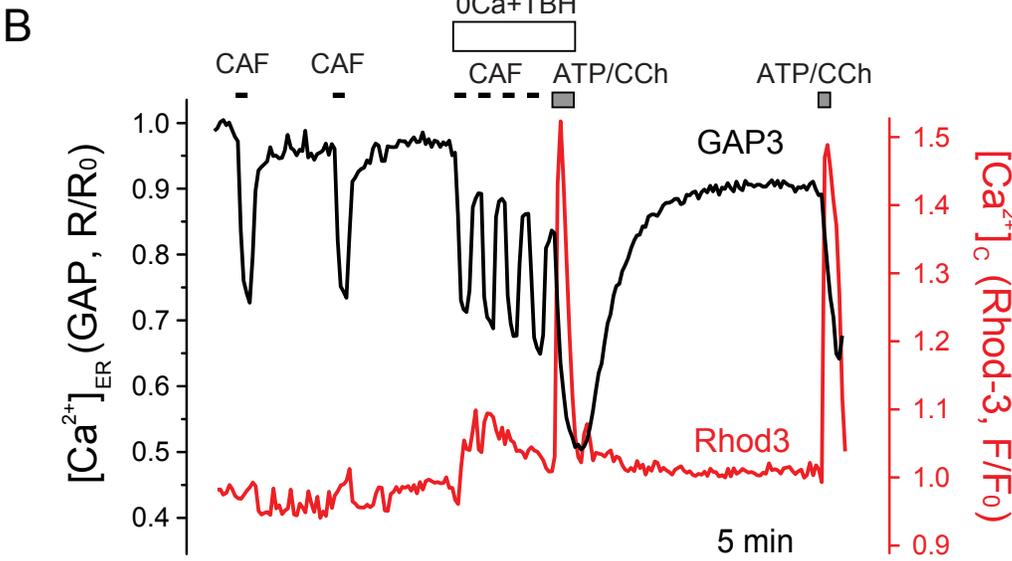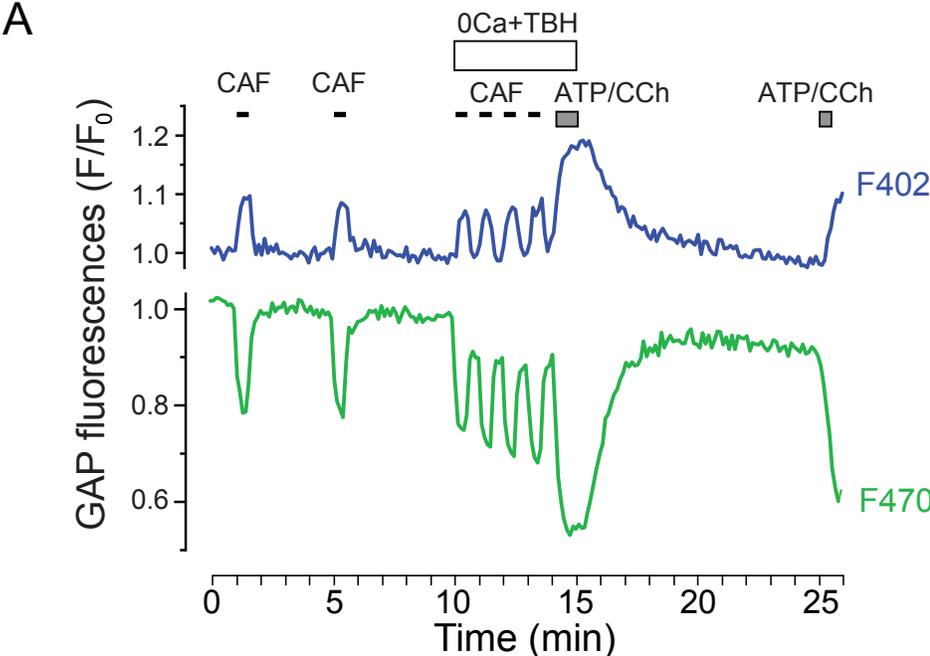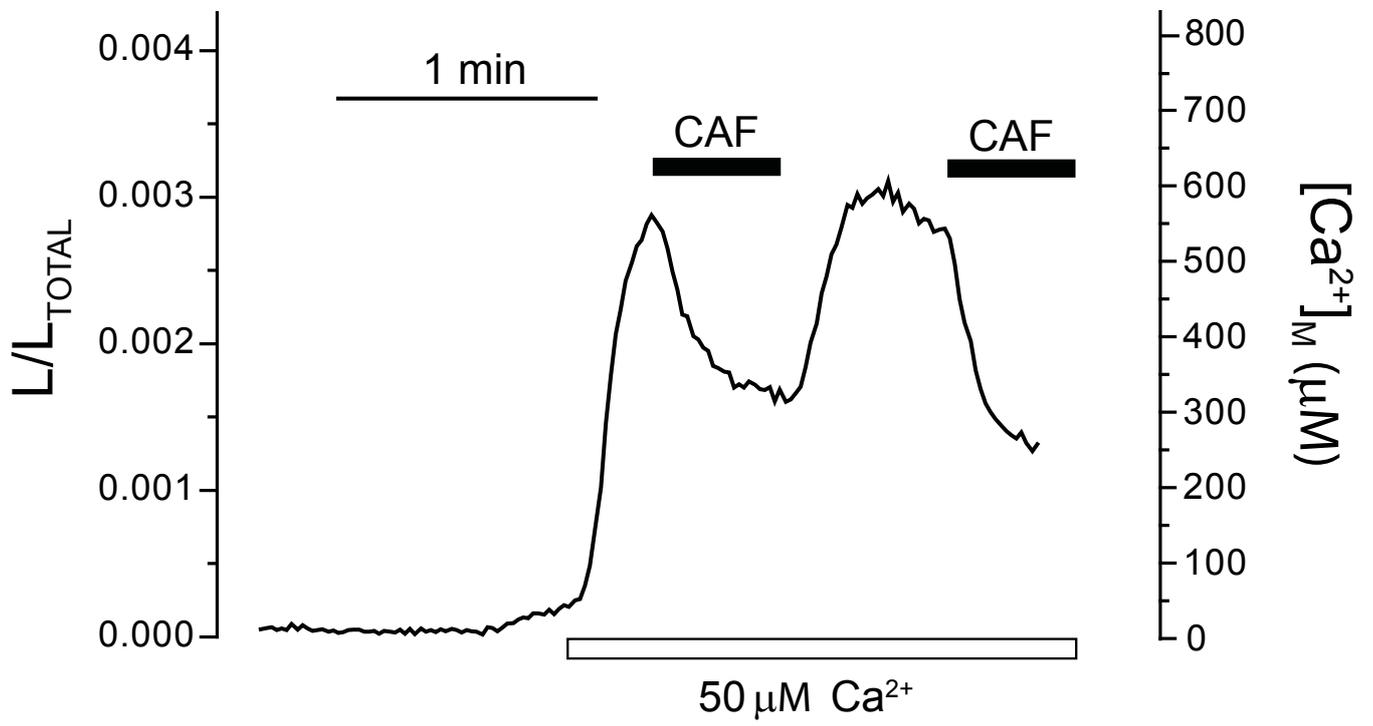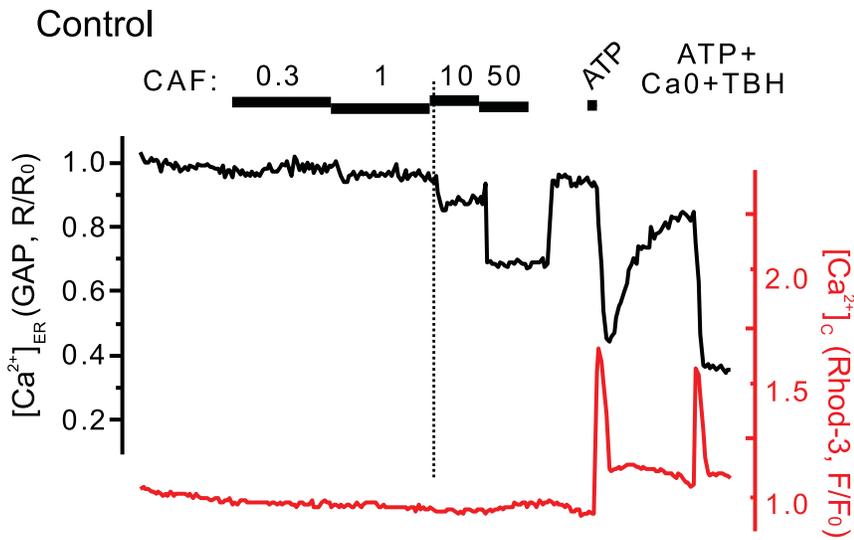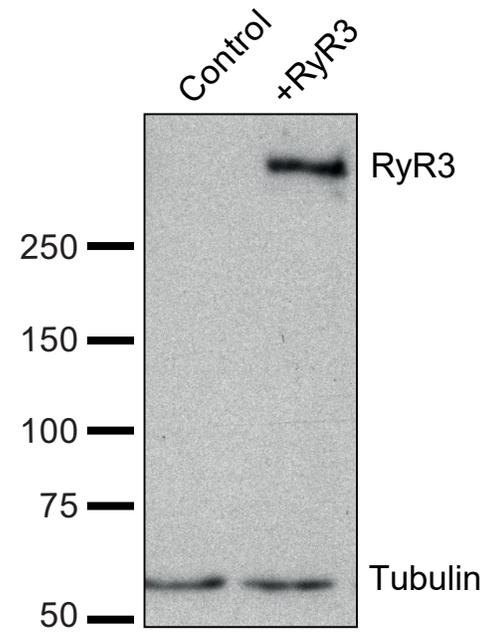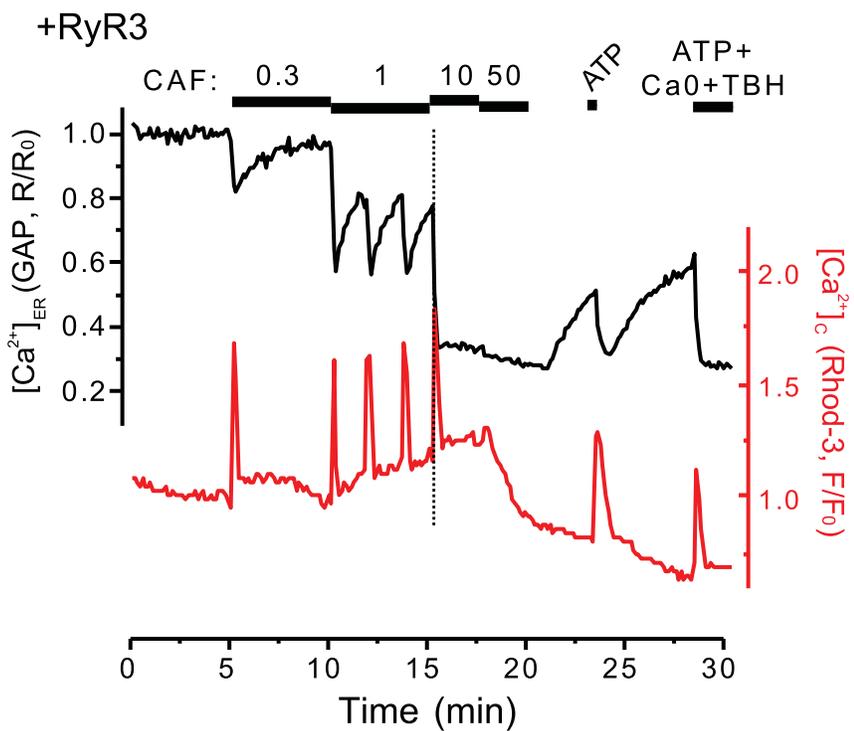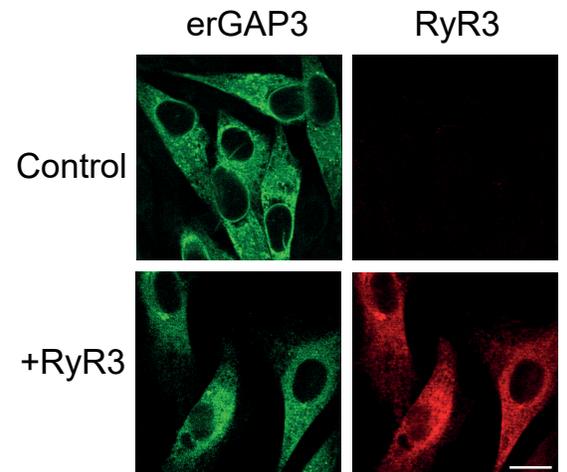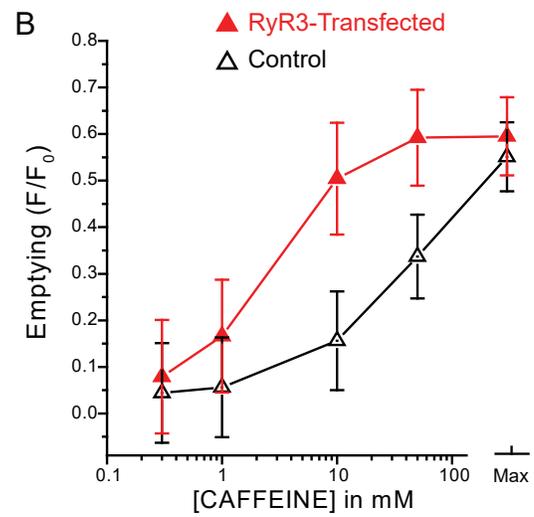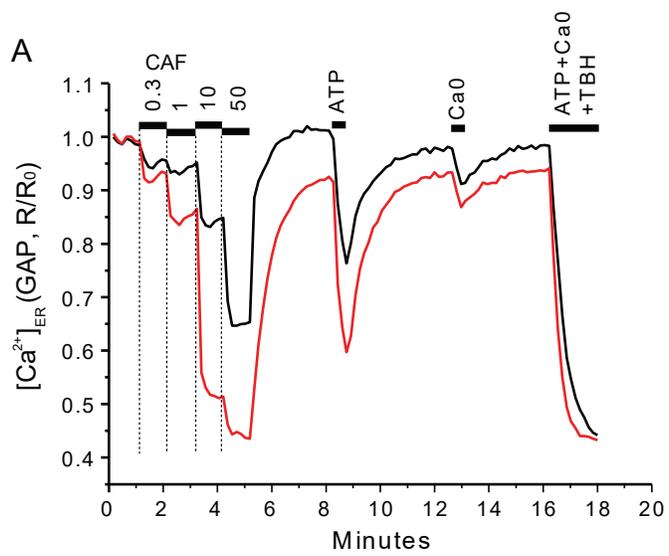
SUPPLEMENTAL MATERIALS:

SUPPLEMENTAL TABLES:

Supplemental Table 1. Comparison of the responses to caffeine in control and RyR3-expressing HeLa cells.

SUPPLEMENTAL FIGURES:

Supplemental Figure 1. Caffeine decreases [Ca^{2+}]ER in HeLa cells measured by aequorin bioluminescence. Cells expressing IgGAP1 (targeted to ER) reconstituted with coelenterazine *n* were used for the measurements. CAF, 50 mM caffeine. ATP, 100 μM ATP.

Supplemental Figure 2. Lack of effect of ryanodine (10 μM) on caffeine-induced ER Ca^{2+} release. The average trace of 20 cells present in the same microscope field is shown. ATP, 100 μM ATP + 100 μM CCh. RND, 10 μM ryanodine. CAF, 50 mM caffeine. Max, Ca^{2+}-free medium containing 10 μM tert butylhydroqinone (TBH), 100 μM ATP and 50 mM caffeine. Note absence of use-dependent inhibition. Representative of 3 similar experiments.

Supplemental Figure 3. Comparison of the decrease of [Ca^{2+}]ER induced by caffeine (20 mM, CAF) and theophylline (20 mM, Theo). Maximal stimulation through the IP3 pathway was induced at the end of the experiment by stimulation with 100 μM ATP + 100 μM carbachol (ATP-CCh). The trace is the average of 104 cells.

Supplemental Table 1. Comparison of the responses to caffeine in control and RyR3-expressing HeLa cells.

Stimuli		$[Ca^{2+}]_{ER}$ decrease		$[Ca^{2+}]_C$ increase	
		Control	+RyR3	Control	+RyR3
Caffeine (mM)	0.3	0.05 ± 0.04	0.11 ± 0.03	<0.05*	3.49 ± 0.03
	1	0.06 ± 0.03	0.24 ± 0.03	<0.05*	4.09 ± 0.03
	10	0.16 ± 0.03	0.61 ± 0.03	<0.05*	4.50± 0.03
	50	0.40 ± 0.03	0.65 ± 0.01	<0.05*	4.45 ± 0.03
ATP		0.45 ± 0.10	0.50 ± 0.01	3.09 ± 0.16	4.57 ± 0.10
ATP + TBH		0.63 ± 0.02	0.63 ± 0.01	2.95 ± 0.14	4.39 ± 0.10

*There was no individual $[Ca^{2+}]_C$ peaks

Results correspond to experiments similar to the one shown in Figure 5. Data collected from 274 control cells (6 experiments) and 70 RyR3-transfected cells. (5 experiments). Mean ± SEM values are shown. Stimulation with caffeine produced a graded $[Ca^{2+}]_{ER}$ decrease with no $[Ca^{2+}]_C$ increase in the controls, whereas a large $[Ca^{2+}]_C$ increase was produced in the RyR3-transfected cells. Stimulation with ATP produced a $[Ca^{2+}]_C$ increase in both controls and RyR3-transfected cells. The increase was somewhat larger in the RyR3-transfected cells, suggesting that the release induced by ATP may be amplified by CICR.
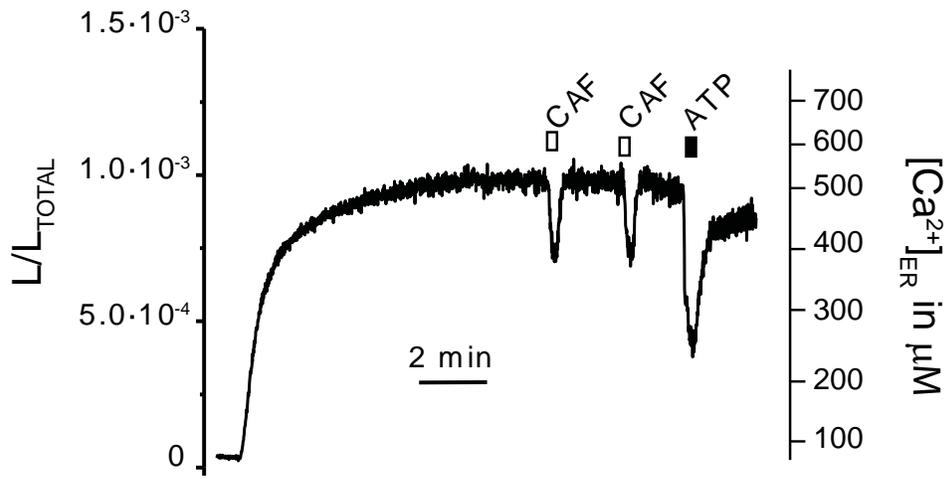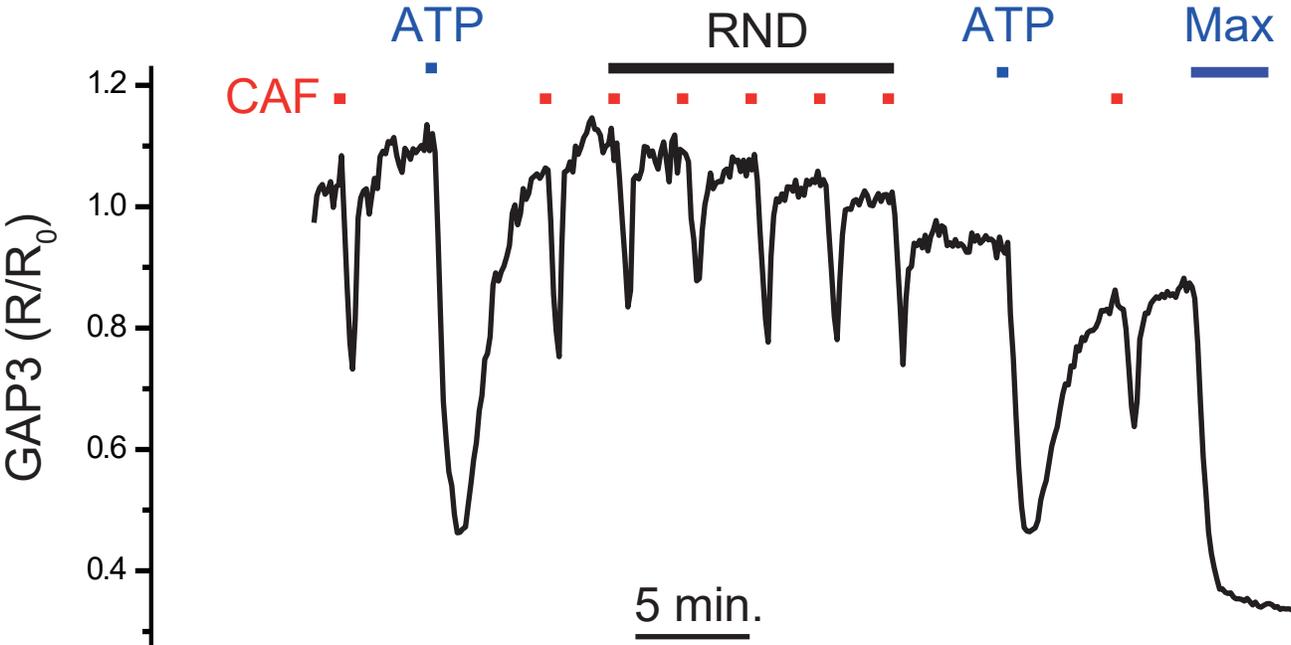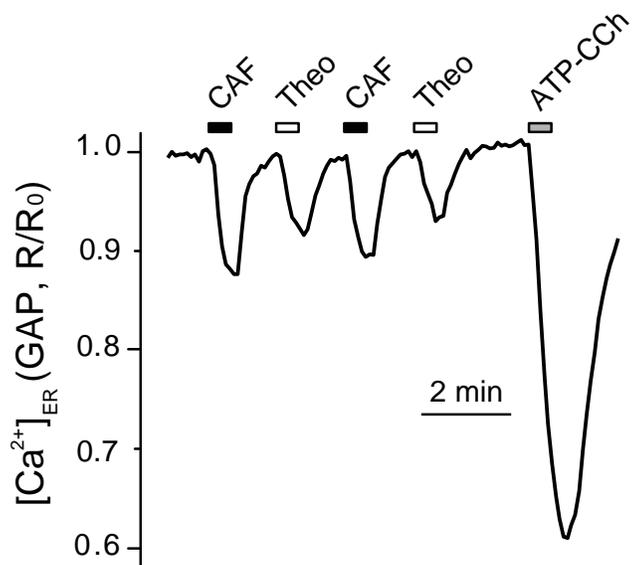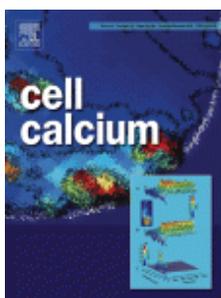
Fig. S1

Fig. S2

Fig. S3

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