RESEARCH COMMUNICATION

Recombinant apoaequorin acting as a pseudo-luciferase reports micromolar changes in the endoplasmic reticulum free Ca^{2+} of intact cells

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We describe a novel method to monitor the endoplasmic reticulum (ER) free Ca^{2+} in intact cells. Continuous perfusion of HeLa cells, expressing ER-targeted apoaequorin, with coelenterazine allowed the apoprotein to act as a pseudo-luciferase capable of reporting free Ca^{2+} from 0.1–100 μM. In intact HeLa cells, addition of ionomycin increased apoaequorin-generated light by 91%, indicating that resting ER free Ca^{2+} was approx. 2 μM. Agonist stimulation decreased the ER apoaequorin signal and proportionally increased cytosolic free Ca^{2+} consistent with agonist-induced release of Ca^{2+} from the ER.

INTRODUCTION

Agonist stimulation of non-excitable cells induces calcium (Ca^{2+}) release from an intracellular store which has been identified as the endoplasmic reticulum (ER) [1]. The intracellular messenger responsible for Ca^{2+} release is D-*myo*-inositol 1,4,5-trisphosphate (IP_3). IP_3-mediated elevation of cytosolic free Ca^{2+} is the primary trigger initiating many cellular processes [1,2].

Considerable progress has been made towards understanding the spatial and temporal organization of cytosolic Ca^{2+} signals using the Ca^{2+}-sensitive photoproteins and fluorescent dyes [1–3]. The free Ca^{2+} within the lumen of the ER is important in controlling both the spatial and temporal aspects of agonist-stimulated Ca^{2+} signals [1,2,4–6]. In order to resolve the mechanisms underlying the Ca^{2+} signals [1–3], regulation of influxes of the ion through the plasma membrane [4,5], the abnormalities in Ca^{2+} signals which occur in the pathology of disease [7] and mechanisms by which Ca^{2+} controls gene expression [8], it is essential to be able to measure the free Ca^{2+} concentration in the ER.

A number of studies have used compartmentalized low-affinity fluorescent Ca^{2+} indicators to monitor free Ca^{2+} inside internal stores. Estimates of ER resting free Ca^{2+} using these indicators (e.g. magfura) range from 60–630 μM in different cell types [6,9–13]. However, these measurements are prone to error as the indicators have poor selectivity for Ca^{2+} compared with Mg^{2+} [14]. Furthermore, the methods used to compartmentalize the dyes do not locate them specifically to the ER [11], and rely on elimination of trapped cytosolic dye by permeabilization of the plasma membrane. These manipulations alter the properties of the ER [13], and may account for some of the disparity in quantification of ER free Ca^{2+}.

Recent developments have allowed measurements of free Ca^{2+} levels in the ER by targeting the Ca^{2+}-sensitive photoprotein to this organelle [15–19]. This overcomes the problems of selective localization of the indicator and allows measurements of free Ca^{2+} in intact, non-permeabilized cells. In our initial studies, we reactivated recombinant aequorin in the ER of intact cells and used this to monitor changes in free Ca^{2+} [15,16]. We reported that the ER free Ca^{2+} of COS7 cells was in the range of 1–5 μM [15,16]. However, this estimate may have been low as it was necessary to cool the cells until measurements were made to prevent the rapid consumption of the aequorin in the relatively high free Ca^{2+} environment of the ER. It was also possible that part of the aequorin had been consumed in a high free Ca^{2+} compartment, leaving a fraction active in a compartment of relatively low free Ca^{2+} [18,19]. Montero et al. [18] used an alternative ER targeting approach and a low-affinity aequorin mutant and concluded, by measuring the surrogate ion strontium, that the concentration of ER free Ca^{2+} was in the millimolar range.

In the present paper, we describe a novel approach to circumvent these problems by using ER-targeted apoaequorin acting as a 'pseudo-luciferase', rather than as the photoprotein aequorin [20]. When Ca^{2+} binds to the photoprotein aequorin, a conformational change within the protein results in the luminescent oxidation of coelenterazine to coelenteramide. However, when coelenterazine is added to apoaequorin in the presence of Ca^{2+}, the coelenteramide is replaced continuously by coelenterazine to regenerate aequorin. Under these conditions, the aequorin formed instantly undergoes the luminescent reaction depending on the relative amount of Ca^{2+} ions already bound to the apoaequorin. The Ca^{2+} bound to the apoprotein, therefore, determines the rate of conversion of coelenterazine to coelenteramide resulting in multiple, rather than a single, photon emissions [20].

When intact HeLa cells expressing the ER-targeted apoaequorin [15–17] were perfused with coelenterazine, a clear correlation was found between decreases in free Ca^{2+} in the lumen of the ER and increases in the cytosol in response to the agonist ATP. Using the Ca^{2+} ionophore ionomycin to modulate cytosolic free Ca^{2+} [21], we estimate that the resting free Ca^{2+} in the ER lumen of HeLa cells is approx. 2 μM. This is in close agreement with our estimates for free Ca^{2+} in the ER of COS7 cells, measured by aequorin in its conventional photoprotein mode [15,16].

Abbreviations used: ER, endoplasmic reticulum; CCD, charge coupled device; IP_3, D-*myo*-inositol 1,4,5-trisphosphate; RAdER, apoaequorin targeted to the ER; RAdLA, cytosolic firefly luciferase–apoaequorin chimaera.

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EXPERIMENTAL

Materials

Coelenterazine and the Ca^{2+}/EGTA calibration buffers were purchased from Molecular Probes (Eugene, OR, U.S.A.). Tissue culture reagents were from Sigma (Poole, Dorset, U.K.) and GibcoBRL (Paisley, Renfrewshire, U.K.). All other chemicals were of
Analar grade.

Cell culture

The HeLa cell-line was cultured as described previously [17]. Cells were seeded onto 25 cm^2 flasks to approx. 80 % confluence (2×10^6 cells) and were infected with recombinant replication-deficient adenovirus vectors [17] expressing either apoaequorin targeted to the ER (RAdER) [15,16], or a cytosolic firefly luciferase–apoaequorin chimaera (RAdLA) [22], at a multiplicity of infection of 100 plaque-forming units/cell.

Calibration of apoaequorin as a luciferase

HeLa cells, infected with the RAdER for 48 h, were washed twice in PBS (120 mM NaCl and 10 mM Na_2HPO_4,$2H_2O$, pH 7.4) containing 1 mM EDTA, and finally with PBS containing 50 μM EDTA. Cells were scraped into a hypo-osmotic extraction buffer (containing 0.5 mM EDTA, 5 mM β-mercaptoethanol and 20 mM Tris/HCl, pH 7.4), and freeze-thawed three times to release proteins. The cell extracts were centrifuged at 20000 ***g*** for 10 min at 22 °C and the proteins contained in the supernatants were dialysed with 0 Ca^{2+} buffer (10 mM EGTA/120 mM KCl/1 mM $MgSO_4$/10 mM Mops) from the Ca^{2+}/EGTA calibration kit (Molecular Probes). Aliquots of the protein were then added to dilutions of the Ca^{2+} calibration buffers (Molecular Probes) or to 1 M $CaCl_2$ diluted in 120 mM KCl/1mM $MgSO_4$/10 mM Mops, pH 7.1 (a buffer similar in composition to that supplied in the Molecular Probes kit), at 37 °C, in the wells of white plastic 96-well tissue-culture plates. Coelenterazine, solubilized in methanol, was added to a final concentration of 2 μM (final methanol concentration < 0.1 %, v/v). Light production was monitored through a 55 mm macro lens attached to a Photek three microchannel plate intensified CCD camera (Photek 216; Photek Ltd., Hastings, U.K.), which stored the photon image as an array of 384×288 pixels at video rates (50 Hz) [23]. Total light production from each well was normalized to that observed in 1 mM Ca^{2+}.

Luminescence measurements from cells

At 24 h post-infection with either RAdER or RAdLA, HeLa cells were detached in cold PBS containing 1mM EDTA and 8×10^4 cells were seeded on to 22×22 mm glass coverslips. To increase expression of the recombinant proteins, 5 mM sodium butyrate was included in the culture medium for approx. 18 h before the experiments [17].

For measurement of cytosolic free Ca^{2+} in cells infected with RAdLA, cytosolic recombinant apoaequorin was converted to the photoprotein by adding coelenterazine (final concentration 2 μM) at least 4 h before the experiments [16]. The coverslips were inverted over the reservoir of a plastic perfusion chamber (maintained at 37 °C in a dark box) and brought into contact with a fibre optics bundle attached to the photon counting camera. Cells were perfused for at least 10 min in Krebs–Ringer–Henseleit solution (containing 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$ and 25 mM Hepes, pH 7.4) at 37 °C, to remove excess coelenterazine. The fractional discharge of aequorin was calculated from the total light emitted by the photoprotein at the end of each experiment by exposing the cells to water containing 5 mM $CaCl_2$ to discharge unconsumed aequorin. This value was used to convert light emission into free Ca^{2+} [24].

Cells expressing the ER-targeted apoaequorin were not exposed to coelenterazine until the start of the experiment, when 2 μM coelenterazine was included in all perfusion medium. All cellular manipulations were delayed for at least 12 min after the addition of coelenterazine to allow stabilization of light production.

RESULTS AND DISCUSSION

Calibration of apoaequorin as a pseudo-luciferase

In order to establish that apoaequorin as a pseudo-luciferase could be used to monitor different concentrations of free Ca^{2+}, the relationship between light emission and free Ca^{2+} was measured. Recombinant apoaequorin was extracted from HeLa cells which had been infected with RAdER. The apoprotein was then added to buffers containing various free Ca^{2+} concentrations, and Ca^{2+}-dependent light production was monitored after the addition of 2 μM coelenterazine. Addition of coelenterazine resulted in immediate light production which remained constant for at least 1000 s (Figure 1, upper trace). Apoaequorin light production was saturated at approx. 100 μM free Ca^{2+}, and was sensitive to 0.1 μM Ca^{2+} (Figure 1). This

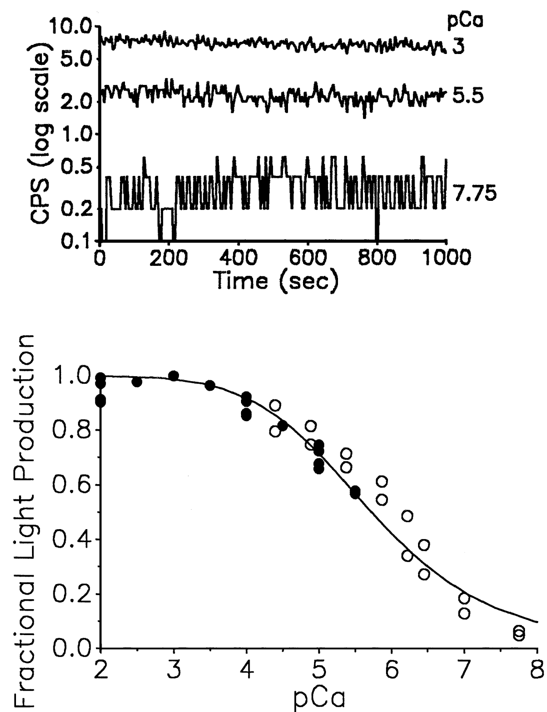

Figure 1 Calibration of apoaequorin

Apoaequorin was extracted from HeLa cells infected with RAdER and mixed with Ca^{2+} calibration buffers. Coelenterazine (2 μM final concentration) was added and light production was monitored by a photon counting CCD camera (Photek). The upper trace shows a typical time course of light production [CPS, chemiluminescent c.p.s. (log scale)], plotted against time for pCa 3, 5.5 and 7.75. Total light production is expressed as a fraction of that at pCa3, and is shown plotted against time (○, Ca^{2+} calibration buffers; ●, dilutions of 1 M $CaCl_2$; see the Experimental section). Each data point represents the mean from an independent experiment in which measurements were conducted in triplicate.

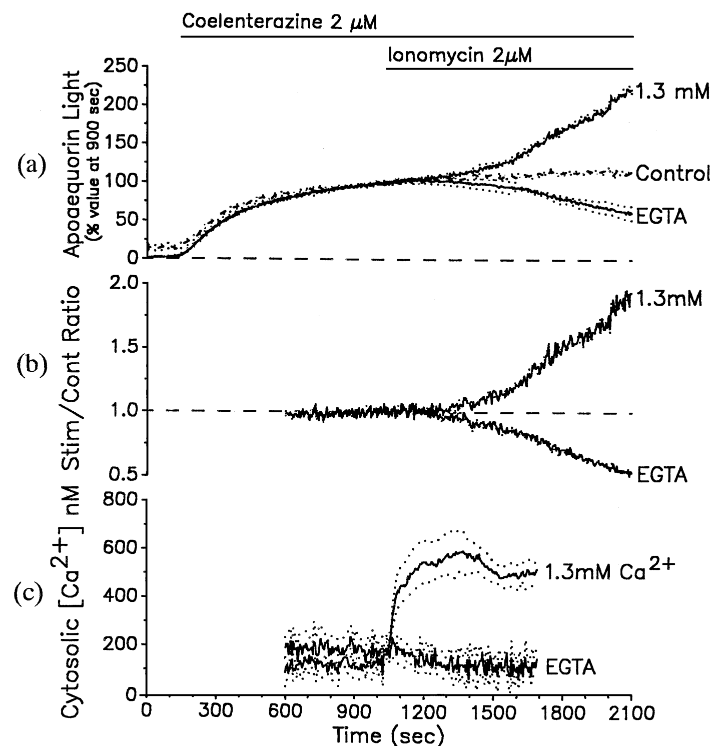

Figure 2 Estimation of ER free Ca^{2+} with targeted apoaequorin

HeLa cells expressing recombinant apoaequorin were cultured on coverslips, and light emission was monitored through a fibre optic probe by a photon counting camera. The cells were perfused with Krebs–Ringer–Henseleit solution containing 2 μM coelenterazine. (**a**) Light emission normalized to that at 900 s after the addition of coelenterazine. Ionomycin (2 μM) was then included in the perfusion medium containing either 1.3 mM free Ca^{2+}, or no free Ca^{2+} containing 1 mM EGTA and mean light production is shown as solid lines. Control cells were not treated with ionomycin and the mean is shown as a dashed line. The dotted lines indicate ± 1 S.E.M. for each data set ($n = 6$). (**b**) Ratio of light production from ionomycin-treated cells to control cells, which corrects for any deviation in the light produced in the control cells. (**c**) Effect of ionophore on cytosolic free Ca^{2+}, assessed in cells expressing the cytosolic luciferase–aequorin chimaera. Aequorin was reconstituted conventionally by addition of coelenterazine to the cells. Free-Ca^{2+} values were derived from luminescence counts obtained from cells as described in the Experimental section. Mean values are shown as a solid line, and the dotted lines indicate ± 1 S.E.M. ($n = 6$).

range of sensitivity is similar to that of the native and recombinant photoproteins [15,24]. However, whereas the photoprotein can only monitor free Ca^{2+} at 10–100 μM for a few seconds, in pseudo-luciferase mode the apoprotein was able to monitor free Ca^{2+} for at least 30 min (see below) [20]. This makes the pseudo-luciferase a suitable indicator in environments of relatively high free Ca^{2+}, such as the ER [15,16,18,19]. Although the sensitivity of pseudo-luciferase and photoprotein are similar, the range of light intensities is narrower for the protein in the luciferase mode [24]. These differences in light intensities may arise as a result of engineering the C-terminus of the photoprotein, which has significant effects on the Ca^{2+}-dependent and -independent light emission [16,25]. However, the use of apoaequorin as the pseudo-luciferase overcomes the decreased stability and increased sensitivity of the photoprotein aequorin associated with engineering the C-terminus [15,16,25].

Estimation of ER free Ca^{2+} using apoaequorin as a pseudo-luciferase

In order to estimate the free Ca^{2+} inside the ER of intact cells, HeLa cells expressing ER-targeted apoaequorin were perfused with coelenterazine. Light emission from the cells was then monitored under conditions where the cytosolic free Ca^{2+} was either elevated or reduced as a result of manipulation with the Ca^{2+} ionophore ionomycin. Perfusion of HeLa cells expressing ER-targeted apoaequorin with 2 μM coelenterazine for approx. 10 min resulted in an immediate rapid increase, followed by a slower increase, in light production (Figure 2a, control trace). Perfusion of cells expressing the cytosolic luciferase–apoaequorin chimaera, or 'sham-infected' cells, resulted in light production which was less than 1 % of that observed in cells expressing the ER-targeted apoprotein (results not shown). This result indicated that the majority of light production was caused by the action of the ER-targeted apoaequorin on coelenterazine.

When cells were perfused with coelenterazine and the Ca^{2+} ionophore ionomycin (2 μM), in the presence of 1.3 mM external Ca^{2+}, an increase in light production was observed. When the signal was compared with the control in the absence of ionophore, apoaequorin light production significantly increased by 91 ± 2 % ($n = 6$) (i.e. the stimulated/control light ratio was 1.91 ± 0.02) (Figure 2a). This indicated that, at rest, ER free Ca^{2+} was below saturation point (100 μM) for apoaequorin in luciferase mode. To assign a value to resting ER free Ca^{2+}, we made the assumption, based on the calibration (Figure 1), that treatment of cells with ionomycin increased the ER free Ca^{2+} towards 100 μM (i.e. an increase of 91 % above resting levels). From this, the estimated resting ER free Ca^{2+} was approx. 2 μM. These increases in ER free Ca^{2+} were consistent with the effect of ionomycin on cytosolic free Ca^{2+} in cells expressing the cytosolic luciferase–aequorin (Figure 2c). In the presence of ionomycin, cytosolic Ca^{2+} reached a plateau level at a faster rate than that of the ER, from a resting level of 122 ± 45 nM to a sustained elevated level of 506 ± 53 nM. As ionomycin disrupts the Ca^{2+}

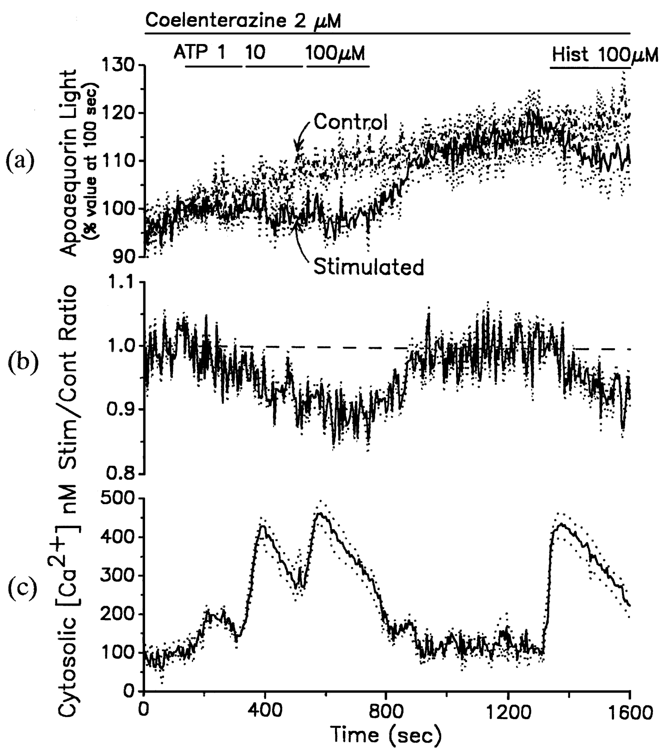

Figure 3 Agonist-induced release of Ca^{2+} from the ER in the presence of extracellular Ca^{2+}

(**a**) Measurement of light production from cells expressing ER-targeted apoaequorin. Coelenterazine (2 μM) was added at zero time. Light production was normalized to that produced 100 s after the addition of coelenterazine. The cells were stimulated by ATP at concentrations shown for the periods indicated by the bars. Control cells were perfused with coelenterazine but not treated with agonists (the mean is shown as a dashed line, dotted lines indicate ±1 S.E.M.). After washout of ATP, both control and experimental cells were stimulated with histamine (Hist) (100 μM). (**b**) Ratio of light production from stimulated (Stim) cells to control (Cont) cells (the dotted lines indicate ±1 S.E.M.; $n = 6$). (**c**) Free Ca^{2+} transients in the cytosol of HeLa cells induced by ATP. ATP-induced changes in cytosolic free Ca^{2+} were monitored as described in the Experimental section and in the legend to Figure 2. Mean values are shown as a solid line, dotted lines indicate ±1 S.E.M.; $n = 6$.

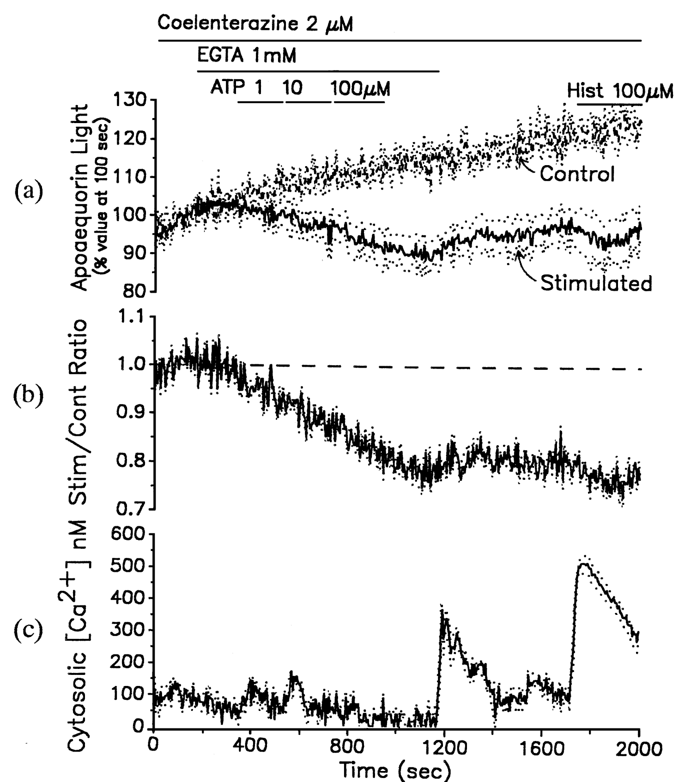

Figure 4 Agonist-induced release of Ca^{2+} from the ER in the absence of extracellular Ca^{2+}

(**a**) Measurement of light production from cells expressing ER-targeted apoaequorin. Coelenterazine (2 μM) was added at zero time. Light production was normalized to that produced 100 s after the addition of coelenterazine. The cells were stimulated by ATP at concentrations shown for the periods indicated by the bars. Control cells were perfused with coelenterazine but not treated with agonists (the mean is shown as a dashed line, dotted lines indicate ±1 S.E.M.). Following washout of ATP both control and experimental cells were stimulated with histamine (Hist) (100 μM). (**b**) Ratio of light production from stimulated (Stim) cells to control (Cont) cells (the dotted lines indicate ±1 S.E.M.; $n = 6$). (**c**) Free Ca^{2+} transients in the cytosol of HeLa cells induced by ATP. ATP-induced changes in cytosolic free Ca^{2+} were monitored as described in the Experimental section and in the legend to Figure 2. Mean values are shown as a solid line, and dotted lines indicate ±1 S.E.M.; $n = 6$.

homoeostasis between the cytosol, ER and extracellular space, the relative activity of the ER Ca^{2+}-dependent ATPase and the plasma membrane Ca^{2+}-pumps will then determine the rate at which a new Ca^{2+} steady-state is established.

In contrast, when HeLa cells were treated with ionomycin in the absence of extracellular free Ca^{2+} (Krebs–Ringer–Henseleit solution with $CaCl_2$ replaced by 1 mM EGTA) there was a decrease of 55±0.8% in the light emission relative to the control (Figure 2b). The treated/control light ratio fell to 0.54±0.01 (Figure 2b). Based on the calibration shown in Figure 1 and the estimated resting ER free Ca^{2+} of approx. 2 μM (see above), ionomycin decreased ER free Ca^{2+} to approx. 0.3 μM. Under these conditions, cytosolic free Ca^{2+} fell from 188±53 nM to 112±43 nM (Figure 2c).

In the presence and absence of extracellular Ca^{2+}, estimates of ER free Ca^{2+} were greater than cytosolic free Ca^{2+}. In the absence of specific inhibitors this is consistent with the known activity of the ER Ca^{2+}-dependent ATPase inducing a gradient across the ER membrane, despite the presence of ionomycin [21].

In our initial studies, using aequorin in conventional photoprotein mode, we estimated the free Ca^{2+} in the ER of COS7 cells to be in the range 1–5 μM [15,16], which is in close agreement with the value reported for HeLa cells. Although this value is significantly lower than the estimates derived from other groups [9–14,18,19,21], it is the first reported measurement of ER free Ca^{2+} in intact cells in which the ionic environment had not been perturbed. In order to assign a value to ER free Ca^{2+}, we assumed ionomycin increased free Ca^{2+} to a concentration approaching 100 μM. We also used a calibration *in vitro* (Figure 1), which may differ significantly from Ca^{2+} sensitivity of the protein in the ER of intact cells, to assign an approximate value of 2 μM. Furthermore, the ER signal in resting cells was not saturated, which implied that ER free Ca^{2+} must lie significantly below 100 μM.

Agonist-induced release of Ca^{2+} from the ER

We measured the fractional change in ER-targeted apoaequorin light emission when HeLa cells were stimulated by the agonists ATP and histamine, which mobilize intracellular Ca^{2+} following the generation of IP_3 [17]. Cells were sequentially stimulated with ATP (1, 10 and 100 μM) in the presence of extracellular Ca^{2+}. There was a decrease in apoaequorin light emission, consistent

with ATP-induced release of Ca^{2+} from the ER (Figures 3a and 3b). Stimulation of cells with 1, 10 and 100 μM ATP significantly decreased apoaequorin light emission to 96 ± 1, 90 ± 1 and 89 ± 1 % ($n = 6$) of control values respectively. From the calibration (Figure 1), and the estimate of 2 μM free Ca^{2+}, the changes in light emission correspond to a new free Ca^{2+} of 1.9 ± 0.1, 1.5 ± 0.1 and 1.4 ± 0.1 μM respectively. Washout of ATP resulted in a return to control levels of free Ca^{2+} (101 ± 2 % of light emission from unstimulated cells). Subsequent stimulation of the cells with 100 μM histamine decreased light emission to 93 ± 2 % of control values (Figures 3a and 3b). These changes in ER free Ca^{2+} were paralleled by typical biphasic increases in cytosolic free Ca^{2+}, measured by the luciferase–aequorin chimaera (Figure 3c).

In the absence of extracellular Ca^{2+}, sequential stimulation with 1, 10 and 100 μM ATP decreased light emission to 92 ± 2, 87 ± 1 and 81 ± 1 % of control values respectively (Figures 4a and 4b). Washout of ATP and the addition of extracellular Ca^{2+} resulted in stabilization of the light signal. Although light emission did not return to pre-stimulation level, it increased above the level observed following the stimulation, which corresponded to an influx of Ca^{2+} into the cytosol, monitored by the cytosolic luciferase–aequorin chimaera (Figure 4c).

In conclusion, the data presented here show that problems associated with the relatively short half-life of aequorin targeted to the ER have been overcome by using apoaequorin as a pseudo-luciferase rather than as a photoprotein. Agonist stimulation of intact HeLa cells decreased apoaequorin-generated light production, consistent with IP_3-induced release of Ca^{2+} from the ER. We have used this method to estimate an ER free Ca^{2+} in resting cells of approx. 2 μM.

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