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Divergent effect of mammalian PLC-zeta in generating Ca^{2+} oscillations in somatic cells versus eggs

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SYNOPSIS

Sperm PLC-zeta (PLC ζ) is a distinct phosphoinositide-specific phospholipase C isoform that is proposed to be the physiological trigger of egg activation and embryo development at mammalian fertilization. Recombinant PLC ζ has the ability to trigger Ca²⁺ oscillations when expressed in eggs, but it is not known how PLC ζ activity is regulated in sperm or eggs. Here, we have transfected CHO cells with PLC ζ fused with either YFP or luciferase and found that PLC ζ -transfected cells did not display cytoplasmic Ca²⁺ oscillations any different from control cells. PLC ζ expression was not associated with changes in CHO cell resting Ca²⁺ levels, nor in a significantly changed Ca²⁺ response to extracellular ATP compared to control cells transfected with either YFP alone, a catalytically-inactive PLC ζ , or luciferase alone. Sperm extracts containing PLC ζ also failed to cause Ca²⁺ oscillations in CHO cells. Despite these findings, PLC ζ -transfected CHO cell extracts exhibited high recombinant protein expression and PLC activity. Further, either PLC ζ -transfected CHO cells, or derived cell extracts, could specifically cause cytoplasmic Ca²⁺ oscillations when microinjected into mouse eggs. This data suggests that PLC ζ -mediated Ca²⁺ oscillations may require specific factors that are only present within the egg cytoplasm or be inhibited by factors present only in somatic cell lines.

INTRODUCTION

Phospholipase C-zeta (PLC ζ) is an isoform of phosphoinositide-specific PLC that is specifically expressed in mammalian sperm cells [1]. Microinjection of mouse PLC ζ cRNA into mouse, human, or bovine eggs triggers a prolonged series of Ca $^{2+}$ oscillations similar to those observed at fertilization [1-5]. PLC ζ has been identified as the protein responsible for causing Ca $^{2+}$ oscillations in mouse eggs when either cytosolic sperm extracts, or whole intact sperm, are microinjected into the egg cytoplasm [1-6]. The critical level of PLC ζ protein required to trigger Ca $^{2+}$ release in mouse eggs is around 10-50fg, which is similar to the estimated levels in a single mouse sperm [1]. Furthermore, reduced expression of PLC ζ during spermatogenesis by transgenic RNA interference approaches has been shown to decrease the number of Ca $^{2+}$ oscillations at fertilization in mouse eggs [7]. These data have led to the proposal that sperm PLC ζ is the physiological activator of mammalian development that is delivered to the egg after sperm-egg membrane fusion [1-5]. PLC ζ mediates its effects in eggs by virtue of its ability to hydrolyse PIP $_2$ and generate InsP $_3$ since mutation of aspartate-210 to an arginine (D210R), which is a critical residue in the PLC ζ catalytic domain, abolishes its ability to cause Ca $^{2+}$ oscillations or activate eggs [1]. Hence, PLC ζ can account for all of the Ca $^{2+}$ oscillations in eggs at fertilization since they are entirely dependent upon the stimulation of Ca $^{2+}$ release via the type 1 InsP $_3$ receptor [4,5,8,9].

It is not known whether PLC ζ activity is regulated either in sperm or eggs. One significant feature of PLC ζ that distinguishes it from many other mammalian PLCs is that its activity *in vitro* is retained in very low concentrations of Ca $^{2+}$ without the requirement for other protein factors [3,10]. PLC ζ is up to 50% active at the resting cytoplasmic Ca $^{2+}$ concentrations [3,10]. This implies that PLC ζ would be autonomously active in generating Ca $^{2+}$ oscillations when it is injected into the egg cytoplasm at low concentrations. In this case it is remarkable that the sperm contains more than a thousand fold higher PLC ζ concentration than the egg, yet sperm have a normal resting Ca $^{2+}$ level and undergo controlled Ca $^{2+}$ oscillations in response to stimuli such as progesterone [11]. This implies that PLC ζ might be inactive in sperm and that its activity is regulated differently in sperm compared to eggs. However, the nature of this differential regulation remains unclear.

Some previous studies have characterized recombinant PLC ζ expressed in cell lines or somatic tissues [12,13]. It was reported that mouse PLC ζ expression in COS cells is associated with the generation of Ca $^{2+}$ oscillations [13]. Injection of boar sperm extracts (containing PLC ζ) into hepatocytes has also been shown to trigger Ca $^{2+}$ oscillations [15]. These data suggest that PLC ζ can be autonomously active in somatic cells. However, another report showed that low levels of PLC ζ expressed in all tissues of transgenic mice has no discernable affect upon somatic cells, despite the fact that it activated oocytes within the ovaries of females [16]. Ca $^{2+}$ changes were not examined in that study but it implies that low levels of PLC ζ may be ineffective in

somatic tissues. The only known mechanism for inactivating PLC ζ is nuclear sequestration which can account for the cessation of Ca²⁺ oscillations after fertilization in the mouse [17,18]. However, it is unclear whether PLC ζ localization accounts for the different reports on the effect of PLC ζ expression in somatic cells. PLC ζ has been reported to be present in either the cytoplasm or the nucleus of somatic cells in a manner that may depend upon time, the cell line used, or species source of PLC ζ [12,19].

Here, we have expressed mouse PLC ζ in CHO cells that have been widely used to study the mechanism of Ca²⁺ oscillations [20]. We find that very high levels of PLC ζ expression in CHO cells has little effect upon Ca²⁺ homeostasis, suggesting it is inactive in these cells. Nuclear localization of PLC ζ depended upon time after transfection, but is unlikely to account for the lack of Ca²⁺ oscillation in these cells. Remarkably we found that, despite the absence of an effect on CHO cell Ca²⁺ level, the PLC ζ -expressing CHO cells are able to cause Ca²⁺ oscillations when introduced into mouse eggs, and can be extracted and shown to have a high intrinsic PLC activity.

EXPERIMENTAL

Cell culture and transfection

Chinese hamster ovary (CHO) cells were cultured in complete F-12 media (Invitrogen Ltd) in 5% CO₂ at 37°C and seeded in 6 well plates or in polylysine coated glass-bottomed culture dishes (World Precision Instruments Inc) 24 hours prior to transfection at ~80-90% confluency. For transient expression, the nuclei of cells maintained in Leibovitz L-15 media were microinjected with plasmid DNA (~10ng/ul) in KCl HEPES buffer [21] with 2.5mg/ml Alexa 594-dextran (10,000 MW) or 0.5mM Oregon Green BAPTA dextran (Invitrogen) using a Femtojet and InjectMan NI2 (Eppendorf) with an injection pressure of 90hPa. The injection system was mounted on an Axiovert 200 fluorescence microscope (Zeiss Ltd). After injecting 50-100 cells the medium was exchanged back to F-12 and cells were returned to the incubator prior to imaging. For stable expression, CHO cells were transfected with DNA Lipofectamine2000 (Invitrogen) and then incubated for 16-18 hours prior to testing for heterologous PLC ζ expression. PLC ζ -expressing cells were selected on the basis of resistance to G418 by using an increasing concentration of G418 (0 to 1 mg/ml in 100 μ g increments) and cells exhibiting appreciable YFP fluorescence were enriched using FACS (DakoCytomation, MoFlo). The PLC ζ -luciferase fusion protein construct was made in a pCR3 vector (Invitrogen) as described previously [10]. The YFP-PLC ζ construct was made in pcDNA3.1 with YFP placed at the N-terminus of PLC ζ . The eYFP-D210R-PLC ζ construct was derived from YFP-PLC ζ by site directed mutagenesis of aspartate 210 to arginine using the Stratagene Quikchange XL SDM kit.

Injection of eggs with extracts or cells

Metaphase II mouse eggs were collected and microinjected with ~10pl of extracts (about 5% of the egg volume) as described previously [10, 21]. To make cell extracts, CHO cells (~ 2 x 10⁷) stably transfected with YFP fusion constructs were harvested by trypsinisation then pelleted and resuspended

several times in KCl Hepes buffer [10]. Cells were lysed with 3 cycles of freeze-thaw using liquid nitrogen, before being subjected to ultracentrifugation at 100,000 x g for 1 hour at 4°C. The resulting supernatant was concentrated using centrifugal filters and stored at -80°C. Microinjection of cells was performed using a Piezo micro-manipulator (PrimeTech Ltd) that mimics the process of somatic cell nuclear transfer [22]. Single CHO cells were drawn into the end of a pipette narrow enough to rupture their cell membrane then immediately injected into a mouse egg by application of low pressure. Before injection, eggs were loaded with either fura red or fura PE3 by incubation for 30 mins in 2-4µM of the AM forms for the dyes. Small groups of eggs were microinjected with cell or sperm extracts, or cells and then rapidly transferred to the imaging microscope. Ca²⁺ was measured in eggs by monitoring fluorescence with a Nikon TiU epifluorescence microscope equipped with a Coolsnap HQ₂ CCD camera (Photometrics), with excitation light of 430nm/490nm (emission measured with a 615nm long pass filter) for fura red and excitation of 350nm/380nm (emission measured at 535nm) for fura PE3, or excitation of 550nm and an emission of 600nm for rhod dextran.

Microinjecting CHO cells with sperm extracts.

Boar sperm extracts (~5mg/ml) were prepared as the cytosolic protein fraction of lysed pig sperm as described previously [15]. Sperm extracts were mixed with an equal volume of 10000MW dextran-conjugated Alexa647 (Invitrogen) and microinjected into confluent fluo3-loaded CHO cells [24] using injection pressures of 120-140hPa. The successful transduction of exogenous material into cells was confirmed by visualising Alexa 647 fluorescence using a 633nm He:Ne laser fitted to an SP2 confocal laser scanning microscope (Leica Microsystems). Following microinjection, Ca²⁺-dependent fluo3 signals were acquired at 1Hz for 20mins as described [15]. Post-Ca²⁺ imaging, data was only analysed from those cells that retained >75% of their initial Alexa647 fluorescence.

PLC assays and Western blots.

PIP₂ hydrolytic activity of PLC present in YFP- or YFP-PLC ζ -transfected CHO cell extracts was assayed as described previously [10]. The assay mixture final volume was 50 µl containing 100 mM NaCl, 0.4% sodium cholate (w/v), 2 mM CaCl₂, 4 mM EGTA, 20 µg of bovine serum albumin, 5 mM 2-mercaptoethanol, and 20 mM Tris-HCl buffer, pH 6.8. The PIP₂ concentration in the reaction mixture was 220 µM, containing 0.05 µCi of [³H]PIP₂. Assay conditions were optimized for linearity, requiring a 10 min, 25 °C incubation with 20 pmol protein. Transfected CHO cell line proteins were separated by SDS-PAGE and immunoblot analysis performed as described previously [10]. Proteins were probed with a peptide affinity-purified rabbit anti-PLC ζ polyclonal antibody (1:10,000 dilution) generated to the EF-hand domain amino acid residues 16-31.

CHO cell imaging

Luciferase expression was monitored from cells incubated in 100µM luciferin using an Axiovert 100 microscope (Carl Zeiss Ltd) with a 40x, 1.4NA oil immersion objective lens and a cooled intensified charge-coupled device

(ICCD) camera (Photek Ltd) [21]. Luminescence was collected for 20 minutes and then Ca^{2+} was monitored in the same cells for ~1 hour using low level fluorescence excitation light from a halogen lamp with a 490nm excitation filter. Fluorescence from these cells was integrated every 10 sec with sensitivity of the ICCD at 10% [21]. Resting Ca^{2+} concentrations were estimated using the equation $\text{Ca}^{2+} = K_d(F_{\text{max}} - F)/(F - F_{\text{min}})$ with F_{min} taken as zero and the K_d for Oregon Green BAPTA in cytoplasm taken as 430nM [23]. The F_{max} was obtained by adding ionomycin to saturate the fluorescence of OGBD or fluo3. Cells expressing YFP fusion proteins were imaged with either a confocal or wide field microscope. For confocal imaging we used a TCS SP5 and a DMI 6000 inverted microscope (Leica). Cells were imaged with a 63x 1.23NA oil immersion objective lens and 512x512 pixel resolution. The YFP, fluo3 and fura red fluorescence was excited with the 488nm laser line with emission at 510-540nm for YFP or fluo3, and at 580-610nm for fura red fluorescence. Alexa-594 fluorescence was visualised using the 561nm He:Ne laser and emission at 600-630nm. For wide field imaging we used a Nikon TiU epifluorescence microscope with a Photometrics Coolsnap HQ₂ CCD camera equipped with excitation and emission filter wheels. Excitation was with a 490nm and 420nm band-pass filter, and emission was monitored with a 530nm band-pass filter (for YFP) or a 620 long-pass filter (for fura red). Where specified, cells were loaded with the Ca^{2+} indicator dyes by incubation in 10 μM of the AM form in un-supplemented F-12 medium for 60- 90mins before being washed and transferred to Krebs-Ringer-Hepes buffer [24,25]. For the confocal analysis of intracellular Ca^{2+} transients, cells were imaged every 100ms for 100 frames before the addition of ATP (100 μM) and for a further 500 frames (1 min total). Signal variability (SV) is an index of the magnitude of amplitude and temporal characteristics of Ca^{2+} fluxes during steady-state Ca^{2+} cycling processes [24,25]. For signal variability analysis, data was acquired every 100ms for 30 seconds. Following an experimental series, 15-20 cells were selected per area (typically 4-6 areas per dish) and data analysed from regions of interest (ROIs) of ~40 μm^2 (>150 pixels) using Leica software. Further analysis was carried out using Microsoft Excel and GraphPad Prism. Derivation of mathematical operations used to calculate signal variability is given in a previous report [23]. For wide field imaging of YFP-transfected cells an initial YFP image was collected, and the fura red images were collected every 10 seconds with a 420nm/490nm excitation and data were analysed using Image-Pro Plus and Sigma Plot. Data was analysed for significance using ANOVA or a student's t test.

RESULTS

Expression of PLC ζ -luc in CHO cells

The PLC ζ -luciferase fusion protein (PLC ζ -luc) has previously been shown to trigger Ca^{2+} oscillations when expressed in mouse eggs [10]. CHO cells were microinjected with cDNA encoding either PLC ζ -luc or luciferase, together with the Ca^{2+} indicator Oregon Green BAPTA dextran (OGBD). Figure 1a shows a luminescence image from a typical group of PLC ζ -luc expression plasmid-injected cells indicating successful transient expression of the fusion protein, as reported by the corresponding fluorescence image of OGBD specifically in injected cells (Fig.1b). Intracellular Ca^{2+} was measured in PLC ζ -luc expressing cells 3, 5 and 24 hours following microinjection and the mean

luminescence in single CHO cells expressing PLC ζ -luc was in the range of 1-10cps, compared with a range of 1-15cps from cells injected with control Luc vector. Fig 1c shows typical recordings of Ca²⁺ levels in PLC ζ -luc and luc control cells as well as OGBD control injected cells 5 hours after microinjection. It can be seen that there were small low frequency Ca²⁺ oscillations in all three type of cells but there was no clear difference between PLC ζ -transfected cells and other cells transfected or not (see Table 1). Since CHO cells express the endogenous purinergic P2Y1, P2Y2 and P2X7 receptors, they were tested for responsiveness to extracellular ATP [26]. All the cells underwent a single large rise in Ca²⁺ in response to ATP (Fig.1c). Table 1 summarizes data from transfected cells analysed at 3, 5 and 24 hours post-microinjection. At all three time points there were no differences in the proportion of cells showing low frequency Ca²⁺ oscillations, or in those showing a Ca²⁺ increase in response to addition of ATP, between PLC ζ -luc and luc control CHO cells and control OGBD injected cells (see Table 1). The resting Ca²⁺ levels in CHO cells were estimated by the addition of Ca²⁺ ionophore. Fig.1d shows that the resting Ca²⁺ levels were not significantly different between PLC ζ -luc or luc control cells, or in comparison to OGBD injected cells, at either 3, 5 or 24 hours after microinjection. These data suggest that transient PLC ζ -luc expression does not have a significant effect upon Ca²⁺ homeostasis in CHO cells. The presence of low frequency, small amplitude Ca²⁺ oscillations in some CHO cells evidently occurs in these cells under certain conditions.

Localization of YFP-PLC ζ in CHO cells

Nuclear sequestration of PLC ζ appears to cause the cessation of Ca²⁺ oscillations in mouse eggs and could account for the lack of CHO cell Ca²⁺ oscillations in the above experiments [17,18]. We examined the distribution of PLC ζ using a YFP-tagged PLC ζ which, similar to VenusGFP-PLC ζ , is also effective in causing Ca²⁺ oscillations in mouse eggs (see Fig.S1a). Figure 2a shows confocal images of CHO cells at 3, 5, 24 and 48 hours after being injected with the YFP-PLC ζ expression plasmid. Distinct levels of recombinant YFP expression were detected as early as 3 hours after DNA microinjection and persisted for at least 48 hours for all of the DNA constructs examined. Expression of YFP alone was homogeneous throughout the nucleus and cytoplasm in all cells. In contrast, YFP-PLC ζ showed two distinct patterns of localisation. At 3 and 5 hours post-injection all cells showed a cytoplasmic distribution, whereas after 24 and 48 hours the majority of cells showed nuclear translocation with enhanced staining in the nucleoli. Some cells that did not show obvious nuclear translocation at 24 and 48 hours maintained a clear cytoplasmic distribution identical to that observed at 3 and 5 hours. Unlike YFP-PLC ζ , YFP-D^{210R}PLC ζ did not show any obvious signs of nuclear accumulation at any time points and the majority of cells (>90%) maintained the same cytoplasmic localisation.

As well as studying transiently transfected CHO cells, we also examined the distribution of PLC ζ in stable cell lines. We prepared CHO cell lines that stably expressed YFP-PLC ζ or YFP alone after two rounds of cell sorting. Fig.2b shows that after such sorting there was a clear difference in YFP

channel fluorescence between selected cells and the wild-type CHO cells. The distribution of YFP-PLC ζ in CHO cells was variable and again could be seen in either the cytoplasm or the nucleus (Fig.2b). Along with the transient transfection studies, these data support the previous observations that PLC ζ can localize to the nucleus of cells [12]. However, since localization does not occur in all cells or takes more than 5 hours, this suggests that nuclear translocation cannot account for its apparent inactivity in CHO cells.

Ca²⁺ homeostasis in YFP-PLC ζ transfected CHO cells

Following microinjection of CHO cells with YFP-PLC ζ we measured cytoplasmic Ca²⁺ changes using confocal microscopy. We studied the response of transduced cells to the addition of ATP, as described above with PLC ζ -luc expressing cells, but the faster rate of image acquisition (10Hz) enabled by confocal microscopy permits a more detailed kinetic analysis of basal Ca²⁺ handling and ATP-evoked Ca²⁺ release in these cells. Cells were loaded with fluo3 and after exposure to ATP the amplitude and relative change in fluorescence was taken as a measure of response for YFP, YFP-PLC ζ and YFP-^{D210R}PLC ζ transfected cells, and for control injected cells. The initial increase in Ca²⁺ was unaffected by removal of extracellular Ca²⁺ indicating that P2Y and InsP₃-dependent intracellular Ca²⁺ store release was involved (unpublished observations). Fig. 3a shows examples of the Ca²⁺ response to a maximal concentration of ATP (at the arrow) for all four cell types, which consisted of a large and rapid response followed by a gradual decline as shown in Fig.1c. The responses in Fig.3a are at 5 and 24 hours but similar results were seen at 3 and 48 hours after DNA injection. Fig.3b summarizes the data from for all four types of cells, at all time points. There was no difference in either the proportion of cells responding to ATP, the relative amplitude of the Ca²⁺ rise, nor in the time to peak of the Ca²⁺ rise evoked by ATP, at any of the different times after microinjection of DNA. These data again suggest that P2Y receptor driven InsP₃ production and Ca²⁺ store release is unaltered by PLC ζ expression.

The use of YFP-PLC ζ and confocal microscopy allowed us to examine more subtle changes in Ca²⁺ homeostasis than may be revealed by the above experiments. Previous studies of cardiac ryanodine receptor (RyR2)-transfected CHO cells have shown that there is an increased variability in resting cytoplasmic Ca²⁺ fluxes associated with heterologous expression of RyR2 [24,25]. Such signal variability (SV) is an index that robustly describes the signal noise in the basal Ca²⁺-dependent fluorescence from Ca²⁺ dyes in the cell cytoplasm and has previously been used to characterize subtle perturbations of steady-state Ca²⁺ cycling and their functional consequences (e.g. induction of apoptosis) [24,25]. To examine whether recombinant expression of YFP-PLC ζ resulted in subtle changes to CHO cell Ca²⁺ handling, we monitored Ca²⁺ with fura red since the emission peaks of YFP and fura red are separated by >100nm. Fig 3b bottom right panel shows the analysis of fura red fluorescence noise as measured by signal variability for YFP, YFP-PLC ζ and YFP-^{D210}PLC ζ transfected cells, and for control injected cells at 5 hours and 24 hours after microinjection. There was no difference

observed in the signal variability from all four groups of cells. These experiments suggest that there are no subtle alterations in Ca^{2+} homeostasis in CHO cells expressing YFP-PLC ζ , supporting the conclusion that the YFP-PLC ζ expressed is functionally inactive in CHO cells.

As well as examining Ca^{2+} dynamics in CHO cells transiently expressing recombinant protein we analysed Ca^{2+} in the YFP-PLC ζ stable cell lines and studied the Ca^{2+} response to a full range of ATP concentrations. Fig.4a shows the Ca^{2+} transients generated by different concentrations of ATP in control CHO cells and in YFP-PLC ζ transfected cells. We categorised the cell populations into those that expressed high or low levels of recombinant protein, based on the FACS YFP fluorescence signals and analysed the populations on this basis. As with the transient transfections, there were no differences in the Ca^{2+} response to high concentrations of ATP. Nevertheless, the data in Fig.4 show that, for low concentrations of ATP, a smaller proportion of both types of YFP-PLC ζ expressing cells responded with a Ca^{2+} transient. The response in YFP-PLC ζ cells was also significantly delayed compared to control CHO cells (Fig.4b). However, this small difference in response was independent of the expression level of YFP-PLC ζ since it was equivalent in those cells expressing either high or low levels of YFP-PLC ζ recombinant protein.

The lack of any dramatic change in the Ca^{2+} homeostasis in YFP-PLC ζ expressing cells (Fig. 3b) did not appear to be due to any degradation of PLC portion of the YFP-PLC ζ fusion protein, nor in any loss of its intrinsic PLC activity. We made cytosolic extracts from YFP-PLC ζ cells and tested then for PLC ζ activity and expression. We specifically assayed PLC activity of cells in a mixed micelle assay with a free Ca^{2+} concentration at 200nM which is near maximal for stimulating PLC ζ , but below the level required for stimulating most somatic PLC isoforms [3, 10]. Fig. 5a shows that cytosolic extracts made from YFP-PLC ζ expressing cells had a much higher PLC activity *in vitro* compared with control YFP-transfected cells. This is consistent with the robust expression of the full length YFP-PLC ζ fusion protein (103kDa), as illustrated by the clear immunoreactive protein of ~100kDa recognised by an affinity-purified anti-PLC ζ antibody on the western blot (Fig.5b).

CHO cell expressed YFP-PLC ζ causes Ca^{2+} oscillations in mouse eggs.

One reason why PLC ζ may not be active in somatic cells could be a requirement for a specific factor(s) present only within the egg. To test this idea we sought to introduce recombinant PLC ζ synthesised in CHO cells into mouse eggs. Cell extracts were prepared from untransfected CHO cells (negative control), or cells exhibiting stable expression of YFP ($73.4 \pm 0.6\%$ positively-labelled), or else YFP-PLC ζ ($97.6 \pm 0.1\%$ positively labelled). These extracts were prepared in the same way as previously described for making cytosolic sperm extracts [1,27]. Fig. 6a shows that injecting ~5% volume of YFP-PLC ζ CHO cell extract into mouse eggs triggered a series of Ca^{2+} oscillations in all injected eggs (n=14). The frequency of the Ca^{2+} oscillations observed was 11.4 ± 1.4 spikes/hour, which persisted for the duration of the recording (typically 1-1.5 hours). These Ca^{2+} oscillations are similar in form to

those previously described after injection of mouse eggs with cytosolic sperm extracts [27]. Injection of extracts from untransfected CHO cells, or YFP-transfected CHO cells did not cause any changes in Ca^{2+} in mouse eggs (Fig. 6b).

We also tested the activity of PLC ζ expressed in CHO cells by directly injecting these cells into the egg cytoplasm in the manner used in the somatic cell transfer procedure [22]. Intracellular Ca^{2+} was monitored immediately following microinjection of either untransfected CHO cells (negative control), or cells expressing YFP-PLC ζ (from an enriched stable cell population). Single CHO cells were injected into single mouse eggs then transferred to the fluorescence imaging system within 1-2 minutes. Fig. 6c shows that the injection of cells expressing YFP-PLC ζ induced a series of repetitive Ca^{2+} oscillations in the majority (15/23) of eggs. As was observed with the CHO cell extract injections, the egg Ca^{2+} response to single CHO cell injection displayed rapid spikes with the initial transient taking place within minutes of the cell injection. The frequency of the Ca^{2+} oscillations observed was 4.3 ± 0.8 per hour which persisted for the duration of the recording and is similar to that seen during mouse egg fertilization [2,4]. In contrast, Fig. 6d shows that no change in intracellular Ca^{2+} occurred following microinjection of negative control cells. These data suggest that the YFP-PLC ζ transfected CHO cells are expressing YFP-PLC ζ at levels capable of inducing Ca^{2+} oscillations in mouse eggs. Furthermore, the rapid onset of Ca^{2+} oscillations following injection into egg cytoplasm suggests that the PLC ζ expressed in CHO cells exhibits full functional activity.

Since PLC ζ appears to be inactive in CHO cells we considered whether this might be due to an intrinsic inability of these cells to respond to PLC ζ . Previously sperm extracts were used as a source of an endogenously active Ca^{2+} releasing factor and they contain high levels of PLC ζ [1]. We made cytosolic sperm boar extracts and microinjected them into CHO cells. Fig. 7a-d shows experiments in which boar sperm extracts were injected into cells along with a fluorescent tracer dye. In all cases, the injection of such sperm extracts failed to cause any changes in resting Ca^{2+} , and their response to sperm extracts was indistinguishable from microinjection of fluorescent tracer dye alone (Fig. 7c and d). The same extracts caused high frequency Ca^{2+} oscillations when injected into mouse eggs (Fig. 7e), so these data suggests that CHO cells are not able to generate respond to native sperm PLC ζ even when it is introduced directly by microinjection into their cytoplasm.

DISCUSSION

Consistent with previous expression studies using Venus- or YFP-tagged PLC ζ in COS cells we found that either YFP- or luciferase-PLC ζ could be expressed in CHO cells [12,13]. The previous reports of PLC ζ expression in non-gametes gave apparently contradictory results in that a study using cell lines showed that PLC ζ expression triggered Ca^{2+} oscillations, whereas transgenic expression of PLC ζ throughout the mouse soma did not lead to any clear phenotypic change [16]. The PLC ζ transgenic whole soma expression levels were probably much lower than our studies with mammalian

cell lines since we could readily detect expression and yet expression in the transgenic mice was not detectable using a Venus-PLC ζ fusion protein [16]. Notably, our data indicates that readily detectable expression of two different PLC ζ fusion proteins can be achieved in a cell line with no apparent perturbation of resting Ca²⁺ levels, no major change in Ca²⁺ homeostasis and no altered response to agonists that increase cytoplasmic Ca²⁺. The absence of any effect of expressed PLC ζ on cytoplasmic Ca²⁺ is surprising since previous work implied that PLC ζ is autonomously activated inside cells. Moreover, our data also demonstrates that the PLC ζ expressed within CHO cells is either already active, or can subsequently become activated, when it is assayed *in vitro* or introduced into eggs, thus implying that PLC ζ may be held in an inactive state in CHO cells. This is also consistent with our findings that cytosolic sperm extracts, that contain an endogenous active PLC ζ [1], were also incapable of causing any Ca²⁺ release when directly microinjected into normal CHO cells.

Previous studies showing expression of mouse PLC ζ in somatic cells lines have reported either nuclear or cytoplasmic localization [12]. These observations could provide an explanation for some of the differences in the reported functional effects of PLC ζ expression. The current data shows that the specific localization of PLC ζ within cells may be dependent upon the elapsed time after transfection. We observed this time-dependence because in our studies we microinjected the plasmid DNA into cells, hence the timing of cDNA transduction was more precisely determined than with other methods. The localization of YFP-PLC ζ is primarily retained within the cytoplasm for the first 3-5 hours after injection and is subsequently found in the nucleus after 24 hours. This time period should be sufficient for some cells to complete a cell cycle and hence may imply that differential localization occurs after a round of mitosis. It was of interest that YFP-D^{210R}PLC ζ did not undergo any nuclear localization in CHO cells, which is similar to what we have seen in mouse eggs (Yu and Swann, unpublished data). Since this mutant form of PLC ζ lacks the ability to bind its substrate this may suggest that PIP₂ binding plays some role in nuclear translocation. Previous studies with Venus-PLC ζ shows that a rapid (<1 hour) localization occurred into the nuclei of mouse zygotes and embryos [17,18], suggesting that the mouse PLC ζ expressed in CHO cells is either very slowly transported into the nucleus or alternatively it may require a mitotic transit to become localized. In either case, the time-dependent localization we observe may explain the variation in previous reports of localization of PLC ζ that may be due to the uncertain time at which transcription starts using liposome-based methods. Nevertheless, the observed nuclear localization is unlikely to account for the lack of PLC ζ activity in CHO cells since it occurs many hours after transfection and with high levels of YFP-PLC ζ protein expression some cytoplasmic localization will invariably overlap with nuclear localization. In stably transfected YFP-PLC ζ cells there was also some variability in nuclear localization, but the homogeneity of Ca²⁺ handling in all cells confirmed that nuclear sequestration cannot readily account for an apparent lack of activity of PLC ζ in cells.

Semi-quantitative estimates suggest we have expressed relatively high levels of recombinant PLC ζ protein in CHO cells. From our previous calibration of luciferase expressed in mouse eggs [10] we can determine that the luminescence from single CHO cells expressing PLC ζ -luc (~10cps) corresponds to >100 fg of the fusion protein. We can also calculate the amount of YFP-PLC ζ protein present in our experiments. The amount of YFP-PLC ζ in the selected high-expression cells is at least ten times the detection threshold level, which is ~1 μ M [28]. So our levels should correspond to around 10 μ M. Given a CHO cell volume of ~2pl, our transfected and selected cells should contain ~2pg of YFP-PLC ζ protein (relative molecular mass ~100 kD). The estimated ~2pg/CHO cell is significantly greater than the 20-50fg of PLC ζ that was previously shown to trigger a physiological pattern of Ca²⁺ oscillations in mouse eggs [1]. Given that CHO cells are only ~1% of the volume of a mouse egg, it appears that CHO cells can tolerate expression levels of ~200-fold PLC ζ -luc and ~2000-fold YFP-PLC ζ higher than is required to cause InsP₃-dependent Ca²⁺ oscillations in mouse eggs.

It is not clear why PLC ζ is apparently inactive in CHO cells. One possibility is that our transfected CHO cells have adapted to high levels of PLC ζ by down-regulating the number of InsP₃ receptors. Given the high levels of expressed PLC ζ this would amount to the effective ablation of InsP₃ receptors. However, this seems unlikely to be occurring in our experiments because such down-regulation only occurs after generation of considerable amounts of InsP₃ that are sufficient to cause high frequency Ca²⁺ oscillations [8,9]. Notably, we failed to detect any Ca²⁺ changes, even at early times after transfection with PLC ζ . There is also insufficient time for InsP₃ receptor downregulation to explain the lack of response to the sperm extracts. Furthermore, any InsP₃ receptor down-regulation leads to a loss of agonist-induced Ca²⁺ increase [29]. We did find that there was a reduction in the Ca²⁺ response to low doses of ATP for stably transfected of YFP-PLC ζ cells. However this effect was not related to the expression level of YFP-PLC ζ . It is also noteworthy that previous work on ATP-induced Ca²⁺ response in CHO cells showed that PKC stimulation causes a marked feedback inhibition of Ca²⁺ transients [30]. Therefore, if PLC ζ was causing sustained PIP₂ hydrolysis and hence DAG production, we would anticipate a substantial block of the ATP Ca²⁺ transients. Since we consistently failed to detect any change in Ca²⁺ response at high ATP concentrations, the simplest interpretation of our data is that the recombinant PLC ζ is inactivated when expressed in CHO cells.

The apparent lack of PLC ζ activity in transfected CHO cells, coupled with clear Ca²⁺ releasing activity when these cells are introduced into eggs, resembles the physiological situation with regards to fertilization. Given the size difference between the sperm and egg, the mammalian sperm should contain levels of PLC ζ that must be at least three orders of magnitude higher than is effective in causing Ca²⁺ release in eggs. The mammalian sperm has a resting Ca²⁺ level similar to that of other cells and can exhibit Ca²⁺ increase in response to agonists, some of which appear to work via InsP₃ generation [11]. This implies that the PLC ζ present in sperm is also inactive. However, PLC ζ is capable of causing the first Ca²⁺ increase at fertilization within

minutes of sperm-egg membrane fusion [31]. Therefore, the experiments where we inject transfected CHO cells into the egg mimics the fertilization of the egg by a sperm in that an inactive PLC ζ rapidly becomes active upon exposure to the egg cytoplasm. The high intrinsic PLC activity of sperm extracts also resembles the high *in vitro* PLC activity we find in of YFP-PLC ζ transfected CHO cell cytosolic extracts. There may be two explanations for such effects. One is that PLC ζ may be held in an inactive state in sperm, and that whatever the mechanism the inhibitory components are also present in CHO cells. Otherwise the activity of PLC ζ observed upon entry into eggs could be because it interacts specifically with a protein(s) that is only expressed in the egg cytoplasm. This possibility would be consistent with the fact that other known mammalian PI-specific PLCs are also stimulated by interaction with accessory proteins [32].

AUTHOR CONTRIBUTION

Sally Phillips carried out most of the experiments on CHO cells, Yuansong Yu carried out experiments on eggs and some of studies on CHO cells. Andreas Rossbach prepared the DNA constructs. Chris George contributed to the analysis of confocal studies of CHO cells and microinjected extracts for Fig 7. Michail Nomikos conducted biochemical analysis of PLC protein, Vyronia Vassilakopoulou, Evangelia Livaniou and Bevan Cumbes prepared the rabbit anti-PLC antibody. F. Anthony Lai contributed towards the conception of the project and drafting of the manuscript. Karl Swann supervised the project and prepared the final version of the manuscript.

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Table 1. Analysis of Ca²⁺ dynamics in CHO cells.

3 hours				
	% of cells oscillating	Frequency (spikes/hr)	% of cells responding to ATP	n number (number of cells)
Luc	35.1 +/- 3.6	6.9 +/- 0.9	99.8 +/- 0.3	4 (47)
PLC ζ -luc	36.0 +/- 3.0	7.9 +/- 1.0	99.3 +/- 0.5	4 (45)
OGBD	31.9 +/- 3.7	7.0 +/- 0.7	100	3 (18)

5 hours				
	% of cells oscillating	Frequency (spikes/hr)	% of cells responding to ATP	n number (number of cells)
Luc	25.1 +/- 8.6	6.0 +/- 0.9	100	4 (29)
PLC ζ -luc	31.0 +/- 3.2	5.7 +/- 0.6	99.8 +/- 0.3	4 (50)
OGBD	31.1 +/- 5.9	6.0 +/- 0.9	100	3 (13)

24 hours				
	% of cells oscillating	Frequency (spikes/hr)	% of cells responding to ATP	n number (number of cells)
Luc	31.0 +/- 4.8	5.9 +/- 0.8	99.0 +/- 0.4	4 (22)
PLC ζ -luc	33.4 +/- 3.9	6.2 +/- 1.0	99.0 +/- 0.6	4 (21)
OGBD	29.0 +/- 2.4	6.5 +/- 0.8	99.3 +/- 0.3	4 (14)

CHO cells were injected with OGBD + plasmid DNA encoding luciferase or PLC ζ -luc or were injected with OGBD alone. Cells were imaged for OGBD fluorescence at 3, 5 and 24 hours following microinjection. Data is given as mean \pm S.E.M. n= the number of experimental runs. The total number of cells analysed is shown in parentheses.

FIGURE LEGENDS

Figure 1. Ca^{2+} dynamics in CHO cells expressing PLC ζ -luc

Luminescence (a) and fluorescence (b) images are shown of a representative group of CHO cells injected with plasmid DNA encoding PLC ζ -luc and OGBD. Note that not all the injected cells (shown by fluorescence) are expressing luciferase (shown by the luminescence). In c) are shown representative recordings of fluorescence (from OGBD in arbitrary units (a.u.) indicating Ca^{2+} changes observed in cells within each experimental group. Cells were imaged for fluorescence for at least 30 minutes prior to the addition of ATP (100 μM , at the arrow). The histogram in d) shows the mean and SEM for the resting Ca^{2+} levels, estimated after addition of ionomycin, for CHO cells injected with luc, or PLC ζ -luc DNA, as well as control cells injected with OGBD only. Bars are coded as indicated on the right of graph. There was no significant difference in the resting Ca^{2+} levels between the different groups of cells at any one time point (ANOVA).

Figure 2. Localization of YFP-PLC ζ expressed in CHO cells

In a) is shown the localisation of YFP, the enzymatically inactive YFP- $\text{D}^{210\text{R}}$ PLC ζ mutant and YFP-PLC ζ , in transiently transfected CHO cells. Confocal images acquired at different times after microinjection are indicated by the times above the panel. Each image is representative of the pattern of localisation observed in the majority of cells at each time point. The number of cells displaying the indicated localisation pattern is shown in white followed by the total number of cells analysed in each instance. The scale bar represents 10 μm . In b) is shown the FACS analysis of cells sorted for expression of YFP-PLC ζ with expressing cells above in grey and control CHO cells below in black. The fluorescence (F) is plotted for cells against the forward scatter (FSC). On the right are two confocal fluorescence images of cells with either nuclear or overall staining of YFP-PLC ζ , with again the proportion of cells with the pattern shown indicated by the numbers on each image.

Figure 3. Expression of PLC ζ has no effect on the ATP-induced Ca^{2+} transient in transiently transfected CHO cells.

In a) are shown groups of sample traces of fluo3 fluorescence increases in response (F in arbitrary units) to 100 μM ATP addition. The responses are shown for control CHO cells and for cell expressing YFP alone, YFP-PLC ζ , or YFP- $\text{D}^{210\text{R}}$ PLC ζ , at 5 hours and 24 hours after injection of DNA. Data obtained from six discrete cells randomly distributed across the field of view is shown (grey) with with the average of that group plotted in black. In b) is shown the analysis of parameters of the ATP-induced Ca^{2+} transient at time points between 3 and 24 hours in control CHO cells (open circles), and for cells expressing YFP (open squares), YFP- $\text{D}^{210\text{R}}$ PLC ζ (black circles) or YFP-PLC ζ (black squares). Data are given as mean \pm SEM (n=4 separate experiments each and the total number of cells analysed is shown in parentheses). Each time point analysis involved 43-480 individual cells, and there were no significant differences between any of the groups of cells at the time points of experiments (ANOVA).

Figure 4. ATP-induced Ca^{2+} transients responses of YFP-PLC ζ stably transfected CHO cells.

In a) stably YFP-PLC ζ transfected and wild type CHO cells were stimulated by the addition of ATP over a range of concentrations and the Ca^{2+} response monitored with fura red fluorescence ratio (in arbitrary units). The concentration of ATP added (at the arrow head), is shown down the left hand side and proportion of cells showing the displayed response indicated above each trace. The YFP-PLC ζ cells were divided into 'high' and 'low' level expression depending upon the level of YFP fluorescence (see Fig. S1b), and the data is plotted for each class separately. In b) the dose response curves are plotted for cells in terms of the percentage of cells showing a Ca^{2+} increase as well as the latency between addition of ATP and the start of the Ca^{2+} transient. Data is plotted for control CHO cells (black circles), and again for cells expressing high (open circles) versus low levels (black triangles) of YFP-PLC ζ . Data with an * is significantly different from control CHO cells (students t test, $p < 0.05$).

Figure 5. PLC enzyme activity and expression of YFP-PLC ζ in stably transfected CHO cells. Cells that had been sorted for stable expression of YFP-PLC ζ were used to make cytosolic extracts. In a) the PLC enzymatic activity in cell extracts is shown for YFP-PLC ζ transfected cells (open bar) and control YFP-transfected CHO cells (closed bar). The specific activity was 165 ± 9 nmol/min/mg for YFP-PLC ζ transfected cells, and 23 ± 10 nmol/min/mg for YFP control-transfected cells. Panel b) shows a Coomassie blue-stained SDS gel (left) and a Western blot (right) for the same batches of extracts. The Western blot was developed following incubation with an affinity-purified rabbit polyclonal antibody to PLC ζ .

Figure 6. Ca^{2+} dynamics in mouse eggs after injection of CHO cell or CHO cell extracts Intracellular Ca^{2+} responses in mouse eggs were monitored following the microinjection of extracts prepared from CHO cells. In a) a representative trace after injection of extract prepared from the YFP-PLC ζ cell line is shown. Ca^{2+} oscillations were seen in 14/14 eggs (11.4 ± 1.4 spikes/hour). In b) extracts from YFP-transfected or non-transfected CHO cells produced no Ca^{2+} response in any of the 6 or 4 eggs injected, respectively. The recording in c) is from an egg injected with a whole CHO cell that was expressing YFP-PLC ζ . Ca^{2+} oscillations were seen in 15/23 such whole CHO cell-injected eggs (4.3 ± 0.8 spikes/hour). In d) is shown an example of an egg injected with a whole CHO cell expressing YFP alone. No Ca^{2+} response was observed in any of the 14 injected eggs. Each trace is a recording from a single egg and is representative of the observations made in each experimental group. Traces a) and b) were recorded with the use of fura-red, while c) and d) used fura-PE3.

Figure 7. Microinjection of YFP-PLC ζ transfected cell extracts into control CHO cells.

Boar sperm extracts were microinjected into control CHO cells and then imaged for 20 mins. The protocol for the experiments are shown in a) and images in b) where taken before (Pre) during and after (Post) the microinjection (MI) of extracts. The sample images in b) show a brightfield image of cells (BF), the Ca²⁺-dependent fluorescence of fluo3 (Ca²⁺), and the fluorescence of the Alexa647 tracer (Alexa 645) that was used to visualise successful injection. In c) the black traces show typical fluo3 fluorescence recordings from the 'Ca²⁺ imaging' phase of the experiment (time 10-30, panel (a)) following microinjection (MI) with boar sperm extract (BSE) mixed with Alexa647 (BSE:dye), or injection of Alexa647 alone (dye only).

Representative traces obtained from those cells that had not been microinjected (non-MI), but were present in the same field of view, are given (grey). In d) are shown the average resting Ca²⁺ levels and the Ca²⁺ noise after microinjection of boar sperm extract or dye alone, compared with the same parameters from CHO cells in the same dish that were not injected (non-MI). >40 injections were performed per coverslip (n=4), and the efficacy of injection, determined by the visualisation and retention of Alexa647 over the 20minute imaging period, exceeded 75%. All data in c) and d) are taken from cells that retained Alexa647 to levels of fluorescence >75% of initial values following the 20minute Ca²⁺ imaging window. In e) a sample of the boar sperm extract mixed with an equal volume of Alexa647 that was microinjected into CHO cells was microinjected into unfertilized mouse eggs. The illustrated pattern of Ca²⁺ oscillations measured with Rhod dextran fluorescence (F) was seen in 6/6 injected eggs.

Fig.1

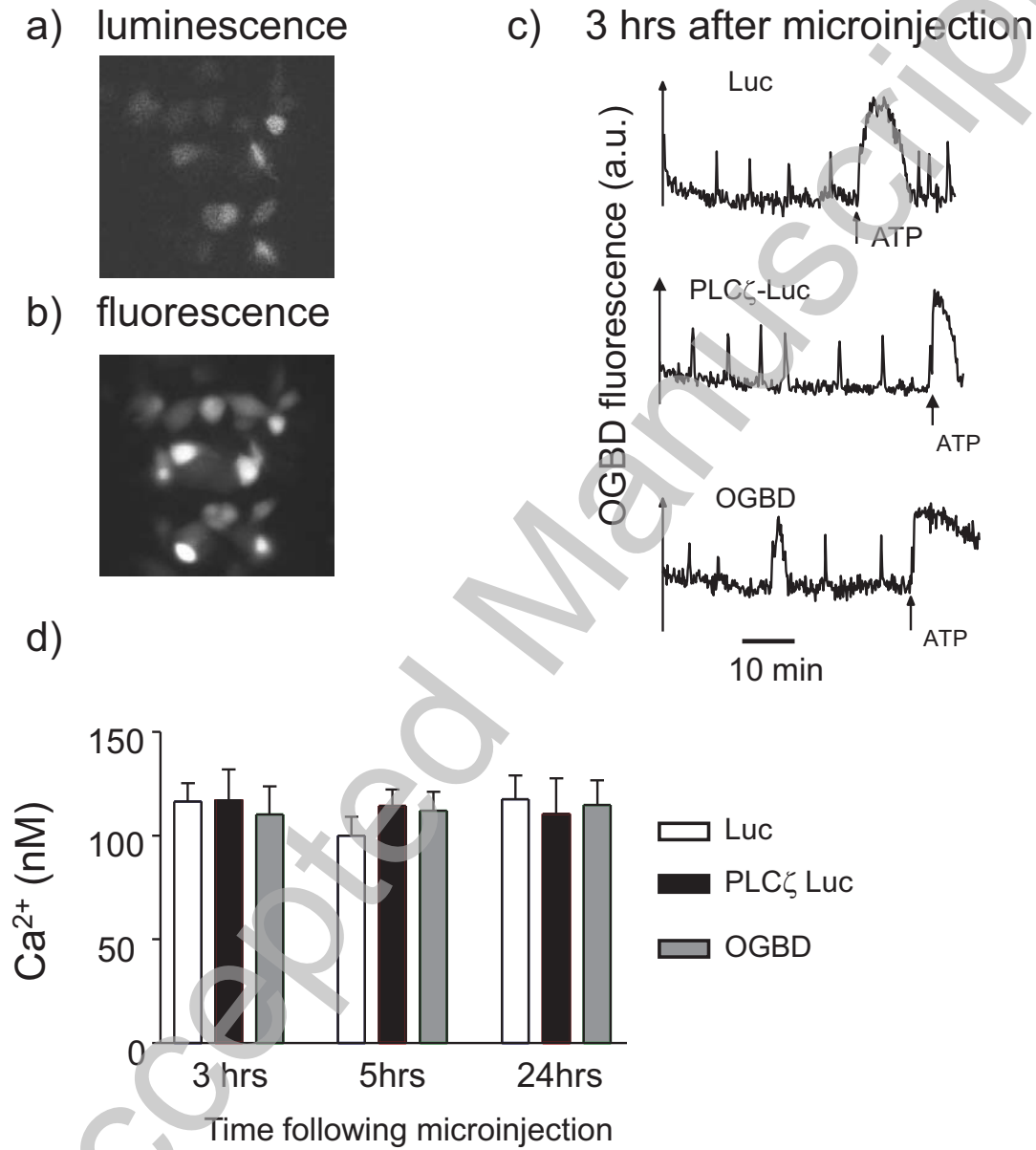
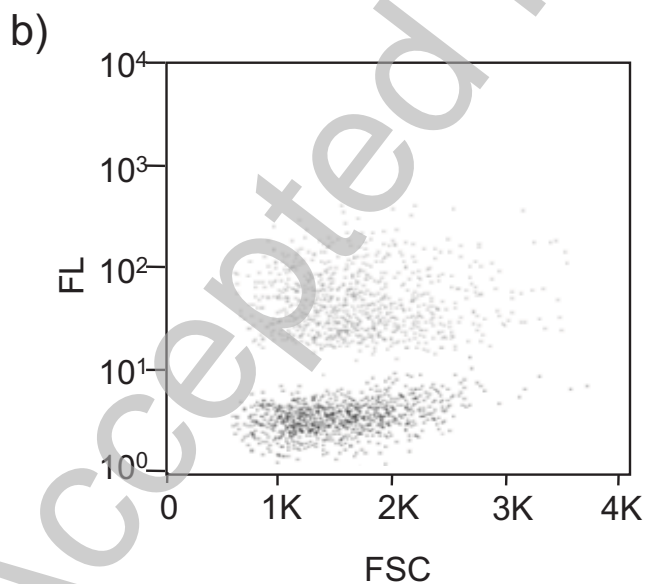
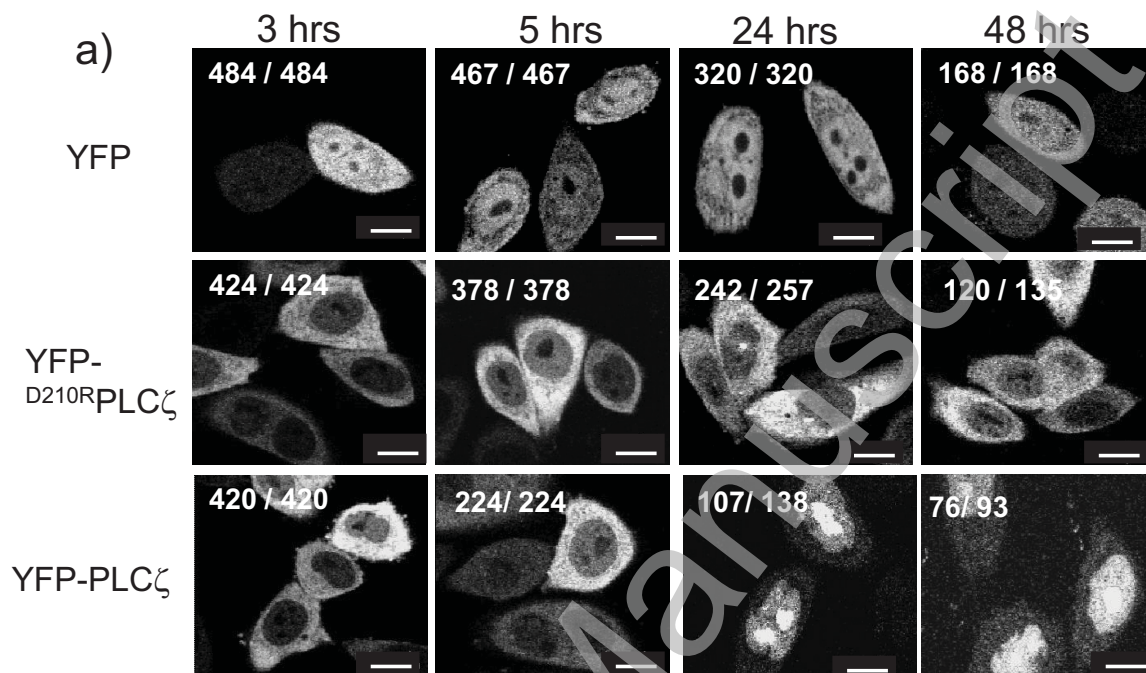


Fig. 2



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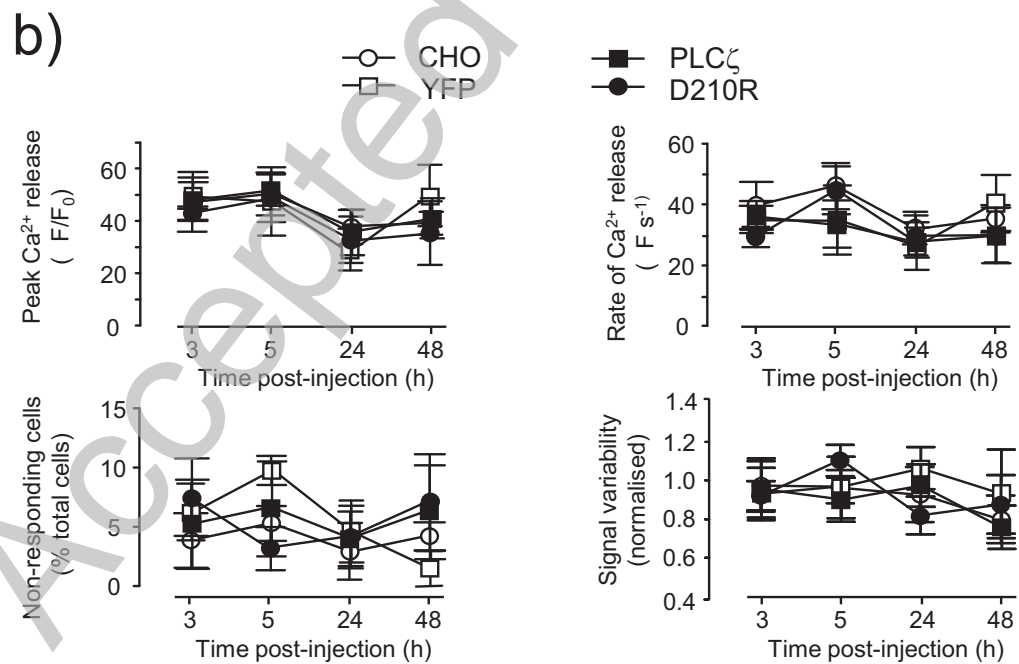
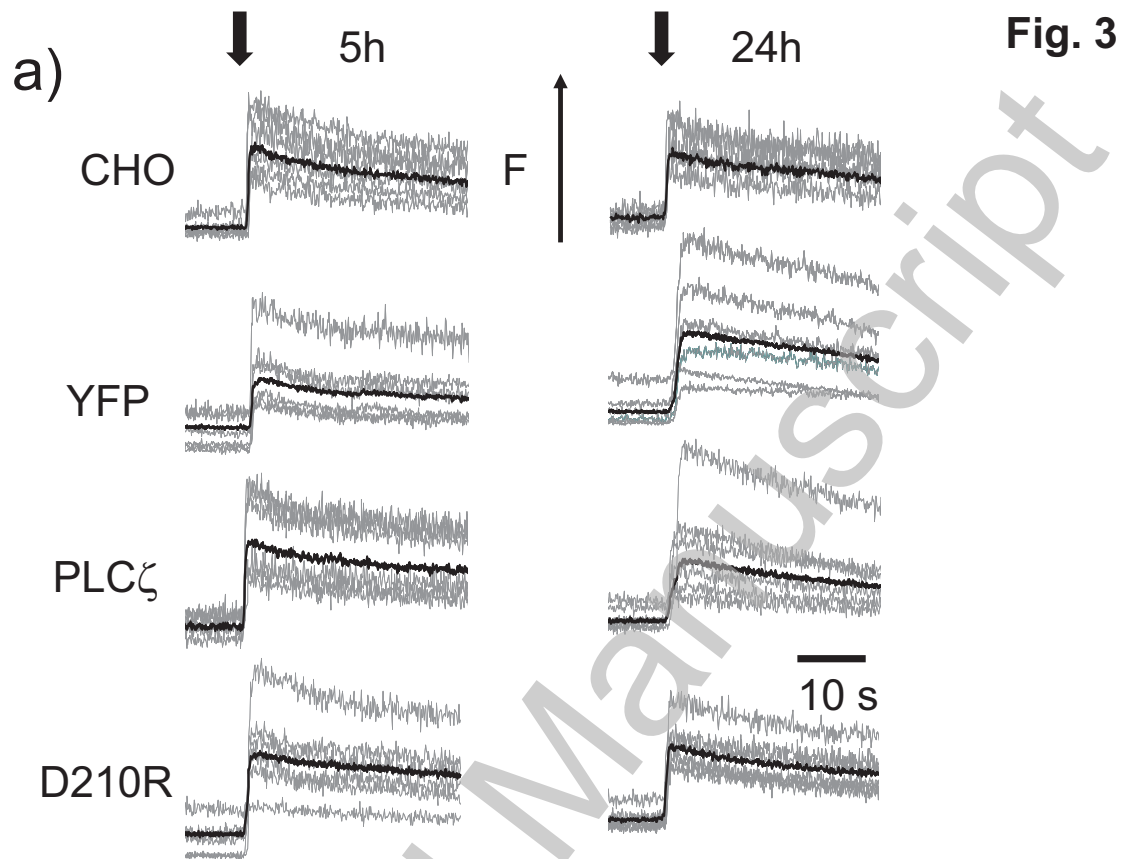
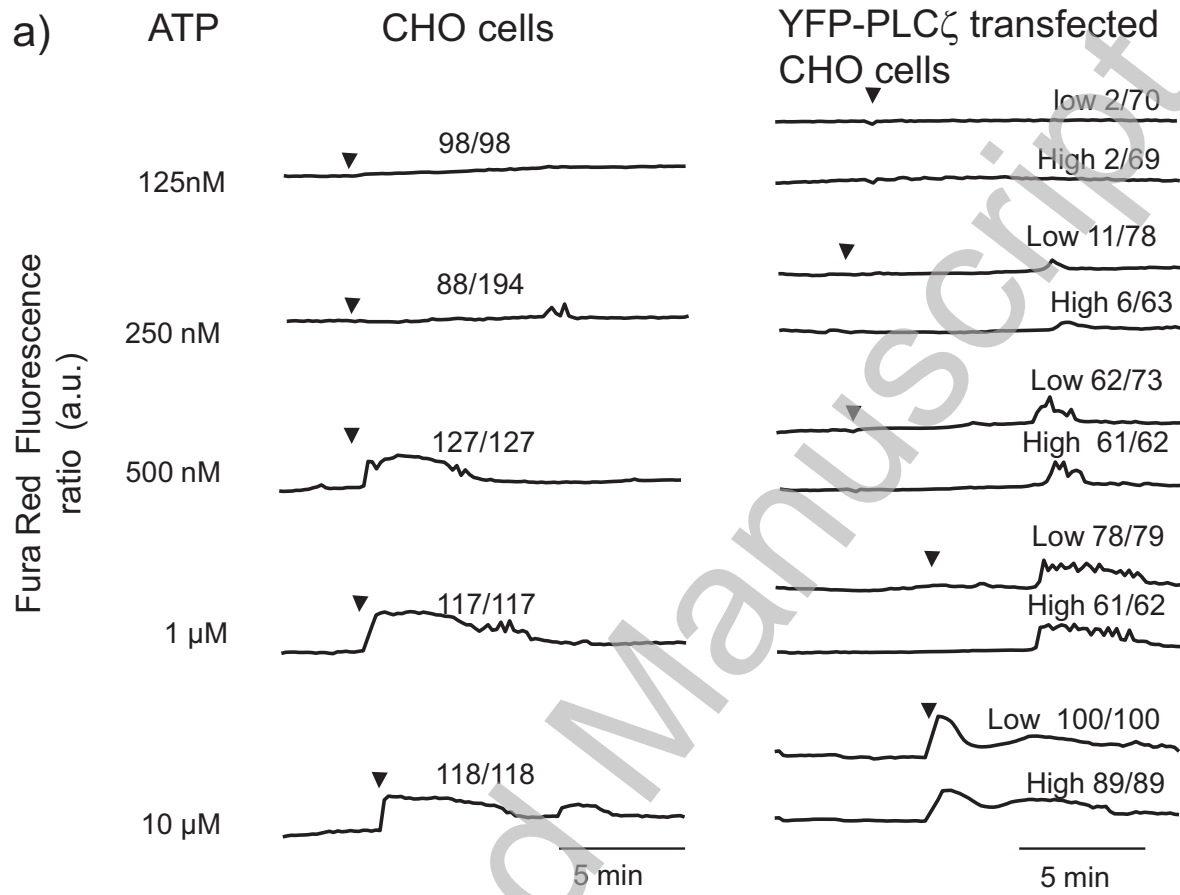
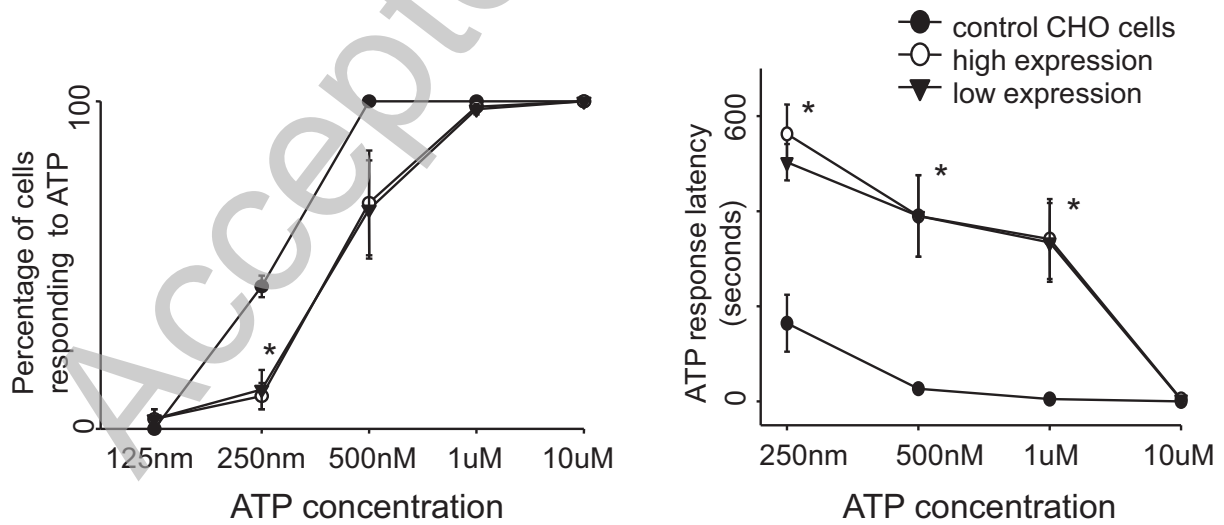


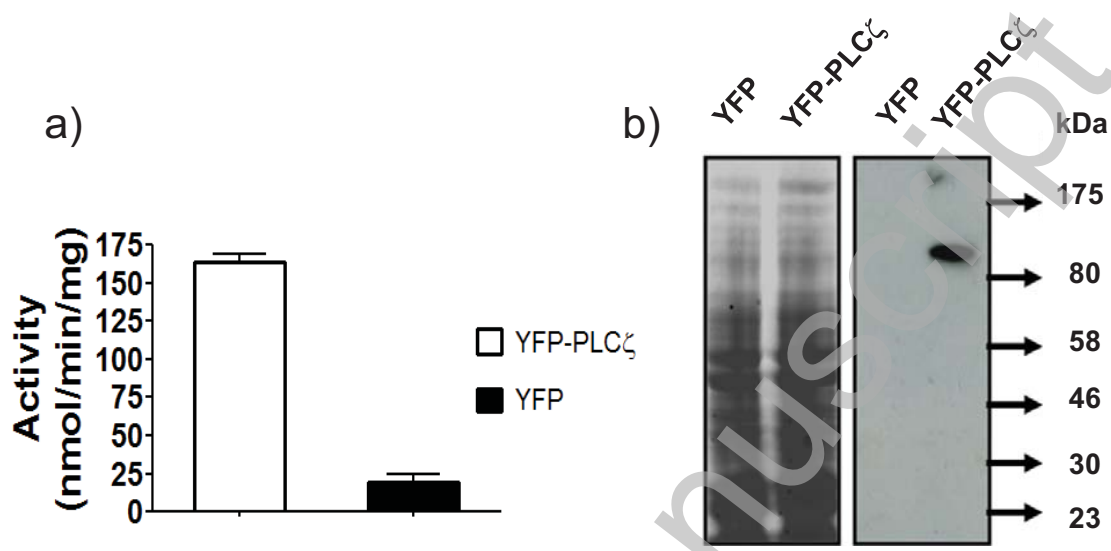
Fig. 4



b) Comparison of cells capacity to respond to ATP



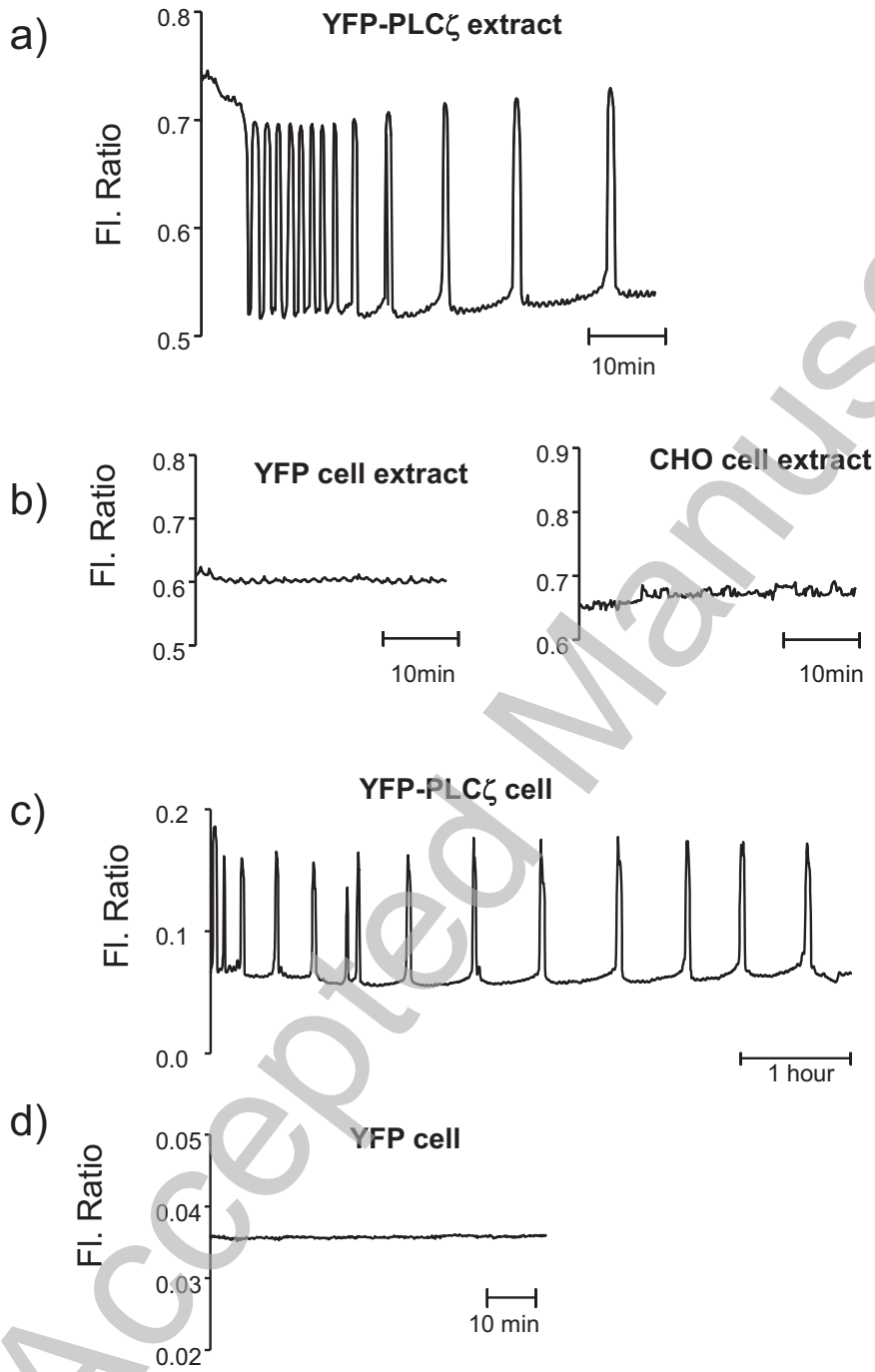
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Fig. 5

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Fig. 6



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