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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 (PLCζ) 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 PLCζ has the ability to trigger Ca^{2+} oscillations when expressed in eggs, but it is not known how PLCζ activity is regulated in sperm or eggs. Here, we have transfected CHO cells with PLCζ fused with either YFP or luciferase and found that PLCζ-transfected cells did not display cytoplasmic Ca^{2+} oscillations any different from control cells. PLCζ expression was not associated with changes in CHO cell resting Ca^{2+} levels, nor in a significantly changed Ca^{2+} response to extracellular ATP compared to control cells transfected with either YFP alone, a catalytically-inactive PLCζ, or luciferase alone. Sperm extracts containing PLCζ also failed to cause Ca^{2+} oscillations in CHO cells. Despite these findings, PLCζ-transfected CHO cell extracts exhibited high recombinant protein expression and PLC activity. Further, either PLCζ-transfected CHO cells, or derived cell extracts, could specifically cause cytoplasmic Ca^{2+} oscillations when microinjected into mouse eggs. This data suggests that PLCζ-mediated Ca^{2+} oscillations may require specific factors that are only present within the egg cytoplasm or be inhibited by factors present only in somatic cell lines.

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INTRODUCTION

Phospholipase C-zeta (PLCζ) is an isoform of phosphoinositide-specific PLC that is specifically expressed in mammalian sperm cells [1]. Microinjection of mouse PLCζ cRNA into mouse, human, or bovine eggs triggers a prolonged series of Ca^{2+} oscillations similar to those observed at fertilization [1-5]. PLCζ 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 PLCζ 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 PLCζ 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 PLCζ is the physiological activator of mammalian development that is delivered to the egg after sperm-egg membrane fusion [1-5]. PLCζ 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 PLCζ catalytic domain, abolishes its ability to cause Ca^{2+} oscillations or activate eggs [1]. Hence, PLCζ 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 PLCζ activity is regulated either in sperm or eggs. One significant feature of PLCζ 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]. PLCζ is up to 50% active at the resting cytoplasmic Ca^{2+} concentrations [3,10]. This implies that PLCζ 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 PLCζ 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 PLCζ 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 PLCζ expressed in cell lines or somatic tissues [12,13]. It was reported that mouse PLCζ expression in COS cells is associated with the generation of Ca^{2+} oscillations [13]. Injection of boar sperm extracts (containing PLCζ) into hepatocytes has also been shown to trigger Ca^{2+} oscillations [15]. These data suggest that PLCζ can be autonomously active in somatic cells. However, another report showed that low levels of PLCζ 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 PLCζ may be ineffective in

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somatic tissues. The only known mechanism for inactivating PLCζ is nuclear sequestration which can account for the cessation of Ca^{2+} oscillations after fertilization in the mouse [17,18]. However, it is unclear whether PLCζ localization accounts for the different reports on the effect of PLCζ expression in somatic cells. PLCζ 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 PLCζ [12,19].

Here, we have expressed mouse PLCζ in CHO cells that have been widely used to study the mechanism of Ca^{2+} oscillations [20] We find that very high levels of PLCζ expression in CHO cells has little affect upon Ca^{2+} homeostasis, suggesting it is inactive in these cells. Nuclear localization of PLCζ depended upon time after transfection, but is unlikely to account for the lack of Ca^{2+} oscillation in these cells. Remarkably we found that, despite the absence of an effect on CHO cell Ca^{2+} level, the PLCζ-expressing CHO cells are able to cause Ca^{2+} 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% CO_2 at 37°C and seeded in 6 well plates or in poly-lysine 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 PLCζ expression. PLCζ–expressing cells were selected on the basis of resistance to G418 by using an increasing concentration of G418 (0 to 1 mg/ml in 100μg increments) and cells exhibiting appreciable YFP fluorescence were enriched using FACS (DakoCytomation, MoFlo). The PLCζ-luciferase fusion protein construct was made in a pCR3 vector (Invitrogen) as described previously [10]. The YFP-PLCζ construct was made in pcDNA3.1 with YFP placed at the N-terminus of PLCζ. The eYFP-^{D210R}PLCζ construct was derived from YFP-PLCζ 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 10^7) stably transfected with YFP fusion constructs were harvested by trypsinisation then pelleted and resuspended

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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. Ca^{2+} was measured in eggs by monitoring fluorescence with a Nikon TiU epifluorescence microscope equipped with a Coolsnap HQ_2 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, Ca^{2+}-dependent fluo3 signals were acquired at 1Hz for 20mins as described [15]. Post-Ca^{2+} imaging, data was only analysed from those cells that retained >75% of their initial Alexa647 fluorescence.

PLC assays and Western blots.
PIP_2 hydrolytic activity of PLC present in YFP- or YFP-PLCζ-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 $CaCl_2$, 4 mM EGTA, 20 µg of bovine serum albumin, 5 mM 2-mercaptoethanol, and 20 mM Tris-HCl buffer, pH 6.8. The PIP_2 concentration in the reaction mixture was 220 µM, containing 0.05 µCi of $[^3H]PIP_2$. 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-PLCζ 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

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(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 $Ca^{2+}=K_d(F_{max}-F)/(F-F_{min})$ 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 HQ_2 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 10μM 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 (100μM) 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 ~40μm^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 PLCζ-luc in CHO cells

The PLCζ-luciferase fusion protein (PLCζ-luc) has previously been shown to trigger Ca^{2+} oscillations when expressed in mouse eggs [10]. CHO cells were microinjected with cDNA encoding either PLCζ-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 PLCζ-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 PLCζ-luc expressing cells 3, 5 and 24 hours following microinjection and the mean

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luminescence in single CHO cells expressing PLCζ-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 Ca^{2+} levels in PLCζ-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 Ca^{2+} oscillations in all three type of cells but there was no clear difference between PLCζ-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 Ca^{2+} 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 Ca^{2+} oscillations, or in those showing a Ca^{2+} increase in response to addition of ATP, between PLCζ-luc and luc control CHO cells and control OGBD injected cells (see Table 1). The resting Ca^{2+} levels in CHO cells were estimated by the addition of Ca^{2+} ionophore . Fig.1d shows that the resting Ca^{2+} levels were not significantly different between PLCζ-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 PLCζ-luc expression does not have a significant effect upon Ca^{2+} homeostasis in CHO cells. The presence of low frequency, small amplitude Ca^{2+} oscillations in some CHO cells evidently occurs in these cells under certain conditions.

Localization of YFP-PLCζ in CHO cells

Nuclear sequestration of PLCζ appears to cause the cessation of Ca^{2+} oscillations in mouse eggs and could account for the lack of CHO cell Ca^{2+} oscillations in the above experiments [17,18]. We examined the distribution of PLCζ using a YFP-tagged PLCζ which, similar to VenusGFP-PLCζ, is also effective in causing Ca^{2+} 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-PLCζ 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-PLCζ 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-PLCζ, YFP-^{D210R}PLCζ 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 PLCζ in stable cell lines. We prepared CHO cell lines that stably expressed YFP-PLCζ or YFP alone after two rounds of cell sorting. Fig.2b shows that after such sorting there was a clear difference in YFP

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channel fluorescence between selected cells and the wild-type CHO cells. The distribution of YFP-PLCζ inCHO 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 PLCζ 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.

Ca^{2+} homeostasis in YFP-PLCζ transfected CHO cells

Following microinjection of CHO cells with YFP-PLCζ we measured cytoplasmic Ca^{2+} changes using confocal microscopy. We studied the response of transduced cells to the addition of ATP, as described above with PLCζ-luc expressing cells, but the faster rate of image acquisition (10Hz) enabled by confocal microscopy permits a more detailed kinetic analysis of basal Ca^{2+} handling and ATP-evoked Ca^{2+} 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-PLCζ and YFP-^{D210R}PLCζ transfected cells, and for control injected cells. The initial increase in Ca^{2+} was unaffected by removal of extracellular Ca^{2+} indicating that P2Y and $InsP_3$-dependent intracellular Ca^{2+} store release was involved (unpublished observations). Fig. 3a shows examples of the Ca^{2+} 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 Ca^{2+} rise, nor in the time to peak of the Ca^{2+} rise evoked by ATP, at any of the different times after microinjection of DNA. These data again suggest that P2Y receptor driven $InsP_3$ production and Ca^{2+} store release is unaltered by PLCζ expression.

The use of YFP-PLCζ and confocal microscopy allowed us to examine more subtle changes in Ca^{2+} 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 Ca^{2+} 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 Ca^{2+}-dependent fluorescence from Ca^{2+} dyes in the cell cytoplasm and has previously been used to characterize subtle perturbations of steady-state Ca^{2+} cycling and their functional consequences (e.g. induction of apoptosis) [24,25]. To examine whether recombinant expression of YFP-PLCζ resulted in subtle changes to CHO cell Ca^{2+} handling, we monitored Ca^{2+} 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-PLCζ and YFP-^{D210}PLCζ transfected cells, and for control injected cells at 5 hours and 24 hours after microinjection. There was no difference

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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-PLCζ, supporting the conclusion that the YFP-PLCζ 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-PLCζ 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-PLCζ 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-PLCζ expressing cells responded with a Ca^{2+} transient. The response in YFP-PLCζ 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-PLCζ since it was equivalent in those cells expressing either high or low levels of YFP-PLCζ recombinant protein.

The lack of any dramatic change in the Ca^{2+} homeostasis in YFP-PLCζ expressing cells (Fig. 3b) did not appear to be due to any degradation of PLC portion of the YFP-PLCζ fusion protein, nor in any loss of its intrinsic PLC activity. We made cytosolic extracts from YFP-PLCζ cells and tested then for PLCζ 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 PLCζ, but below the level required for stimulating most somatic PLC isoforms [3,10]. Fig. 5a shows that cytosolic extracts made from YFP-PLCζ 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-PLCζ fusion protein (103kDa), as illustrated by the clear immunoreactive protein of ~100kDa recognised by an affinity-purified anti-PLCζ antibody on the western blot (Fig.5b).

CHO cell expressed YFP-PLCζ causes Ca^{2+} oscillations in mouse eggs. One reason why PLCζ 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 PLCζ 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 ± 0.6% positively-labelled), or else YFP-PLCζ (97.6 ± 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-PLCζ 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

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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 PLCζ 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-PLCζ (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-PLCζ 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-PLCζ transfected CHO cells are expressing YFP-PLCζ 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 PLCζ expressed in CHO cells exhibits full functional activity.

Since PLCζ appears to be inactive in CHO cells we considered whether this might be due to an intrinsic inability of these cells to respond to PLCζ. Previously sperm extracts were used as a source of an endogenously active Ca^{2+} releasing factor and they contain high levels of PLCζ [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 PLCζ even when it is introduced directly by microinjection into their cytoplasm.

DISCUSSION

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

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cell lines since we could readily detect expression and yet expression in the transgenic mice was not detectable using a Venus-PLCζ fusion protein [16]. Notably, our data indicates that readily detectable expression of two different PLCζ fusion proteins can be achieved in a cell line with no apparent perturbation of resting Ca^{2+} levels, no major change in Ca^{2+} homeostasis and no altered response to agonists that increase cytoplasmic Ca^{2+}. The absence of any effect of expressed PLCζ on cytoplasmic Ca^{2+} is surprising since previous work implied that PLCζ is autonomously activated inside cells. Moreover, our data also demonstrates that the PLCζ 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 PLCζ 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 PLCζ [1], were also incapable of causing any Ca^{2+} release when directly microinjected into normal CHO cells.

Previous studies showing expression of mouse PLCζ 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 PLCζ expression. The current data shows that the specific localization of PLCζ 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-PLCζ 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-^{D210R}PLCζ 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 PLCζ lacks the ability to bind its substrate this may suggest that PIP_2 binding plays some role in nuclear translocation. Previous studies with Venus-PLCζ shows that a rapid (<1 hour) localization occurred into the nuclei of mouse zygotes and embryos [17,18], suggesting that the mouse PLCζ 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 PLCζ 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 PLCζ activity in CHO cells since it occurs many hours after transfection and with high levels of YFP-PLCζ protein expression some cytoplasmic localization will invariably overlap with nuclear localization. In stably transfected YFP-PLCζ cells there was also some variability in nuclear localization, but the homogeneity of Ca^{2+} handling in all cells confirmed that nuclear sequestration cannot readily account for an apparent lack of activity of PLCζ in cells.

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Semi-quantitative estimates suggest we have expressed relatively high levels of recombinant PLCζ 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 PLCζ–luc (~10cps) corresponds to >100 fg of the fusion protein. We can also calculate the amount of YFP-PLCζ protein present in our experiments. The amount of YFP-PLCζ in the selected high-expression cells is at least ten times the detection threshold level, which is ~1μM [28]. So our levels should correspond to around 10μM. Given a CHO cell volume of ~2pl, our transfected and selected cells should contain ~2pg of YFP-PLCζ protein (relative molecular mass ~100 kD). The estimated ~2pg/CHO cell is significantly greater than the 20-50fg of PLCζ that was previously shown to trigger a physiological pattern of Ca^{2+} 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 PLCζ-luc and ~2000-fold YFP-PLCζ higher than is required to cause $InsP_3$-dependent Ca^{2+} oscillations in mouse eggs.

It is not clear why PLCζ is apparently inactive in CHO cells. One possibility is that our transfected CHO cells have adapted to high levels of PLCζ by down-regulating the number of $InsP_3$ receptors. Given the high levels of expressed PLCζ this would amount to the effective ablation of $InsP_3$ receptors. However, this seems unlikely to be occurring in our experiments because such down-regulation only occurs after generation of considerable amounts of $InsP_3$ that are sufficient to cause high frequency Ca^{2+} oscillations [8,9]. Notably, we failed to detect any Ca^{2+} changes, even at early times after transfection with PLCζ. There is also insufficient time for $InsP_3$ receptor downrgulation to explain the lack of response to the sperm extracts. Furthermore, any $InsP_3$ receptor down-regulation leads to a loss of agonist-induced Ca^{2+} increase [29]. We did find that there was a reduction in the Ca^{2+} response to low doses of ATP for stably transfected of YFP-PLCζ cells. However this effect was not related to the expression level of YFP-PLCζ. It is also noteworthy that previous work on ATP-induced Ca^{2+} response in CHO cells showed that PKC stimulation causes a marked feedback inhibition of Ca^{2+} transients [30]. Therefore, if PLCζ was causing sustained PIP_2 hydrolysis and hence DAG production, we would anticipate a substantial block of the ATP Ca^{2+} transients. Since we consistently failed to detect any change in Ca^{2+} response at high ATP concentrations, the simplest interpretation of our data is that the recombinant PLCζ is inactivated when expressed in CHO cells.

The apparent lack of PLCζ activity in transfected CHO cells, coupled with clear Ca^{2+} 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 PLCζ that must be at least three orders of magnitude higher than is effective in causing Ca^{2+} release in eggs. The mammalian sperm has a resting Ca^{2+} level similar to that of other cells and can exhibit Ca^{2+} increase in response to agonists, some of which appear to work via $InsP_3$ generation [11]. This implies that the PLCζ present in sperm is also inactive. However, PLCζ is capable of causing the first Ca^{2+} increase at fertilization within

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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 PLCζ 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-PLCζ transfected CHO cell cytosolic extracts. There may be two explanations for such effects. One is that PLCζ 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 PLCζ 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.

ACKNOWLEDGMENTS.

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Table 1. Analysis of Ca^{2+} 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)
PLCζ-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)
PLCζ-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)
PLCζ-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 PLCζ-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 ± S.E.M. n= the number of experimental runs. The total number of cells analysed is shown in parentheses.

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FIGURE LEGENDS

Figure 1. Ca^{2+} dynamics in CHO cells expressing PLCζ-luc
Luminescence (a) and fluorescence (b) images are shown of a representative group of CHO cells injected with plasmid DNA encoding PLCζ-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 (100μM, 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 PLCζ-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 Ca2+ levels between the different groups of cells at any one time point (ANOVA).

Figure 2. Localization of YFP-PLCζ expressed in CHO cells
In a) is shown the localisation of YFP, the enzymatically inactive YFP-^{D210R}PLCζ mutant and YFP-PLCζ, 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 10μm. In b) is shown the FACS analysis of cells sorted for expression of YFP-PLCζ 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-PLCζ, with again the proportion of cells with the pattern shown indicated by the numbers on each image.

Figure 3. Expression of PLCζ 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 100μM ATP addition. The responses are shown for control CHO cells and for cell expressing YFP alone, YFP-PLCζ , or YFP-^{D210R}PLCζ, 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-^{D210R}PLCζ (black circles) or YFP-PLCζ (black squares). Data are given as mean ± 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).

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Figure 4. ATP-induced Ca^{2+} transients responses of YFP-PLCζ stably transfected CHO cells.
In a) stably YFP-PLCζ 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-PLCζ 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-PLCζ. 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-PLCζ in stably transfected CHO cells. Cells that had been sorted for stable expression of YFP-PLCζ were used to make cytosolic extracts. In a) the PLC enzymatic activity in cell extracts is shown for YFP-PLCζ transfected cells (open bar) and control YFP-transfected CHO cells (closed bar). The specific activity was 165 ± 9 nmol/min/mg for YFP-PLCζ 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 PLCζ.

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-PLCζ 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-PLCζ. 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.

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Figure 7. Microinjection of YFP-PLCζ 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 Ca^{2+}-dependent fluorescence of fluo3 (Ca^{2+}), 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 'Ca^{2+} 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 Ca^{2+} levels and the Ca^{2+} 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 Ca^{2+} oscillations measured with Rhod dextran fluorescence (F) was seen in 6/6 injected eggs.

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Fig.1

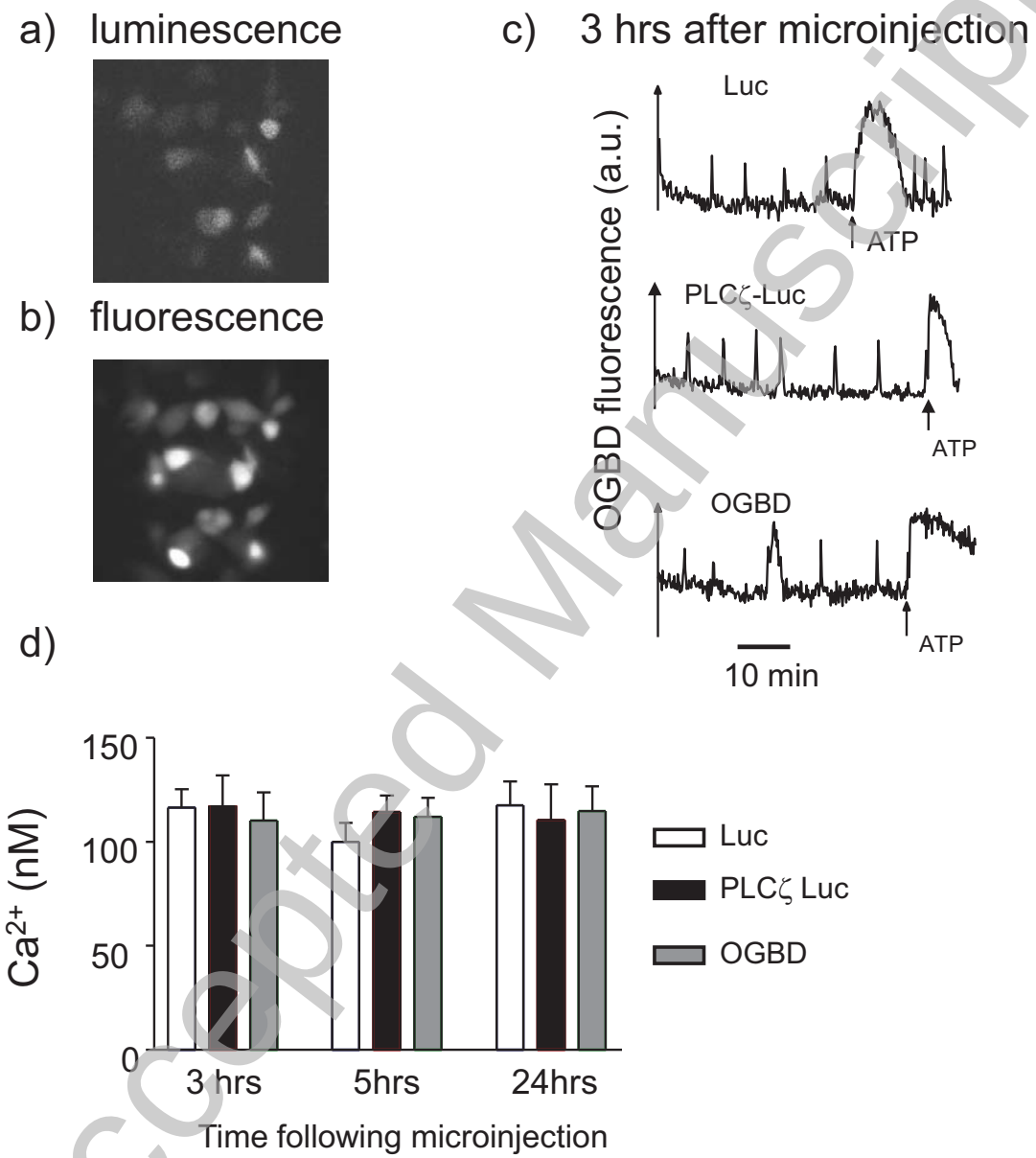

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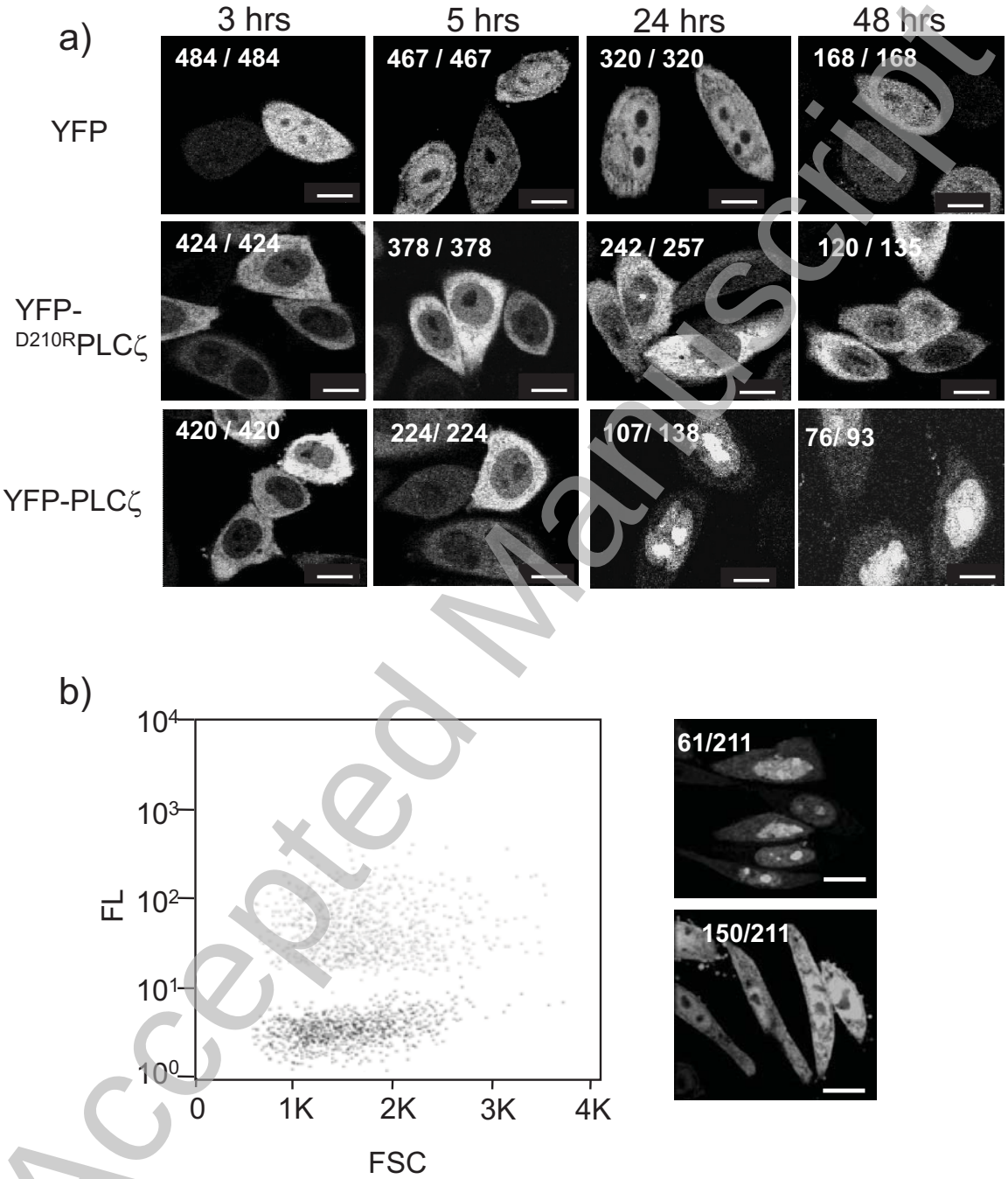
Fig. 2



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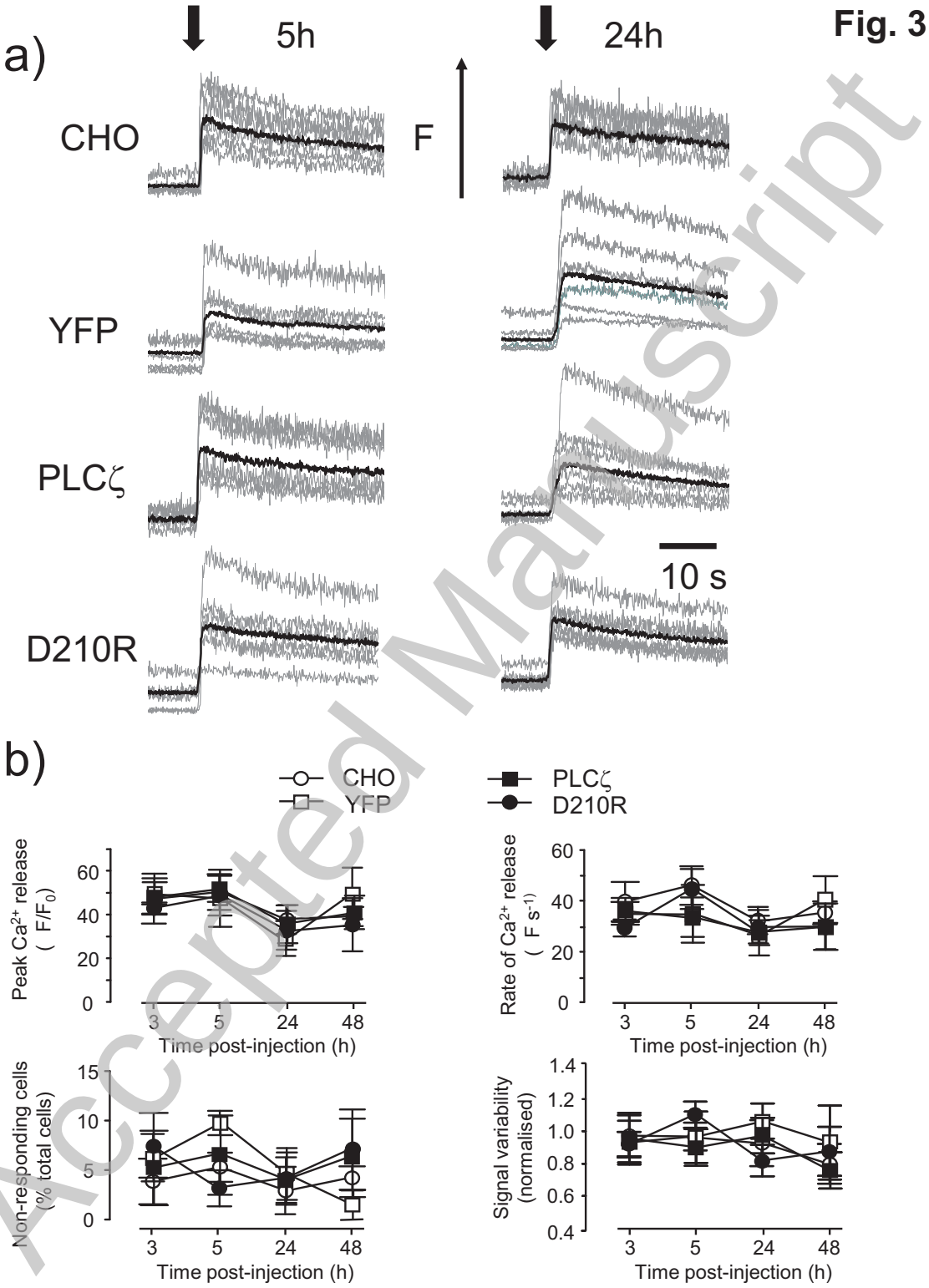

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Fig. 4

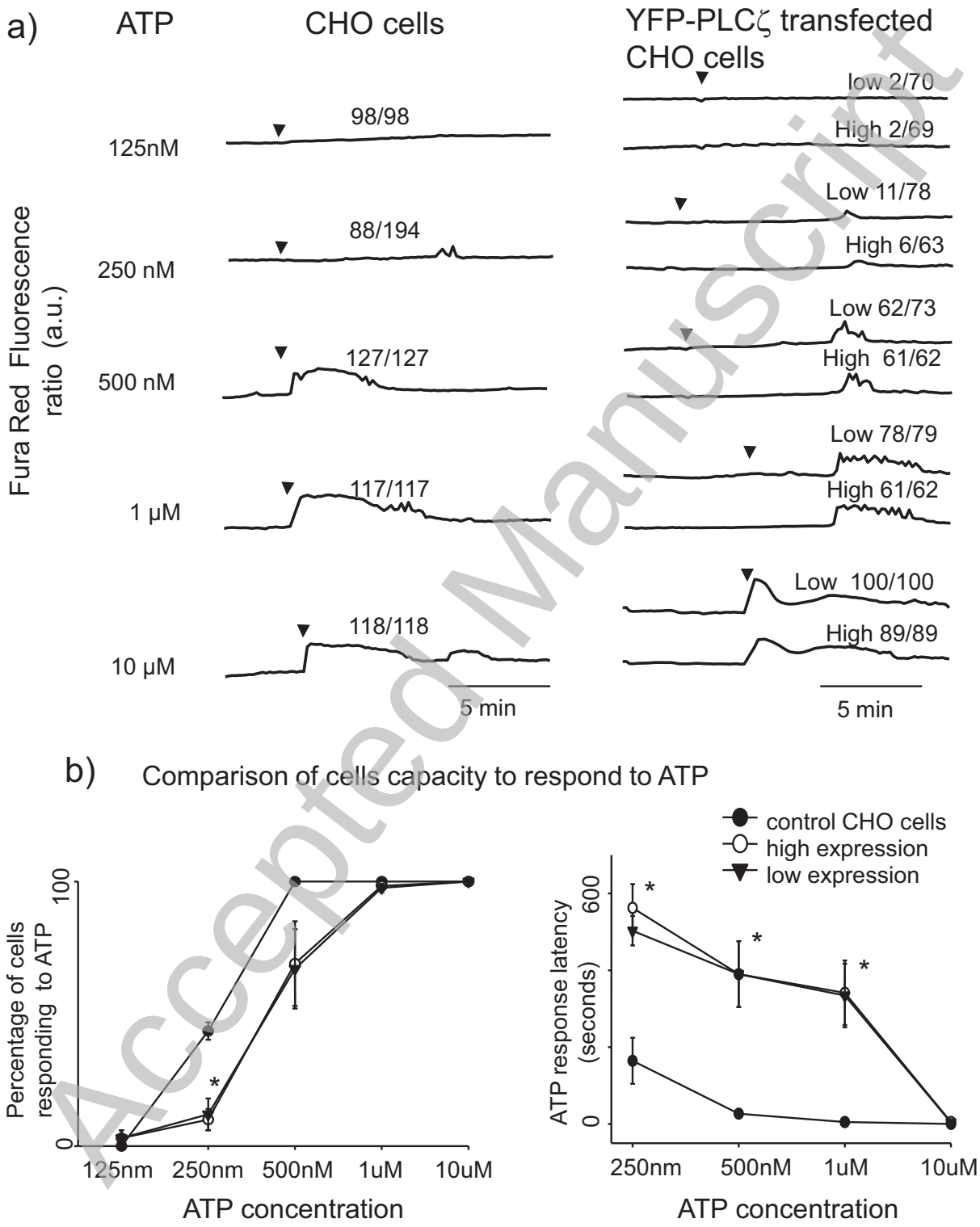

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Fig. 5

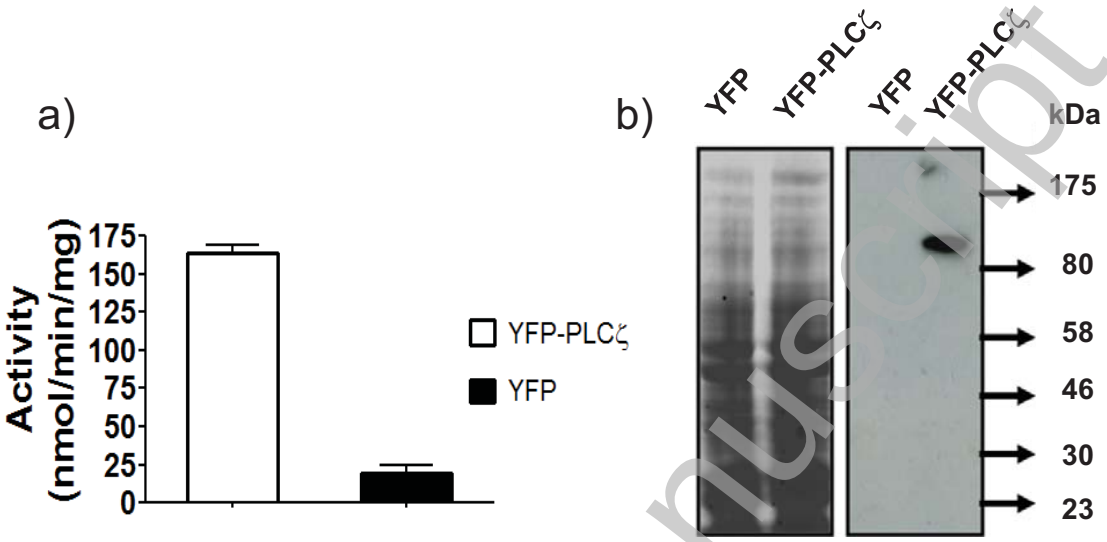

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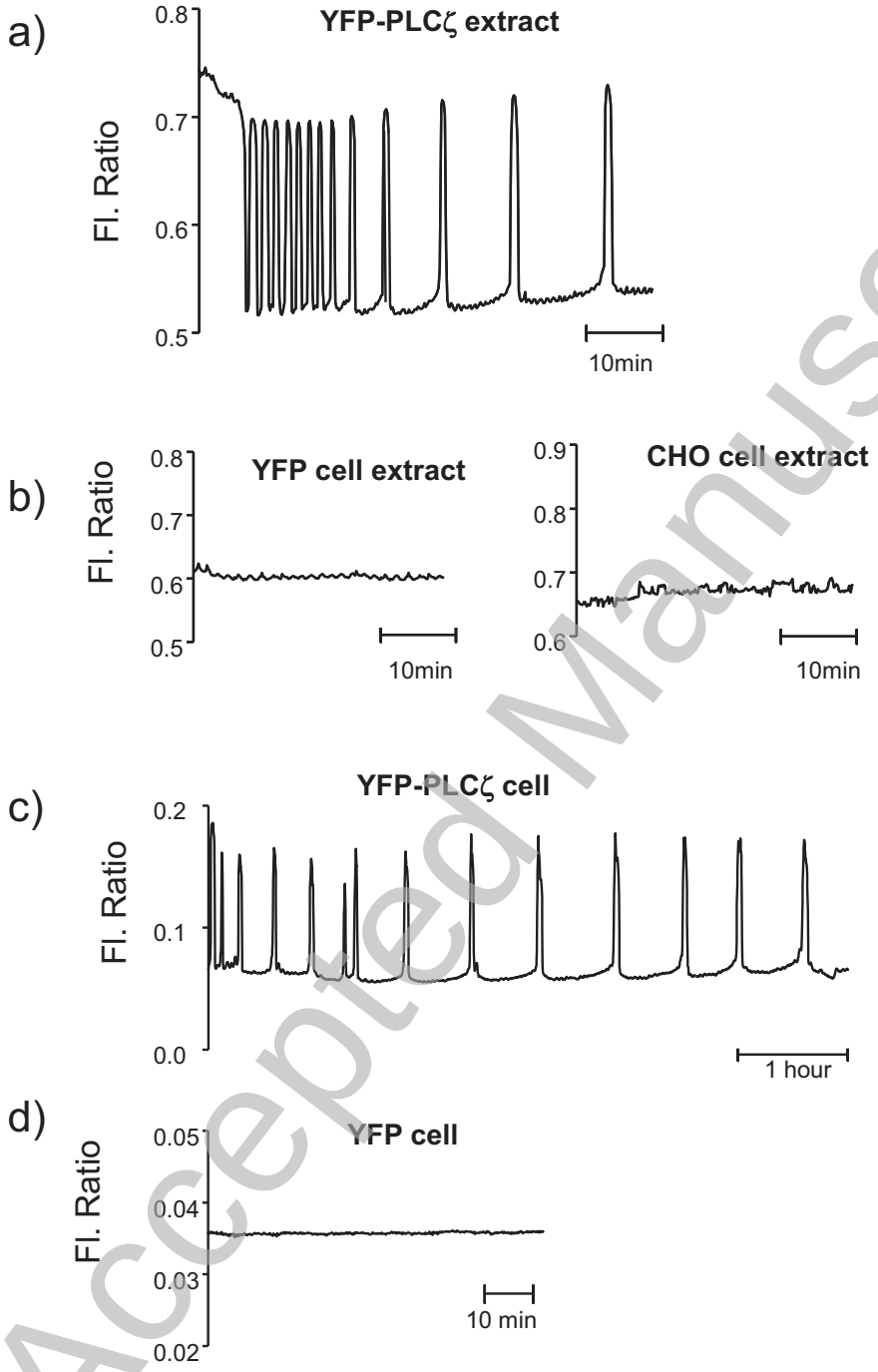
Fig. 6



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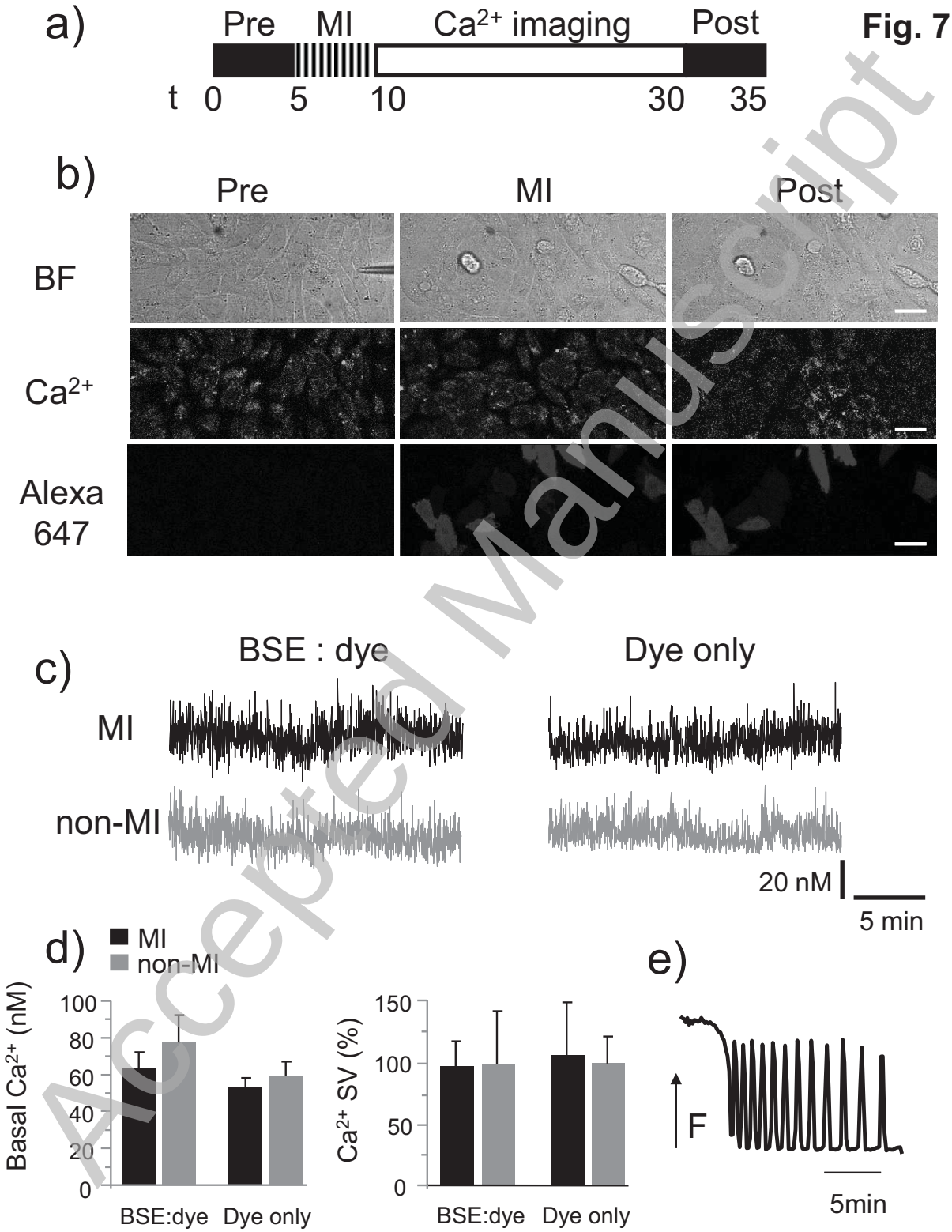

Fig. 7

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