Calcium signaling in Hela cells. IP3/RyR receptors and SERCA pump. K. Essin.

Introduction:

Cells have two major types of intracellular Ca^{2+} release channels – inositol 1,4,5-triphosphate (IP3) and ryanodine receptors (RyRs)[1]. IP3 receptors are activated by IP3, following G protein-coupled receptor (GPCRs) receptor stimulation. Human cervical cancer cell line (HeLa cells) expresses endogenous GPCRs - histamine H1 [2] and muscarinic cholinergic receptors (Schonbrunn A. and Steffen D., "Endogenous GPCR list in common cell lines", http://www.tumor-gene.org/GPCR/gpcr.html). Therefore, application of histamine and carbachol (CCh), a non-hydrolysable cholinergic agonist, provokes a remarkable release of Ca^{2+} from intracellular stores in HeLa cells (Fig. 5). RyRs are activated by external Ca^{2+}, entering the cells upon depolarization through voltage-dependent Ca^{2+} channels, and mediate a massive Ca^{2+}-induced Ca^{2+} release (CICR) in cardiac and muscle cells [3]. In non-excitable cells, like HeLa cells, the level of RyRs expression is modest. Correspondingly, caffeine, an agonist of RyRs, does not elicit a detectable increase in intracellular Ca^{2+} in HeLa cells [4].

Ca^{2+} is a mediator of many processes, from contraction to apoptosis and cells at resting state keep the internal $[Ca^{2+}]$ at a low level (~100nM). The major source of Ca^{2+} contributing to cytoplasmic Ca^{2+} elevation is sarcoplasmic/ endoplasmic reticulum (SR/ER). To maintain high $[Ca^{2+}]$ inside the SR/ER cells possess sarco/endoplasmic reticulum Ca^{2+} – ATPase (SERCA) pumps [5]. SERCA pump locates in the SR/ER membrane and couples the transport of Ca^{2+} from cytosolic to lumen spaces. HeLa cells expresses SERCA2b and SERCA3a isoforms of SERCA pump [6,7]. Pharmacological block of SERCA by cyclopiazonic acid (CPA) or thapsigargin empties the ER Ca^{2+} stores and prevents Ca^{2+} release evoked by GPCRs activators histamine and CCh in HeLa cells[8].

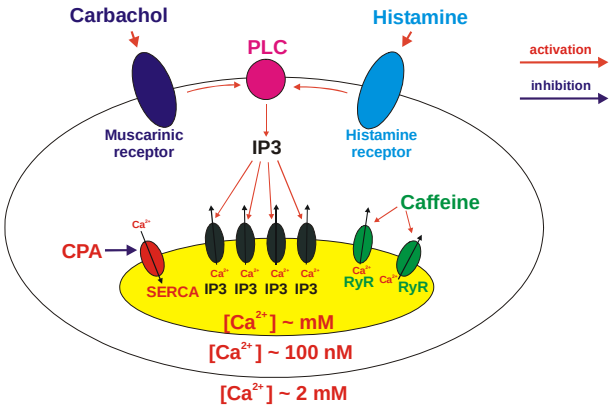

Fig 1. Calcium signaling in Hela cells. IP3/RyR receptors and SERCA pump.
A schematic of the various ways the intracellular Ca^{2+} concentration can increase. GPCRs activation is the most accepted mechanism for Ca^{2+} increase in Hela cells. The depletion of Ca^{2+} stores by CPA, a blocker of SERCA pump, prevents Ca^{2+} release evoked by GPCR agonists carbachol and histamine.

Experiments:

Ca^{2+} signals in the HeLa cells will be measured with the Fluo-4 AM, a Ca^{2+} fluorescent indicator dye (Fig. 2). Fluo-4 AM posseses acetoxymethyl ester group which makes it membrane permeable. After penetration into the cells, dyes undergo de-esterification by intrinsic esterases and remain trapped. Ca^{2+} bound Fluo-4 can be excited by blue light and emits green light [9]. The intensity of the emitted light reflects the level of $[Ca^{2+}]$ in the cytoplasm, where Fluo-4 was accumulated. Therefore an increase in intracellular $[Ca^{2+}]$ evoked by application of different substances can be detected by rise of the fluorescence signal in Fluo-4 loaded cells.

Three experiments should be performed (Fig. 3B):

1) CCh/ histamine (1mM) induced Ca^{2+} release (IP3 receptors)
2) Caffeine (10mM) induced changes in intracellular $[Ca^{2+}]$ (RyR receptors)
3) CPA (10µM) mediated block of the CCh/ histamine induced Ca^{2+} release (SERCA pump).

The experiments are designed to address the question which type of the Ca^{2+} release receptors - IP3 or RyR dominates in HeLa cells.

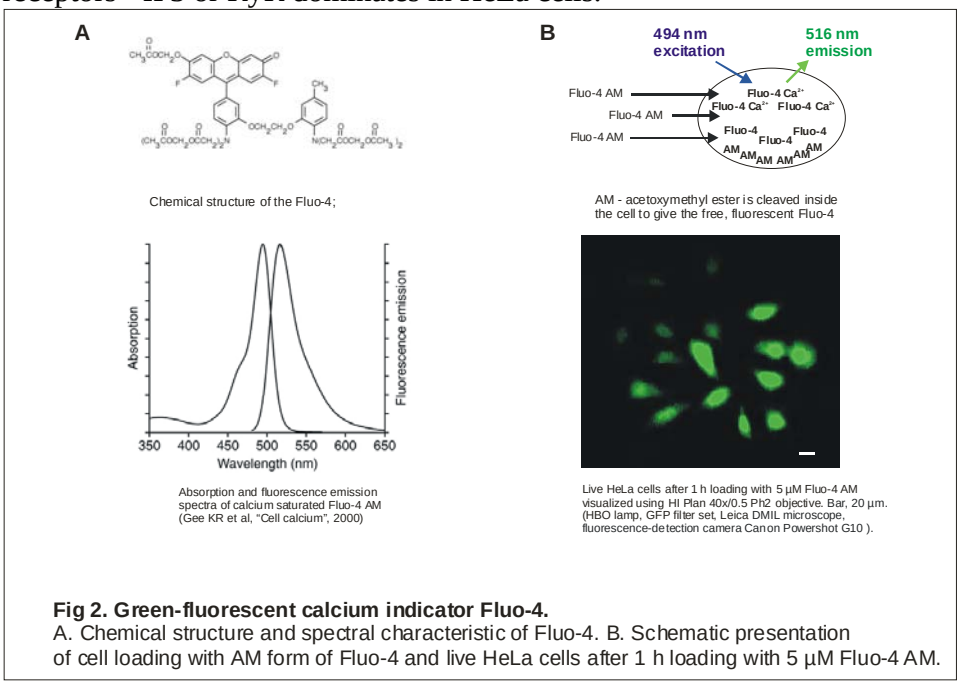

Fig 2. Green-fluorescent calcium indicator Fluo-4.
A. Chemical structure and spectral characteristic of Fluo-4. B. Schematic presentation of cell loading with AM form of Fluo-4 and live HeLa cells after 1 h loading with 5 µM Fluo-4 AM.

Experimental procedure:

One day before the experiment:

1) Prepare the cells: Take a coverslip with the forceps, dip it into 99% ethanol and flame to sterilize. Place three coverslips per one 35 mm Petri dish. Prepare four of such a Petri dishes. Seed cells on coverslips placed in Petri dishes (2 ml of cell suspension of density 0.1 x 10^6 cells/ml per 35 mm Petri dish).

2) Prepare the PSS and stocks of Fluo-4 AM, CCh, histamine, caffeine and CPA:

PSS				
	CatN	FW	MM	**g/liter**
NaCl	6781.1, Roth	58.44	140	**8.18**
KCL	3957.2, Roth	74.55	3	**0.22**
CaCl2	2382, Merck	147.02	3	**0.44**
MgCl2	A375333, Merck	203.3	1	**0.20**
HEPES	9105.3, Roth	238.3	10	**2.38**
D-mannitol	M-4125, Sigma	182.2	50	**9.11**
pH 7.4	NaOH			

Substance	CatN	FW	mM	Solvent	**mg/ml**
CCh	C4382	182.65	100	water	**18.27**
Histamine	H7125	111.15	100	water	**11.12**
CPA	C1530	336.38	10	DMSO	**3.36**
Caffeine	C0750	194.19	100	PSS	**19.42**
Fluo-4 AM	F-14201	1096.95	2.5	DMSO	**2.74**

CCh	aliquote to 100 µl, freeze at -20°C
Histamine	aliquote to 100 µl, freeze at -20°C
CPA	aliquote to 100 µl, freeze at -20°C
Caffeine	keep at +4°C (no more than 24h)
Fluo-4 AM	18.2 µl DMSO per 50µg, feeze at -20°C

3) Obtain from your instructor or download the software for data analysis and install it at your laptops if possible ("Pazera Free MOV to AVI Convertor" www.pazera-software.com, "VirtualDub" www.virtualdub.org and "ImageJ" http://rsbweb.nih.gov/ij/). The manual for Canon Powershot G10 is available at www.canon.com.

4) Transfer all data from the SD memory cards located in your cameras to computers.

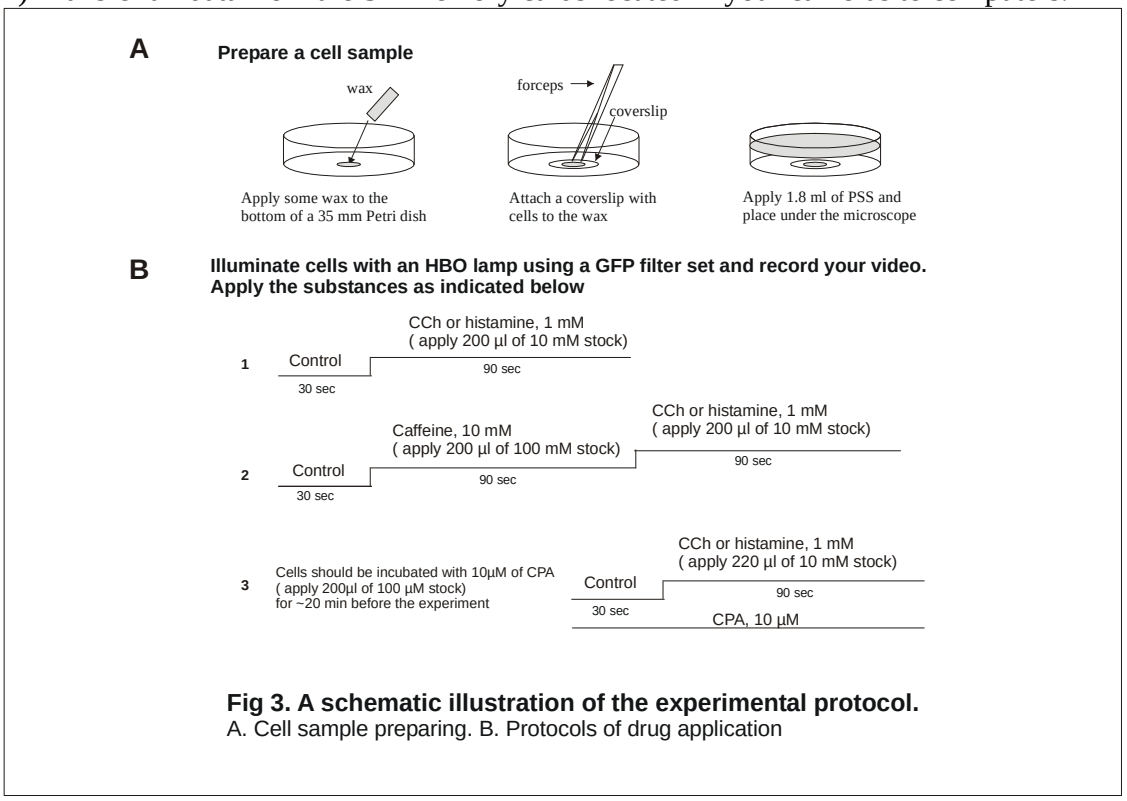

Fig 3. A schematic illustration of the experimental protocol.
A. Cell sample preparing. B. Protocols of drug application

Experimental day:

Loading of the cells with the Fluo-4 AM (1.2 h):

Dissolve 16 µl of 2.5 mM Fluo-4 AM stock solution in 8 ml of PSS in a 50ml tube. Then, cover this tube with aluminum foil to prevent photobleaching from unwanted exposure to ambient light. Take the cells seeded on coverslips out of the incubator. Remove the culture medium and replace it with PSS containing Fluo-4 AM (2 ml per 35

mm Petri dish). Cover the Petri dishes with aluminum foil and keep them for 1 h at room temperature (RT). After 1 h of incubation with Fluo-4 AM, wash cells twice with PSS. Before recording, keep cells in the darkness for 20 min to allow de-esterification of the dye.

During incubation and de-esterification processes :

Prepare your drugs. Take the caffeine stock out of the refrigerator and let it warm to RT. Dilute stock solutions. Take a 1.5 ml eppendorf tube and add 900 µl of PSS and 100 µl of 100 mM histamine stock to obtain 1 ml of the solution containing 10 mM histamine in PSS. Dilute CCh and CPA stocks solutions to 10 and 0.1 mM correspondently.

Dilutions	Initial stock	Mix of		Resulted stock
	conc., mM	Init stock,µl	PSS, µl	conc., mM
Histamine	100	900	100	10
CCh	100	900	100	10
CPA	10	990	10	0.1

Prepaire 30 nonsterile Petri dishes with the wax attached to the bottom (Fig. 3A). Do not apply a lot of wax as this could cause difficulties at fluorescence measurements. Switch the HBO lamp ON (~20 min before the experiment).

Experiments (2 h):

Take a 35 mm Petri dish with a wax layer at the bottom (Fig. 3A). Attach a coverslip with the Fluo-4 loaded cells to the wax and add 1.8 ml of PSS. Ensure that the coverslip is attached properly and does not move upon application of PSS. Reattach it if necessary with the forceps or take another Petri dish. Place your Petri dish under the microscope and visualize cells under 40x objective. Illuminate cells with the blue light using HBO lamp and GFP filter set. Evaluation under blue light should reveal the presence of green-fluorescent cells successfully loaded with Fluo-4 (Fig. 2B, lower part). Redirect the fluorescence signal to the Canon camera coupled to your microscope. Start video acquisition of fluorescent signal with the camera.(Operating with the Canon PowerShot G10: Switch on the camera; set video mode; set video resolution at 640x480 pixels; set ISO to 1600; focus on the cells; press star button at the top right corner of the camera body; set gain to the maximum value using a control ring located at the right middle part of the camera body; start video acquisition.)

Experiment 1: Collect a video for 30 seconds to monitor Ca^{2+} level before drug application. Add 200 µl of 10 mM CCh or histamine stock to reach a final concentration of 1mM in Petri dish. Continue the video capture for the next 1.5 min to detect CCh/histamine induced Ca^{2+} release (Fig 3B, experimental protocol 1). Repeat the experiment 3 times taking each time a fresh cell sample.

Experiment 2: Collect a video for 30 seconds to monitor Ca^{2+} level before drug application. Add 200 µl of 100 mM caffeine stock to reach a final concentration of 10mM in Petri dish. Continue the video capture for the next 1.5 min to detect caffeine induced changes in $[Ca^{2+}]$, if any (Fig 3B, experimental protocol 2). Add 200 µl of 10 mM CCh or histamine stock to reach a final concentration of 1mM in Petri dish. Continue video capture for 1.5 min to detect CCh/histamine induced Ca^{2+} release. Repeat the experiment 3 times taking each time a fresh cell sample.

Experiment 3: Before starting this experiment, cells should be incubated with 10 µM of CPA for ~20 min. Prepare 3 cell samples and add 200 µl of 0.1 mM CPA stock. During

the incubation period, replace previously collected videos from the SD memory cards located in your cameras to computers. After CPA incubation, collect a video for 30 seconds to monitor Ca^{2+} level before application of CCh or histamine (Fig 3B, experimental protocol 3). Add 220 µl of 10 mM CCh or histamine stock to reach a final concentration of 1mM in Petri dish. Continue the video capture for the next 1.5 min to detect CCh/histamine induced changes in [Ca^{2+}], if any. Repeat the experiment 3 times taking each time a fresh cell sample.
The videos will be taken at 30 frames per second (fps) with the resolution of 640x480 pixels. The capacity of your SD memory card is restricted to about 2 Gb. This equates to 23 minutes of videos (Canon PowerShort manual, p 283).

Experimental video size		# of experiments	length of the video, min	total length, min
Experiment 1	CCh/histamine	3	2	6
Experiment 2	Caffeine and CCh/histamine	3	3.5	10.5
Experiment 3	CPA and CCh/histamine	3	2	6
			Total, min	**22.5**

Transfer the resulted videos from the SD memory cards to computer for further analysis.

Analysis of the results: *(analyze 1-2 videos with your instructor what will require ~0.5 h; analyze the rest videos on your own)*

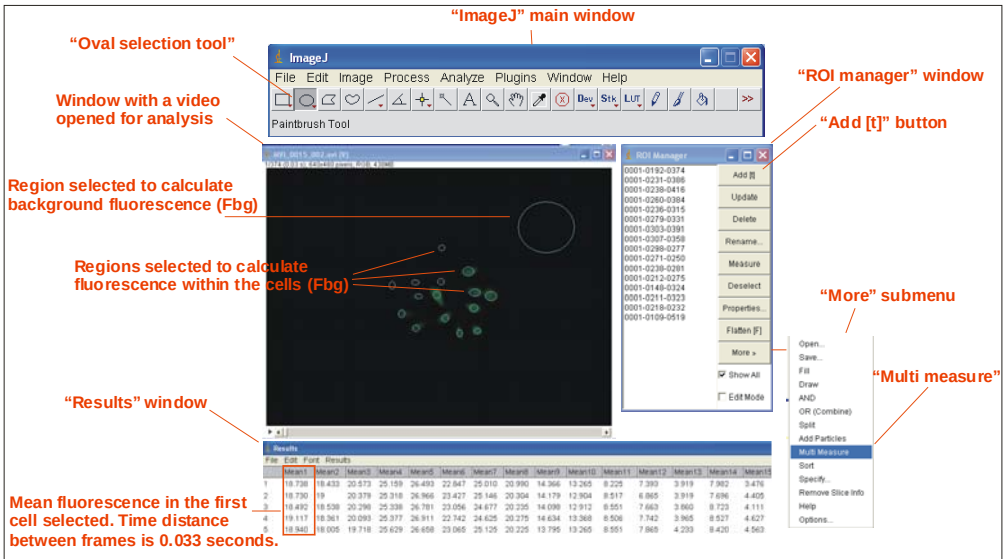

Fig 4. Analysis of the result using "ImageJ" software.
Open a video converted to an old .AVI format. Select the areas within the cells using "ROI manager". Calculate the fluorescence intensities within the cells over time using the "Multi measure" plugin. Save the result of the analysis ("Result window") in a file. Open the resulted file with "MS Excel" for the final analysis and graphical presentation.

1) Convert videos obtained using Canon Powershot G10 from the .MOV to .AVI format (software "Pazera Free MOV to AVI Convertor"). Set output format as MPEG4.
2) Convert further videos to the old .AVI format using "VirtualDub" utility. The resulted videos can be opened with "ImageJ" software.

3) Analyze the fluorescent signal traces of the cells using ImageJ.
   3.1) Open a video with the ImageJ (menu item File->Open). The program activates “AVI Reader” window by file opening. To speed up file opening process, please check mark box “Use Virtual Stack”. Confirm your choice pressing “OK” button.
   3.2) Activate “Set Measurements” window (menu item Analyze->Set Measurements). To speed up the analysis process, please check mark box “Mean gray value” only. Confirm your choice pressing “OK” button.
   3.3) Activate “ROI manager” window (menu item Analyze->Tools->ROI manager). Check mark box “Show All” located at the bottom of “ROI manager” window. Click on the button “Oval selection tool” located under the menu item “File”. Select an area inside of a cell, so-called region of interest (ROI) (Fig. 4). Confirm your selection by clicking on the button “Add [t]” located at the right up corner of “ROI manager” window. Select all the cells of interest confirming each time your choice. At the end select an area outside of the cells to calculate background fluorescence level during your experiment.
   3.4) Activate “Multi Measure” window (menu item More-> Multi Measure in “ROI manager” window). Check mark boxes “Measure All Slices” and “One Row per Slice”. Confirm your choice clicking on the button “OK”. After a while, the program activates “Results” window (Fig. 4). Save the result of analysis (menu item File-> Save as… in “Results” window). The saved file can be opened by Microsoft Excel for final analysis.
4) Open the resulted file with Excel. The file contains columns of numbers representing fluorescence levels detected in cells during your experiment. The last column is background fluorescence. First, subtract the background fluorescence from the fluorescence measured in cells. After that, calculate the fluorescence in cells before drug applications (Fo). Normalize the fluorescence intensities (F) to Fo. Present the result as a graph of the background corrected and normalized fluorescence intensities versus time as shown in Fig. 5. When you will calculate time scale, keep in mind that videos were taken at 30 fps.

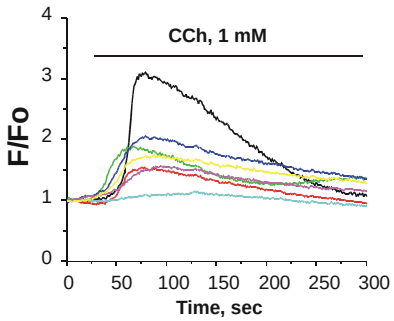

Fig 5. Example of carbachol-evoked calcium release in several individual HeLa cells. Cells were loaded for 1 h with 5 μM of Fluo-4 AM. Fluo4 loaded cells were excited by blue light, using the HBO lamp and the GFP filter set of Leica DMIL inverted microscope

Questions :

1) What mechanism is responsible for the CCh and histamine induced Ca^{2+} releases in HeLa cells. Why CPA prevents these releases?
2) Which type of the Ca^{2+} release receptors IP3 or RyR dominates in HeLa cells? Describe how your experiment might prove it?

Materials, reagents, equipment and software:

***Materials*:**

1) Round borosilicate glass coverslips *(*No.1*;* 12 mm diameter; cod. 0111520) purchased from Marienfeld GmbH
2) 5 sterile Petri dishes, 35 mm diameter
3) 50 nonsterile Petri dishes, 35 mm diameter
4) Wax (White Wax Square Ropes, "J.A.W. Products, Inc.", CatN 4101-0209)
5) Forceps (Inox, CatN 11200-10, "FST")
6) Ethanol 99%
7) A 50 ml tube
8) 1.5 ml eppendorfs (10 pcs) and 0.5 ml eppendorfs (30 pcs)
9) Aluminum foil

***Reagents*:**

10) PSS (in mM: NaCl 140, KCl 3, $CaCl_2$ 3, $MgCl_2$ 1, HEPES 10, D-mannitol 50; pH 7.4 with th NaOH), stored at +4°C
11) Fluo-4AM, „Invitrogen", # F14201, MW1096.95, DMSO soluble, stored at –20°C
12) CCh, „Sigma", # C4382, MW 182.65, water soluble, stored at RT
13) Histamine, „Sigma", # H7125, MW 111.15, water soluble, stored at –20°C
14) CPA, "Sigma", #C1530, MW 336.38, DMSO soluble, stored at –20°C
15) Caffeine, "Sigma", #C0750, MW 194.19, soluble in PSS, stored at RT

***Equipment*:**

Leica DMIL inverted microscope equipped with transmitted and fluorescent light source (HBO lamphouse ebq 50 ac), with a GFP filter set, an objective HI Plan 40x/0.5 Ph2 and a fluorescence-detection camera Canon Powershot G10 coupled to the microscope using a homemade adapter. A laboratory timer.

***Software and manuals*:**

1) Pazera Free MOV to AVI Convertor (free at www.pazera-software.com)
2) VirtualDub (free at www.virtualdub.org)
3) ImageJ (free at http://rsbweb.nih.gov/ij/)
4) Microsoft Excel
5) Manual for the Canon PowerShot G10 (free at http://www.canon.com/)

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