Scientific Research Publishing

Advances in Microbiology, 2022, 12, 555-567
https://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

Luminometric Studies of Yeast Response to Complex Environmental Calcium Variations Demonstrate Sensing of External Calcium Ion Changes

Augustus C. Parker, David A. Mintz, Francisco J. Solis, Pamela A. Marshall*

School of Mathematical and Natural Sciences, Arizona State University, Glendale, USA
Email: *pamela.marshall@asu.edu

How to cite this paper: Parker, A.C., Mintz, D.A., Solis, F.J. and Marshall, P.A. (2022) Luminometric Studies of Yeast Response to Complex Environmental Calcium Variations Demonstrate Sensing of External Calcium Ion Changes. *Advances in Microbiology*, **12**, 555-567.
https://doi.org/10.4236/aim.2022.129038

Received: August 22, 2022
Accepted: September 27, 2022
Published: September 30, 2022

Abstract

The response of yeast to sharp environmental increases in calcium concentration has been extensively studied. However, systematic studies of the response under more general changes are still lacking. Only limited exploration of cellular responses has been conducted where calcium concentration is decreased. This article describes a set of luminometric experiments that monitor the cytosolic calcium concentration under changing external concentration conditions. As a decrease in external calcium concentrations requires the use of large sample volumes, the experiments require the use of equipment adapted for this purpose. We describe the modification of commercial luminometric equipment to make the exploration possible. We explore the yeast cellular behavior when an increase in external calcium concentration is followed by a decrease in external calcium concentration. We compare these results with those from the case of a double pulse of concentration increase. Results from the experiment show that the first, concentration increasing pulse produces the well-known sharp increase in cytosolic calcium followed by calcium sequestration to return to a cytosolic concentration near its initial condition. Surprisingly, the calcium decrease step shows similar results with a cytosolic increase followed by a return to lower levels. The results suggest the presence of a calcium sensing mechanism regulating calcium influx from external sources. This mechanism would produce channel opening as a response to any changes in external concentration, be it an enhancement or a depletion.

Keywords

Saccharomyces cerevisiae, Calcium, Homeostasis, Extracellular Ion Changes

1. Introduction

The response of yeast to changes in external calcium concentration has been extensively investigated using diverse experimental techniques as well as modeling approaches [1] [2] [3] [4]. Key components of the network that regulates cytosolic calcium have been identified [5] [6] though important details remain to be clarified. The nature of the calcium channels of yeast has been clarified [7], but questions remain as to whether different channels can transfer these ions into the cytosol.

An important gap in the understanding of the yeast response is the lack of systematic experiments where the external concentration is controlled and has changes that go beyond simple step-pulses. Such investigations are required for several reasons. Experimental work where the external change is not an increasing pulse has already provided interesting and somewhat unexpected results and has helped elucidate channels and feedback loops [1] [8]. It is known that increasing the external calcium concentration temporarily increases the cytosolic concentration (for example see [9]). However, it has also been found that decreasing the external calcium concentration can as well increase the cytosolic concentration instead of simply maintaining or decreasing this concentration [10] [11]. Though the effect is well established, it calls for further investigation as it can provide insight into the structure of the calcium system in yeast and other organisms. A second reason for investigation of complex variations is the need to validate models of the calcium regulation system [2] [3] [4] [12] [13] [14]. Comparison of simulations for a wide variety of external inputs presents more stringent tests of model soundness than contrast to just pulse increases. In turn, validation of models using this approach will produce models that can readily investigate more complex scenarios.

The systematic change of environmental concentrations is difficult to perform experimentally but can occur quite readily in real-life systems. Sharp incremental increases in concentration simply require the addition of calcium salts to the experimental environment. Decreases in concentration, in contrast, require either the sequestration of calcium or change of calcium in the whole environment. Both methods are challenging to implement systematically. Substantial dilution of external calcium concentrations during luminometry requires the use of volumes much larger than the size of the sample itself and is difficult to perform in a standard luminometer bounded by the volume of a test tube, for example. The present work develops a concrete approach towards this goal.

In addition to the demonstration of feasibility of complex changes in external concentrations, the work described here provides a concrete new insight into the yeast response. In the original observation of response under a direct hypotonic shock [11], a transient calcium rise in the yeast cytosol is quickly induced after hypotonic shock. The calcium is believed to be extracellular in origin. A second increase in cytosolic calcium after hypotonic shock is inhibited by gadolinium and seems to originate from internal stores. Later work revealed that the second

calcium influx is mediated by the endoplasmic reticulum, potentially by the Flc2p transmembrane protein transporter [10], but that the origin of the first extremely fast rise is extracellular, and its pathway is unknown. Our work shows that the extrusion of calcium, presumably at the plasma membrane since it is a very rapid response, as a response to decreased extracellular tonicity also occurs even after an initial external concentration increase is first performed. It is therefore the case that this is a generic response to external calcium decrease and is not tied to the absolute value of the external or internal conditions.

The main obstacle to this type of experiment is the need for the decrease of external calcium concentrations. A step from, for example, 10 mM to 5 mM requires the doubling of sample volume. Such changes are not easily handled by typical luminometric systems that utilize tubes of approximately 5 - 7 ml but with quite a small volume of injection, typically less than 500 ml; modern equipment utilizes experiments with low sample volumes. Thus, we have modified equipment with high volume capability (up to 5 ml) and replaced the system injectors with external devices capable of injecting or flowing large volumes of solutions.

2. Materials and Methods

2.1. Overnight Preparation of Yeast Cells

SEY6210 (*MATa leu*2-3,112 *ura*3-52 *his*3-Δ200 *trp*1-Δ901 *lys*2-801 *suc*2-Δ9) was transformed with the pEVp11-Aeq plasmid (kind gift of Dr. Martha Cyert, Stanford University) using the Frozen EZ Yeast Transformation II Kit (Zymo Research) according to the manufacturer's instructions and plated on selective media lacking leucine. Overnight cultures at 30C were made to amplify yeast numbers and the following morning growth was monitored spectrophotometrically to ensure adequate amounts of cells.

2.2. Preparation of Yeast Solutions

Cells were prepared similarly to Coleman *et al.* [9] and coleatrazine was added to reconstitute aequorin as indicated. Each overnight culture was separated into three, 15 mL centrifuge tubes in 5 mL portions for a total of six tubes. The yeast samples were centrifuged at maximum speed for approximately five minutes. Supernatant was decanted for disposal and 400 µL of YNB glucose was added to each centrifuge tube. The mixtures were then vortexed until the contents homogenized.

2.3. Assembly and Preparation of Luminometry Trials

The yeast samples were removed from the shaker and incubated at 30C. The cells were removed individually at the start of each trial to prevent the other samples from deteriorating. Each microcentrifuge tube was vortexed to resuspend the cells, and then the entire 200 µL of solution was pipetted into a 13 × 100 test tube.

2.4. Luminometry System

The equipment necessary for experimentation consisted of a luminometer, appropriately customized computer software, two peristaltic pumps, and silicone tubing. We have modified a Berthold, Gen-Probe Leader I luminometer. The modification consists in the replacement of the two injection pumps of the model by external, larger capacity peristaltic pumps. The original pumps can only inject smaller solution volumes. Using the same injection opening the peristaltic pumps can inject any required volume. We use two separate pumps to be able to sequentially inject different solutions into the probe tube. The pumps were connected to the luminometer with silicone tubing. A silicone cap was casted around the input silicon tubing to fit into test tubes containing samples. This cap maintained fixed the tubing during experiments and ensured correct positioning of the test tubes inside the luminometer. The tubing was threaded through the mechanical arm that presses down the test tubes into the luminometer. In the original system the arm blocks any external light from the sample. In the modified system the arm does not fully block light and dark cloth is placed over the system before data is acquired. The background signal with the cloth cover is identical to the original background signal without modification. The data generated by the luminometer was recorded in a computer connected to it through its data port. A sketch of the system is shown in Figure 1.

2.5. Luminometric Analysis of Yeast Calcium Response

The yeast samples prepared as described above were placed into test tubes and closed with a casted cap. The tubes were lowered into the luminometer and covered to assure accurate measurements. Luminosity readings were checked to match the observed baseline prior to starting the experiment. The initial calcium pulse uses a solution of $CaCl_2$ with a calcium concentration C_A. The solution is pumped through the tubing via peristalsis to achieve the desired extracellular concentration C_1 in the extracellular environment. The calcium concentration in the sample is small (it has been estimated as 0.9 mM in [15] but we simply considered as negligible) and thus the environmental concentration after this first pulse is determined by the added calcium from solution C_A divided by the total volume of the sample plus added solution. When the initial volume is V_0 and the

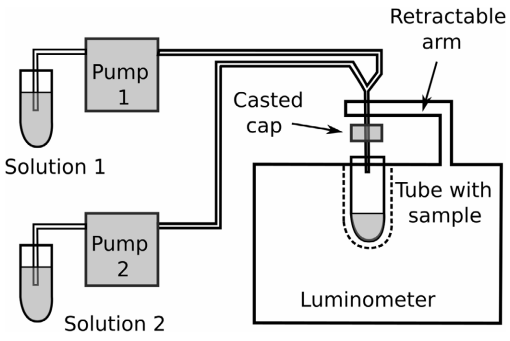

Figure 1. Scheme of modified luminometer system.

added volume is V_A, the total volume at the end of this step is $V_1 = V_A + V_0$, so that the concentration is $C_1 = C_A V_A / V_1$. The injection lasts 20 seconds. After a waiting period, the second calcium pulse with concentration C_B was added to establish the intended final calcium concentration. The injection also lasts 20 seconds. The total time between the starting time of both pulses was 60 seconds. The volume at the end of the second injection is $V_2 = V_1 + V_B$, so that the concentration is $C_2 = (C_1 V_1 + C_B V_B)/V_2$. Between each trial, the silicone tubing was flushed with water to avoid contamination between experiments. Figure 2 shows a scheme of the time variation of the external calcium concentration to which the yeast is exposed. The figure corresponds to a case where the second change is an external decrease. In the system we use a large volume of solution added to samples, so that the change in concentration cannot be considered instantaneous.

Table 1 and Table 2 summarize the calcium concentrations and volumes in the two types of experiments. The double increase can be considered as a reference system while the key experiments are two-step processes with a concentration increase followed by a decrease. The tables show the parameters for a set of the recorded experiments. Other concentrations and timings were also explored. The data below is representative of the broader set of experiments. Similar results

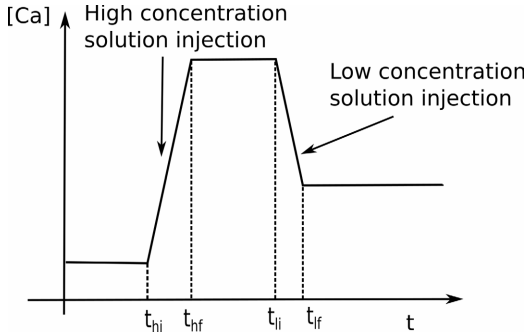

Figure 2. Scheme of time sequence of [Ca] (calcium) in the sample solution for an increase-decrease experiment. The time intervals of injection and their time separation are indicated for concrete runs in the text.

Table 1. Double increase experiments parameters. A yeast sample of volume V_0 is placed in the luminometer. A volume V_A of salt solution, with calcium concentration C_A, is injected to obtain a calcium concentration C_1 in the sample (yeast environment). A second injection of salt solution of volume V_B, with calcium concentration C_B, leads to a final calcium concentration C_2 for the sample.

Double increase						
Injection information					Yeast environment concentrations	
V_0 (mL)	V_A (mL)	C_A (mM)	V_B (mL)	C_B (mM)	C_1 (mM)	C_2 (mM)
0.200	0.200	1.0	1.6	10.0	0.5	8.1
0.200	0.200	3.0	1.6	30.0	1.5	24.3
0.200	0.200	10.0	1.6	100.0	5.0	81.0

Table 2. Increase-decrease experiments parameters. A yeast sample of volume V_0 is placed in the luminometer. A volume V_A of salt solution, with calcium concentration C_A, is injected to obtain a calcium concentration C_1 in the sample (yeast environment). A second injection of salt solution of volume V_B, with calcium concentration C_B, leads to a final calcium concentration C_2 for the sample.

Increase-Decrease						
Injection information					Yeast environment concentrations	
V_0 (mL)	V_A (mL)	C_A (mM)	V_B (mL)	C_B (mM)	C_1 (mM)	C_2 (mM)
0.200	0.200	1.0	1.6	0.1	0.5	0.18
0.200	0.200	3.0	1.6	0.1	1.5	0.38
0.200	0.200	10.0	1.6	0.1	5.0	1.08

(increased cytosolic calcium followed by decrease in cytosolic calcium) were observed for all settings explored.

3. Results

The central result of our experimental work is the observation of yeast responses to a sequence of two changes in extracellular calcium concentration. The first change is an increase in concentration that produces a quick rise in the cytosolic calcium, which then stabilizes at a level closer to its initial value, value as the calcium is quickly sequestered away in the yeast vacuole and endomembrane system [1]. For the second change we have explored both extracellular calcium concentration increases as well as concentration decreases. In experiments with double concentration increases, the second pulse results in behavior similar to the first, namely, a quick rise followed by a slower leveling. In experiments where the second step is a decrease, the result is surprisingly similar. A sharp increase is followed by a leveling off even though the environmental concentration is being reduced. This observation is consistent, within some expected variations, for all initial concentrations increments and all decreases performed.

The graphs in Figures 3-8, are representative of our results. Figures 3-5 show results for double increase experiments for conditions described in Table 1. Figure 6-8 show results for the increase-decrease experiments described in Table 2. The observations are qualitatively similar in all cases. The luminosity, considered as an indirect measure of the cytosolic calcium concentration, is presented in relative units. Though less systematic, our observations for other concentrations (not included in Table 1 and Table 2) reflect the same pattern. Namely, both increases and decreases in concentration lead to a transient increase in the cytosolic concentration.

In all experiments the change in cytosolic concentration coincides with the solution injection in the system. We note that the period of cytosolic calcium elevation is short, lasting about 10 seconds, consistent with established results from single pulse experiments. The full injection lasts about 20 seconds so that

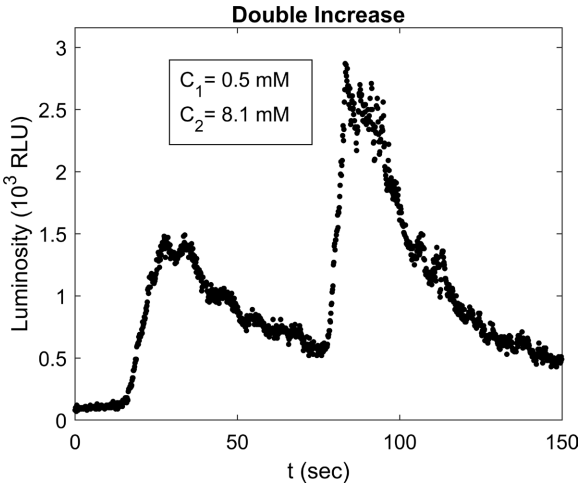

Figure 3. Example of results for a double concentration increase experiment with parameters as in the first row of **Table 1**.

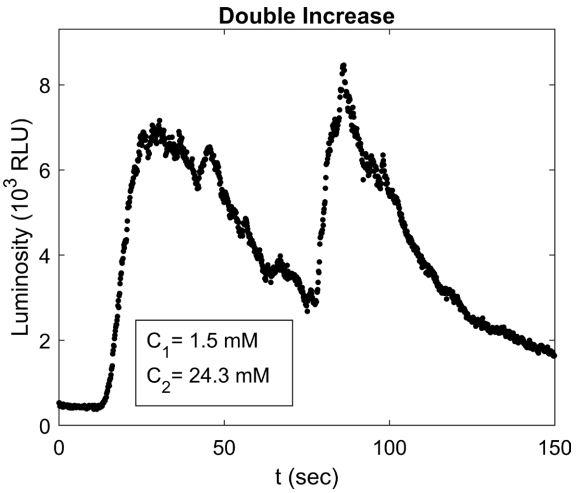

Figure 4. Example of results for a double concentration increase experiment with parameters as in the second row of **Table 1**.

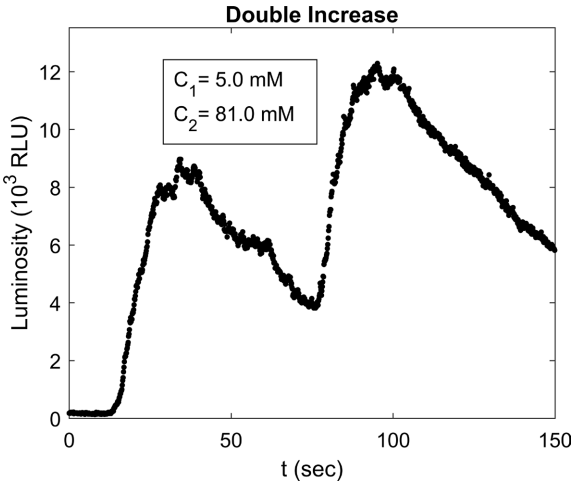

Figure 5. Example of results for a double concentration increase experiment with parameters as in the third row of **Table 1**.

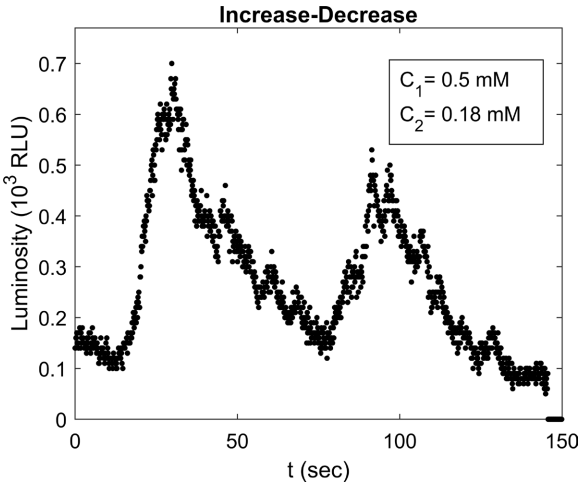

Figure 6. Example of results for an increase-decrease experiment with parameters as in the first row of **Table 2**.

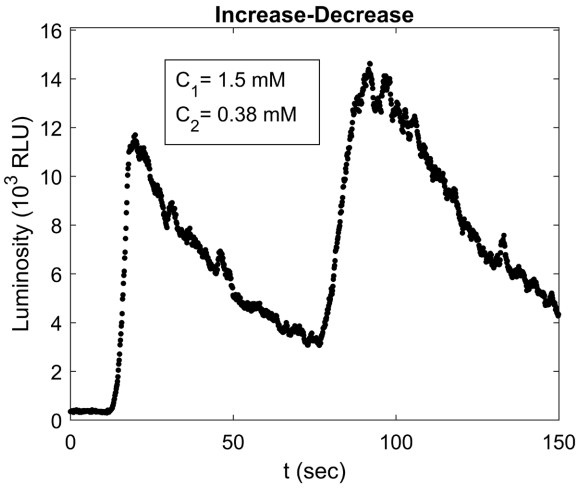

Figure 7. Example of results for an increase-decrease experiment with parameters as in the second row of **Table 2**.

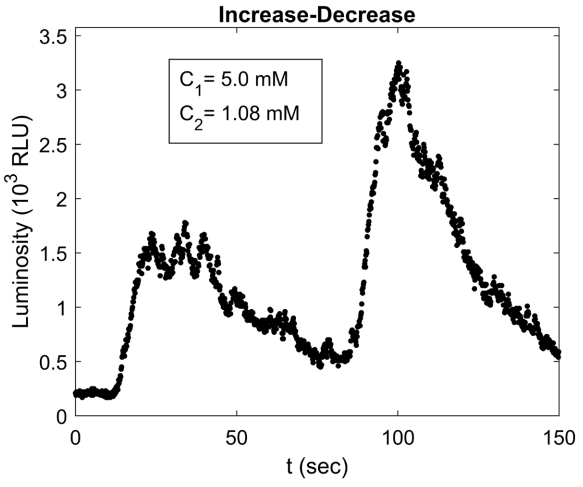

Figure 8. Example of results for an increase-decrease experiment with parameters as in the third row of **Table 2**.

the mechanisms that stop the increase in the cytosolic concentration acts faster than the process that, in this case, externally changes the concentration.

Our experimental results show a large variation in absolute magnitude. We attribute these to the variations of fractions of active yeast within the samples. The use of relatively large volumes of samples and solution leads to inhomogeneous samples where parts of the sample might be in a different life cycle period.

Our experimental results show a large variation in absolute magnitude. We attribute these to the variations of fractions of active yeast within the samples. The use of relatively large volumes of samples and solution leads to inhomogeneous samples where parts of the sample might be in a different life cycle period.

4. Discussion

The use of large volumes brings new challenges to the experimental set up and protocols, which is presumably why this is the first time this type of experiment has been reported. The exposure of yeast to specific sequences of concentrations requires longer timeframes and extends the length of experiments; the cytosolic calcium sensor, aequorin, can lose activity during this period and yeast can also lose viability. For proper exposure to the environmental conditions the individual yeast cells must be properly suspended and not flocculated, concentrated, or precipitated in the medium, though this condition is difficult to monitor. However, our results are consistent with prior literature but extend our understanding of yeast calcium homeostasis.

We propose the following interpretation of these experimental observations. We can first consider the standard step increase in external calcium. The cytosolic calcium can change as a result of influx of external ions though dedicated channels, as a result of ejection/injection into/from the vacuole and other organelles. In the case of external increases in calcium concentration, it has been established that the raise in concentration in the cytosol is predominantly a result of the influx of external ions through a channel. The subsequent decrease reflects, mainly, transport into the vacuole though a fraction of the excess ions might travel to the ER, nucleus or other organelles [1]. Equilibrium of influx and outflux can produce a steady value of the cytosolic concentration [3] [12]). However, this scenario requires a continuous increase in calcium vacuole content, and it is not clear that this can be continued indefinitely. As calcium and other channels are most often regulated rather than passive transporters (see for example [13]), it seems likely that the increase in cytosolic concentration eventually leads to the closing of the channel. Once the channel is closed, sequestration into the vacuole can restore a cytosolic calcium concentration close to its original value [14]. A subsequent increase in external calcium concentration can produce a new, large, ion influx [9]. As we are proposing that the channels are closed after the first influx, the change in external ion concentration would be responsible for reopening the channel and the restart of the same process as before. In this model, a key element is then played by a sensing mechanism that

opens the channels upon changes in external concentrations and a second mechanism that leads to the channel closing. As we additionally observe the very quick influx when the external concentration is diluted, this indicates that there is a sensing mechanism that detects perturbation away from an equilibrium state regardless of direction of change. We also note that under this interpretation, the calcium channel is predominantly closed under homeostatic conditions. This picture is summarized in the scheme of **Figure 9**.

In previous work, models for calcium homeostasis have been developed based on assumptions regarding the nature of calcium channels and the components of this homeostatic system, for example in [11] [12]. Our lab has further explored these ideas with mathematical modeling and contrast with experiments [3] [16]. In short, these models consider a passive calcium channel, and the equilibrium is obtained by sequestration into the vacuole enhanced by internal mechanisms, activated by the excess cytosol calcium. As noted above, our current observations suggest that the calcium channel is not passive. The proposed interpretation furthermore requires that the feedback system of the cell should produce not only enhanced sequestration of calcium but the closing of the channels. Mathematically, the passive channel is described by a Michaelis-Menten model. The flux rate J is written as

$$J_{\max} = C_{ext} \Big/ \left(K + C_{ext} \right) \tag{1}$$

where C_{ext} is the external calcium concentration, K is an association constant and $J_{\max}$ is the maximum flux rate. In this expression, the flux is permanent and increases with external concentration increments. Our observations indicate that the use of the model does not account for important features of the system and,

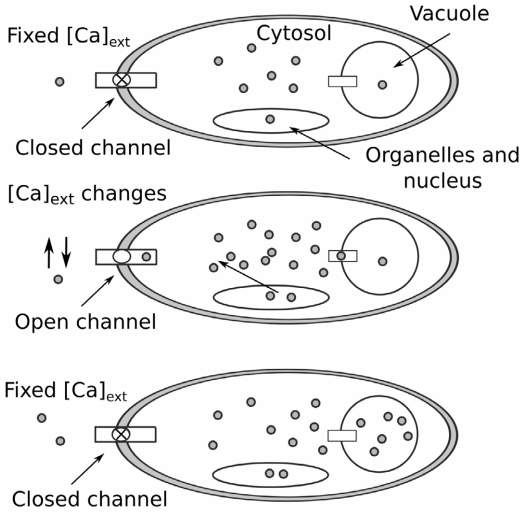

Figure 9. Scheme of channel states and calcium distributions during exposure of yeast to changes in external calcium concentration. At top, when external calcium concentrations do not change, the calcium channels are closed. In the center figure an external change in concentration (either increase or decrease) leads to the opening of the channel and increase in cytosolic calcium. At bottom, once the external concentration is again constant, the channel eventually closes again.

in particular, for the possibility of closing. A recent work has put forward a model where the channel can be in a closed or open state [4]. In the model the probability of transition between states is determined by the difference in osmotic pressure between the environment and the cytosol. A broader alternative is to include in models activated channels where the flux is dependent on the external concentration but where the maximum rate amplitude $J_{\max}$ is dependent on an internal parameter describing the state of the channel. Such models require the specification of the controlling system. In Ref. [4] the pressure difference is the key factor, but many other mechanisms are plausible. We will explore these models in future work.

While we have presented an interpretation of the results based on the idea of a regulated channels, we can point to other possibilities. First, we note that while we assume that the cytosolic calcium observed appears from external sources, it is possible that the observation reflets ejection from internal sources, activated by a mechanism triggered by the external calcium decrease. The broad shape of the time evolution of the cytosolic calcium suggests, however, that the mechanisms of response against concentration increases or decreases are essentially the same.

A second possibility worth considering is the contribution of multiple types of channels to the response. The existence of high-affinity and low-affinity calcium carrying channels has been investigated in the literature [1]. If two or more channels exist, the conditions for their activation might correspond to different types of stimuli. For example, one might transiently open as a response to increases in the external concentration while the second responds to decreases. As with the previous comment regarding the source of the calcium, we note that the features observed in the time evolution of the response peaks suggest one single response rather than the presence of multiple mechanisms.

5. Conclusions

This work shows the value of experiments where complex time-dependent stimuli are used, such as external ion concentration changes of different characteristics. The responses observed allow us to more carefully discern features of the underlying mechanisms. In the present work, the surprising similarity of responses to external changes of different directions has been interpreted as indicators of a switching mechanism for channel control.

From a methodological point of view, we note that a key difficulty in the type of experiment completed is the creation of diluted concentrations. This requires the use of relatively large volumes. Only new equipment or novel techniques can successfully investigate these scenarios. Some possibilities, like the use of continuous flowing systems will be considered in future research.

Acknowledgements

ACP and DAM were partially supported by Barrett, the Honors College, at Ari-

zona State University. ACP was supported by an SRP Scholarship. Both ACP and DAM were supported additionally by NCUIRE in the New College of Interdisciplinary Arts and Sciences.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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