Characterizing the Heavy Metal Chelator, Tpen, as a Ca2+ Tool in the Mammalian Oocyte

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CHARACTERIZING THE HEAVY METAL CHELATOR, TPEN, AS A CA$^{2+}$ TOOL
IN THE MAMMALIAN OOCYTE

A Thesis Presented

By

ROBERT A. AGREDA MCCAUHIN

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
Of the requirements for the degree of

MASTER OF SCIENCE

February 2013

Molecular and Cellular Biology
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To my family and Caitlin Cotter for their never ending support
ACKNOWLEDGMENTS

First and foremost I would like to thank Dr. Rafael Fissore for granting me the opportunity to join his research lab and allow me to pursue my own research project. Throughout the later years of my time at the University of Massachusetts Amherst he has been an invaluable resource and mentor who helped me get to where I am today.

I would like to thank the “Fissore Lab” for the training they have bestowed upon me as well as assisting me in my laboratory projects: Banyoon Cheon, Hoi Chang Lee, Changli, Nan Zhang, Changli He, Eufrocina Atabay and Takuya Wakai.

And to the faculty and staff of the Molecular and Cellular Biology Program who have made my time at the University of Massachusetts Amherst a truly enlightening and valuable experience.
ABSTRACT
CHARACTERIZING THE HEAVY METAL CHELATOR, TPEN, AS A CA\textsuperscript{2+} TOOL IN THE MAMMALIAN OOCYTE
February 2013
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N,N,N’,N’-tetrakis-(2-Pyridylmethyl) ethylenediamine (TPEN) is a heavy metal chelator with high affinity for zinc. TPEN causes important responses in mammalian eggs. For example, these eggs are arrested at the MII stage by the Endogenous Mitotic Inhibitor 2 (Emi2), which prevents activation of the Anaphase Promoting Complex (APC) and degradation of Cyclin B, a component of MPF, whose degradation is required to exit meiosis and mitosis. By chelating zinc, TPEN inactivates Emi2 and eggs undergo spontaneous exit of meiosis and egg activation. TPEN chelates Ca\textsuperscript{2+} with lower affinity, although in the Endoplasmic Reticulum (ER), where Ca\textsuperscript{2+} concentrations are high, TPEN may sequester Ca\textsuperscript{2+} preventing release into the cytoplasm. Upon dose titration studies, we confirmed that TPEN does indeed cause activation of MII eggs. In addition, initial exposure of TPEN to MII eggs failed to cause spontaneous intracellular Ca\textsuperscript{2+} release from the ER. Interestingly, in the case of GV oocytes, addition of TPEN caused Ca\textsuperscript{2+} influx. This influx could be blocked via the addition of 2-APB, a plasma membrane Ca\textsuperscript{2+} channel blocker. To determine the possible role of TPEN on chelation of ER Ca\textsuperscript{2+}, MII and GV cells were incubated in TPEN and ER Ca\textsuperscript{2+} released was by exposure to
Cyclopiazonic Acid (CPA), a sarco/endoplasmic reticulum (SERCA) pump inhibitor, or Ionomycin (IO), a Ca\textsuperscript{2+} ionophore. In MII oocytes, the amplitude of the rises caused by CPA and IO, in TPEN-treated oocytes, was smaller than controls and experienced a delay in return to baseline. In GV oocytes, the responses were highly variable. TPEN enhanced rather than reduced Ca\textsuperscript{2+} responses to CPA and IO. In all cases, TPEN reduced the speed of Ca\textsuperscript{2+} release, time to peak. The presence of TPEN did not significantly increase Ca\textsuperscript{2+} influx at either stage. Curiously, in the presence of TPEN, SrCl\textsubscript{2} failed to initiate oscillations in both GV and MII oocytes. Thus our results show that TPEN can reduce available Ca\textsuperscript{2+} from internal stores, especially in MII eggs. However, given the highly variable effects and its inability to fully chelate ER Ca\textsuperscript{2+}, the use of TPEN as a tool to study Ca\textsuperscript{2+} homeostasis in mouse oocytes needs additional investigation.
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CHAPTER 1

INTRODUCTION

Mammalian oocyte maturation consists of two primary checkpoints. The first is at Prophase I, or more commonly known as germinal vesicle (GV) stage. This stage will persist in the ovarian follicle until a surge of luteinizing hormone (LH) occurs, which causes ovulation (Hartshorne et al., 1994; Fissore et al., 2002). At the conclusion of maturation, the oocyte is arrested at the metaphase II (MII) stage of meiosis. The MII arrest will persist until fertilization occurs (Ducibella and Fissore, 2008). The oocyte maintains this physiological state due to the presence of a cytostatic factor (CSF) (Madgwick and Jones, 2007). Recent publications have stated that one of the components of this elusive factor is known as Early Mitotic Inhibitor 2 (Emi2). This molecule inhibits the Anaphase Promoting Complex (APC; Thornton and Toczyski, 2006; Pesin and Orr-Weaver, 2008). The APC’s purpose is to make possible the transition from the MII stage to the anaphase stage by regulating the stability of Maturation Promoting Factor (MPF) components. The function of MPF is to drive fully grown GV oocytes arrested at the first checkpoint of the first meiosis to the MII stage (Taylor et al., 2004). This complex is made up of the CDK1 and cyclin B (Masui, 2001; Dorée and Hunt, 2002; Jones, 2004). The activity of MPF fluctuates throughout the various stages of meiosis and is at its highest point during MII arrest due to inhibition of cyclin degradation by the CSF as well as new synthesis of this molecule. The release from MII arrest is initiated either by fertilization or induction of artificial calcium (Ca^{2+}) oscillations. Either instance makes possible the degradation of Emi2 molecules, thereby lifting inhibition of the APC and permitting cyclin B degradation. The release of the MII
arrest allows the oocyte to complete meiosis and initiate embryo development if fertilized. Further information can be found in the reviews of (Berridge et al., 2003; Brunet and Maro, 2005).

$\text{Ca}^{2+}$ oscillations are initiated by the sperm during fertilization. Upon interaction and fusion of the sperm with the oocyte, a signaling cascade is initiated that leads to the production of 1,4,5-Inositol Trisphosphate, also known as IP$_3$. IP$_3$ then binds its cognate receptor in oocytes, mostly the IP$_3$ receptor type 1 (IP$_3$R1), which is predominantly located on the surface of the endoplasmic reticulum (ER), the $\text{Ca}^{2+}$ store of the cell. IP$_3$ induces a conformational change in IP$_3$R that causes the channel to open and release $\text{Ca}^{2+}$ into the ooplasm. In the presence of IP$_3$, low concentrations of $\text{Ca}^{2+}$ activate the receptor, while high concentrations of $\text{Ca}^{2+}$ inhibit the conductivity of the receptor. In mammals, the $\text{Ca}^{2+}$ responses occur as repetitive oscillations (Kline et al., 1999), which are initiated by the release into the ooplasm of the so called “Sperm Factor,” that is now known to be a sperm-specific phospholipase C zeta (Saunders et al., 2002).

$\text{Ca}^{2+}$ homeostasis is crucial for oocyte survival. $\text{Ca}^{2+}$ enters the ER via the Sarco/endoplasmic reticulum $\text{Ca}^{2+}$ ATPase (SERCA) pump. Using ATP hydrolysis, $\text{Ca}^{2+}$ ions are translocated from the cytosol, against the concentration gradient, into the ER. How $\text{Ca}^{2+}$ enters the cells to fill the ER has been a matter of speculation. A recently discovered ER $\text{Ca}^{2+}$ sensor is the stromal interaction molecule 1 (STIM1; Jonathan S., 2005; Gill et al., 2006; Hogan et al., 2010), which may play a critical role in mediating $\text{Ca}^{2+}$ influx. It contains an EF hand domain on the interior of the ER that “senses” the $\text{Ca}^{2+}$ concentration. When the ER $\text{Ca}^{2+}$ store is emptied, for instance due to IP$_3$-mediated $\text{Ca}^{2+}$ release, STIM1 forms puncta with other STIM1 molecules near the plasma
membrane. This puncta formation interacts with the Ca\textsuperscript{2+} release activated channel (CRAC) ORAI1, resulting in Ca\textsuperscript{2+} uptake into the cytosol from the extracellular space.

While progress has been made in the understanding of the molecules that regulate MII arrest and those that are part of the Ca\textsuperscript{2+} homeostatic machinery of the mammalian oocytes, much still remains to be learned. For example, we know little of the molecules that regulate Ca\textsuperscript{2+} influx and that control the levels of Ca\textsuperscript{2+} in the ER. A drug that has recently been used in oocytes and might be a useful tool to examine Ca\textsuperscript{2+} homeostasis is N,N,N',N'-tetakis-(2-Pyridylmethyl) ethylenediamine, otherwise known as TPEN. TPEN is a heavy metal chelator with a very strong affinity for zinc. When oocytes are incubated in a solution containing TPEN, the MII arrest is relieved and oocytes initiate parthenogenetic development (Kim et al., 2010). Subsequent research has shown that TPEN inhibits Emi2 due to its Zn\textsuperscript{2+} chelating properties (Suzuki et al., 2010), which makes possible cyclin B degradation and initiation of embryo development. Interestingly, besides causing activation without inducing Ca\textsuperscript{2+} oscillations, TPEN, reportedly affects ER Ca\textsuperscript{2+} levels in somatic cells, as it is able to chelate Ca\textsuperscript{2+} in the ER lumen (Arnaudeau et al., 2002). Thus, given the absence of tools to evaluate the regulation of ER Ca\textsuperscript{2+} levels, this study will focus on understanding whether TPEN affects Ca\textsuperscript{2+} homeostasis in mouse oocytes.
CHAPTER 2

MATERIALS AND METHODS

2.1 Egg Recovery and Culture

Both GV and MII oocytes were collected from female CD-1 mice ranging in age from 5-8 weeks as described in prior publications from our laboratory (Wu et al., 1998). For GV oocyte collection, mice were injected with 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma) 44-48 hours prior to collection. In the case of MII oocyte collection, females were injected with 5 IU of PMSG 60 hours prior to collection followed by 5 IU of human chorionic gonadotropin (HcG; Sigma, St Louis, MO; all chemicals were purchased from Sigma unless otherwise specified) 48 hours later to promote ovulation. Following the Institution of Animal Care and Use Committee (IACUC) guidelines, female mice were euthanized via pressurized CO$_2$ treatment. Cervical dislocation was then performed to ensure euthanasia. Ovaries were harvested into HEPES-buffered Tyrode-Lactate solution (TL-HEPES) supplemented with 5% heat treated fetal calf serum (FCS). Cumulus enclosed GV oocytes were recovered directly from the ovaries and denuded via repeated mechanical aspiration with a micro pipette followed by culture in CZB media (Chatot et al., 1990). MII oocytes were collected from the oviducts and exposed to 0.1% bovine testes hyaluronidase to remove cumulus cells prior to culture in CZB media. In all cases, the CZB media had been pre-incubated under paraffin oil at 37°C in an atmosphere of 5% CO$_2$ for a minimum of 2 hours. The media used to handle and culture GV oocytes was also supplemented with 100µM isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor (Essayan, 2001), unless stated otherwise, so that the GV stage could be maintained.
2.2 TPEN preparation

N,N,N',N'-Tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN; Calbiochem, Billerica, MA) was dissolved using dimethyl sulfoxide (DMSO) and aliquots were diluted into Ca\(^{2+}\) containing CZB supplemented with 0.1% polyvinyl alcohol (PVA) unless stated otherwise. The concentrations of TPEN and time of treatment were dictated by the nature of the experimental conditions.

2.3 Oocyte Activation

Oocyte activation studies were performed using the following TPEN concentrations (µM): 20, 50, 100, 200, and 500. All of the incubations were 60 minutes long. Due to TPEN’s nature of being soluble in oil, exposure was carried out in ~1mL wells (Nunc International, Rochester, NY). Prior to and after exposure, all MII oocytes were incubated at 37°C supplemented with 5% CO\(_2\) in Ca\(^{2+}\) containing CZB. Oocytes activated with 10mM strontium chloride (SrCl\(_2\)) in Ca\(^{2+}\) free CZB media supplemented with 0.1% PVA were utilized as positive controls. Treatment with DMSO was used as a negative control. Activation success rates were scored based on the appearance of the following activation events and embryo development: 2\(^{nd}\) polar body extrusion, pronuclear (PN) formation, 2-Cell, Morula, and Blastocyst. Oocytes and zygotes were observed under a Nikon Diaphot scope. Statistical comparisons were performed using Chi-square analysis.

2.4 Ca\(^{2+}\) Monitoring
For Ca\(^{2+}\) visualization during monitoring, the Ca\(^{2+}\) sensitive Fura-2-acetyoxymethyl (Fura 2AM) ester was utilized (Gryniewicz et al., 1985). The dye was loaded into oocytes by incubation with a mixture of 1.25\(\mu\)M Fura-2AM + 0.02% pluronic acid (Molecular Probes, Eugene, OR) for 20 minutes at room temperature in a dark room due to the photosensitivity of the dye. Recording of Ca\(^{2+}\) responses was performed as previously described (Wu et al., 1998; Gordo et al., 2002). Oocytes were placed in drops of TL-HEPES without fetal calf serum (FCS) to allow oocytes to adhere to the glass-bottomed dish (MatTek Corp, Ashland, MA). Fluorescence ratios of 340/380nm were obtained every 20 seconds. UV light was provided via a 75W Xenon arc lamp and modulated by neutral density filters. The emitted light above 510nm was collected by a cooled Photometrics SenSys CCD camera (Roper Scientific, Tucson, AZ). [Ca\(^{2+}\)] changes and filter wheel changes were captured and controlled using NIS-Elements software (Nikon Instruments Inc., Melville, NY) unless stated otherwise. The collected wavelengths were compiled in a 340/380nm ratio sequence and plotted on a line graph for further analysis.

[Ca\(^{2+}\)]\(_{ER}\) content was estimated after addition of the sarco/endoplasmic reticulum (SERCA) pump inhibitor, Cyclopiazonic Acid (CPA, Sigma; Lawrence and Cuthbertson, 1995). 20\(\mu\)M CPA was added to a 200\(\mu\)L dish of media containing the oocytes. The oocytes rested on a glass-bottomed monitoring dish and CPA was added five minutes after the initiation of monitoring. The monitoring media consisted of nominal Ca\(^{2+}\) free TL-HEPES with 100\(\mu\)M IBMX and no FCS. Ca\(^{2+}\) responses were scored by comparing the amplitude for each test group. Ca\(^{2+}\) influx after use of CPA was induced via the addition of 5mM CaCl\(_2\) and after basal Ca\(^{2+}\) values had returned to normal after addition
of CPA. The monitoring media used was FCS free TL-HEPES supplemented with 1mM EGTA and 100µM IBMX. The CaCl$_2$ was added five minutes after the start of monitoring. Ca$^{2+}$ responses were tabulated via the quantity of oocytes responding to the treatment as well as the magnitude of the response.

Total oocyte [Ca$^{2+}$] was visualized by the addition of Ionomycin, a Ca$^{2+}$ ionophore. 2.5µM Ionomycin was added to oocytes five minutes after the start of monitoring. The monitoring media consisted of FCS free TL-HEPES supplemented with 1mM EGTA and 100µM IBMX. Ca$^{2+}$ responses were scored by comparing the amplitude for each TPEN concentration group.

Ca$^{2+}$ oscillations were induced via the addition SrCl$_2$. The monitoring media, containing nominal Ca$^{2+}$ free TL-HEPES with IBMX and no FCS, was supplemented with 10mM SrCl$_2$ from the start. Ca$^{2+}$ responses were tabulated via the quantity of oocytes responding to the treatment as well as the magnitude of the responses.

2.5 TPEN Exposure and effects of Ca$^{2+}$ homeostasis

GV and MII oocytes were exposed to either 100 or 500µM TPEN, depending on experimental conditions. Oocytes were incubated in 1mL CZB containing 0.1% PVA, 1mM CaCl$_2$, and then with the indicated TPEN concentration. The media was not covered in oil due to TPEN’s ability to dissolve into the oil. Thus, a four well dish was used to house the media and oocytes. The incubation lasted for a total of thirty minutes. Ten minutes of the total time took place at 37°C with an environment of 5% CO$_2$. The remaining twenty minutes occurred while oocytes were being loaded with Fura 2AM in Ca$^{2+}$ containing TL-HEPES w/ 5% FCS at room temperature as mentioned above.
2.6 Statistical Analysis

Data were collected from two or more experiments performed on different days to
determine statistical significance. At least 20 eggs per treatment were used to perform
statistical analysis. The data were graphed and analyzed using Graphpad’s Prism
software (GraphPad Software, Inc. LA Jolla, CA). Depending on the experiment, either
the Student t-test or chi-square analysis was performed and values are shown as mean ±
SEM. Statistical significance was considered at P values <0.05.
CHAPTER 3

RESULTS

3.1 Parthenogenetic Activation by TPEN

The first step of this study was to determine the suitable concentrations of TPEN that oocytes could tolerate. This allowed for maximum potential Ca\(^{2+}\) chelation via TPEN, which was the original goal of this study, with as little side effects as possible. Because TPEN exposure can activate eggs, something that is easily observable, we chose to use this method to ascertain whether the concentrations that we used did not harm the eggs. As it can be seen in Table 1, exposure to 100µM TPEN produced the highest activation rates in regards to 2\(^{nd}\) Polar Body (93.5 ± 3.3) and PN (88.9 ± 5.6) formations. The highest concentration tested was 500 µM, which while still caused egg activation, it did so at lower rates. However, the morphology of the TPEN treated eggs at any of the concentrations tested was similar to those treated with SrCl\(_2\) (Fig. 1), suggesting that they could all be used to test the chelating capacity of TPEN on ER Ca\(^{2+}\). The generated zygotes cleaved to the 2-cell stage, although we did not evaluate for pre-implantation development.

3.2 TPEN does not induce Ca\(^{2+}\) release

If TPEN were to be a suitable ER-Ca\(^{2+}\) chelating tool, it would be desirable that it would not cause Ca\(^{2+}\) release upon exposure. As can be seen in Fig. 2A, addition of TPEN (n=12) or DMSO (n=11) in Ca\(^{2+}\) containing media not only failed to increase Ca\(^{2+}\) baseline levels in MII eggs, but may even have caused a slight reduction in basal Ca\(^{2+}\) levels. Importantly, when the same procedure was performed in nominal Ca\(^{2+}\) free
conditions (Fig. 2B), the addition of TPEN (n=12) caused a marked decrease in basal 
Ca\textsuperscript{2+} levels, which did not recover over time, while the control group (n=7) did not show 
any changes in Ca\textsuperscript{2+} baseline levels upon addition of DMSO. Thus, the fall in baseline 
levels when TPEN was added may indicate that TPEN is sequestering intracellular Ca\textsuperscript{2+} and that in the absence of Ca\textsuperscript{2+} influx, this causes a sustained drop in basal Ca\textsuperscript{2+}.

Following up the MII data above, the role of TPEN stimulation on Ca\textsuperscript{2+} changes 
in GV oocytes was examined. In Ca\textsuperscript{2+} containing media (Fig. 2C), the addition of TPEN 
(n=36) resulted in a brief Ca\textsuperscript{2+} spike of varying amplitude lasting only a couple of 
minutes before returning to baseline levels for the remainder of the monitoring period, 
while the addition of DMSO alone (n=16) failed to produce any changes in Ca\textsuperscript{2+} levels. 
In nominal Ca\textsuperscript{2+} free media (Fig. 2D), as shown in MII eggs, the addition of TPEN 
(n=13) caused a drop in basal Ca\textsuperscript{2+}, although DMSO (n=21) did not cause any changes in 
basal Ca\textsuperscript{2+} levels.

To ascertain if the increase in basal Ca\textsuperscript{2+} caused in GV oocytes by addition of 
TPEN in Ca\textsuperscript{2+} containing media was due to Ca\textsuperscript{2+} influx, the experimental conditions were 
repeated but the media was supplemented with 100µM 2-APB. 2-APB is a plasma 
membrane Ca\textsuperscript{2+} channel blocker (Bootman et al., 2002) and the expectation was that if 
the Ca\textsuperscript{2+} rise is due to influx, the presence of the inhibitor will prevent the rise. In the 
presence of 2-APB (Fig. 2E), addition of TPEN (n= 36) did not cause a Ca\textsuperscript{2+} spike and 
further, following its addition, an immediate drop in baseline was observed, which was 
similar to the effect caused by addition of TPEN to GV oocytes in nominal Ca\textsuperscript{2+} free 
media (Fig. 2D). Together, these results show that TPEN has an effect on Ca\textsuperscript{2+} 
homeostasis and that the effects are very different between GV and MII oocytes.
3.3 TPEN affects Ca\textsuperscript{2+} ER content

The previous results suggested that TPEN had an effect on some aspect of Ca\textsuperscript{2+} homeostasis. To test whether TPEN was able to affect the Ca\textsuperscript{2+} content of the ER in both MII (Fig. 3A,B) and GV (Fig. 3C,D) oocytes, oocytes at these stages were incubated in TPEN for 30 minutes prior to being monitored for Ca\textsuperscript{2+} responses induced by 20µM CPA, a SERCA inhibitor. Monitoring was performed in nominal Ca\textsuperscript{2+} free media to prevent Ca\textsuperscript{2+} influx. TPEN treated MII oocytes (Fig. 3E) displayed lower Ca\textsuperscript{2+} amplitudes (p < 0.05) and longer Ca\textsuperscript{2+} release durations (p < 0.05) compared to controls. Interestingly, the time from the baseline to the peak of the Ca\textsuperscript{2+} response was significantly longer when the eggs were treated with TPEN. From these data it can be concluded that while TPEN may not be chelating the ER Ca\textsuperscript{2+}, it does have an effect on the speed at which the Ca\textsuperscript{2+} is released. This could occur from a possible interaction of TPEN with the SERCA pump.

GV oocytes, on the other hand, show surprising results (Fig. 3F). The amplitude of release was significantly higher in the 100µM TPEN group than in the control group while the reverse was true for the 500µM TPEN group. Similarly to their MII counterparts, TPEN caused significantly longer Ca\textsuperscript{2+} release events than DMSO. Unlike its MII counterpart, 100µM TPEN treated GV oocytes yielded a slight increase in Ca\textsuperscript{2+} store content. This can further be explained via the direct result of a longer duration and larger amplitude of Ca\textsuperscript{2+} response. Strangely, this trend was not seen in the 500µM TPEN treated group as no significant distinction between TPEN and control was observed. As seen in the MII group above, TPEN treatment appeared to result in a longer
time from baseline to peak of the Ca\textsuperscript{2+} response. This affect was not absolute as it was significant for the 100\textmu M TPEN group but not the 500\textmu M TPEN group.

3.4 TPEN affects cell Ca\textsuperscript{2+} content in oocytes and eggs

As CPA induces release of Ca\textsuperscript{2+} from the ER, it was our interest to estimate the impact on the whole Ca\textsuperscript{2+} content in the cell. To do this, we used Ionomycin (IO), a Ca\textsuperscript{2+} ionophore that in the absence of external Ca\textsuperscript{2+} causes a Ca\textsuperscript{2+} release from all internal stores. Oocytes were incubated in TPEN as indicated before, and then exposed to 2.5\textmu M IO. The effects were most discernible in MII oocytes (Fig. 4A,C) where it caused a release of smaller amplitude, shorter duration, and shorter time from baseline to peak than DMSO groups. Together these data indicated that TPEN does in fact soak up Ca\textsuperscript{2+} from some location within the MII oocyte.

In GV oocytes (Fig. 4D), on the other hand, pre-treatment with TPEN seemed to increase the response to IO, at least in amplitude.

3.5 TPEN and Ca\textsuperscript{2+} influx

We next examined whether incubation in the presence of TPEN promoted Ca\textsuperscript{2+} influx both in MII or GV oocytes following addition of CaCl\textsubscript{2}. The addition of CaCl\textsubscript{2} to DMSO (n = 13) treated MII oocytes did not promote Ca\textsuperscript{2+} influx, although a minor increase was observed in TPEN-treated oocytes (n = 10) (Fig. 5A). In GV oocytes however, the effect where not as clear, as in either group a few oocytes showed Ca\textsuperscript{2+} influx following addition of 5mM CaCl\textsubscript{2} (Fig. 5B). Nevertheless, the proportion of
oocytes showing influx was similar and it is known that GV oocytes influx Ca^{2+} at higher rates than in MII oocytes.

3.6 SrCl$_2$ induced oscillations are inhibited by incubation in TPEN

GV and MII oocytes are known to display oscillations, stemming from the ER, when exposed to SrCl$_2$-containing media (Kline and Kline, 1992; Bos-Mikich et al., 1995). SrCl$_2$ induced oscillations also have the ability of inducing activation in MII oocytes, thus lifting the meiotic arrest. To further characterize TPEN’s effect on Ca$^{2+}$, the metal chelator was added while in the presence of SrCl$_2$. In both GV (n = 45, Fig. 6A) and MII (n = 40, Fig. 6B) groups exposed to TPEN, oscillations were not observed. The baseline for each group remained steady and did not deviate for the entirety of the monitoring process. The DMSO groups on the other hand, displayed characteristic oscillations, although not all oocytes at either of the stages showed oscillations, which is expected (Fig. 6A, B). Further, the nature of the Ca$^{2+}$ oscillations differed between the two oocyte states, with GV oocytes consistently producing oscillations of smaller amplitude and rapid start, while MII oocytes displayed delayed oscillations and of higher amplitudes.
CHAPTER 4

DISCUSSION

Past research has identified TPEN as both a zinc chelating agent able to induce parthenogenetic activation in MII eggs (Suzuki et al., 2010) as well as to chelate Ca\(^{2+}\) in intracellular organelles in various cell types (Hofer et al., 1998; Arnaudeau et al., 2002). In this current study, TPEN was assessed as a potential ER Ca\(^{2+}\) chelating candidate in mouse oocytes. Due to the vast differences in Ca\(^{2+}\) homeostasis between eggs and oocytes (Jones et al., 1995a; Kline, 2000), both MII and GV oocytes were utilized for this study. Based on the resulting Ca\(^{2+}\) profiles from TPEN incubations and Ca\(^{2+}\) oscillations, it appears that TPEN is not effective at completely chelating ER Ca\(^{2+}\) stores in MII and GV oocytes. Thus, while some of the results from both groups reveal interesting effects on Ca\(^{2+}\) homeostasis, the lack of consistency challenges the reliability of using TPEN as a Ca\(^{2+}\) chelating tool.

While studies of Ca\(^{2+}\) homeostasis in mouse oocytes have relied on the use of SERCA inhibitors such as thapsigargin and CPA to empty the Ca\(^{2+}\) content of the ER (Jones et al., 1995b; Lawrence and Cuthbertson, 1995), these products in addition to emptying [Ca\(^{2+}\)]\(_{\text{ER}}\) also cause a Ca\(^{2+}\) rise. Thus, we became interested in looking for a product that could chelate Ca\(^{2+}\) without increasing cytosolic Ca\(^{2+}\). TPEN has been used in mouse eggs and Xenopus oocytes to study effects on Ca\(^{2+}\) homeostasis (Lawrence et al., 1998; Yao et al., 1999), although the effects on [Ca\(^{2+}\)]\(_{\text{ER}}\) in mouse oocytes were not carefully examined. Therefore, before proceeding with ER Ca\(^{2+}\) chelation studies, we performed parthenogenetic activation tests as recently carried out by Perry and colleagues (Suzuki et al., 2010). In performing this, a wide range of TPEN concentrations were
tested for activation rates and developmental competency. While the resulting activation rates were not as high as those reported by Suzuki et al. (2010), the data show that MII eggs are both activated by TPEN and able to survive through the PN stage of development. For the purpose of this study, development rates past the PN stage were not of interest. From the resulting activation data, it was determined that both 100 and 500µM TPEN concentrations were suitable for use throughout the duration of the study, as they both induced high activation rates, as measured by 2nd PB extrusion and PN formation and did not cause high rates of fragmentation or cell death.

Our TPEN studies reveal a broad spectrum of effects in oocytes and eggs that complicates its use as a [Ca^{2+}]_{ER}-chelating agent. Nonetheless, several interesting results were observed that provide insight into some of the mechanisms involved in Ca^{2+} homeostasis in oocytes and eggs. The most striking early effect was the finding that while in MII eggs addition of TPEN into Ca^{2+} containing media did not cause an obvious change in baseline values, its addition into GV oocytes caused an immediate Ca^{2+} rise. These results were unexpected and have not been reported in the literature thus far. Further, we show that the increase is due to Ca^{2+} influx from the extracellular media, as it was inhibited by addition of 2-APB or did not occur in the absence of external Ca^{2+}. While the channels that mediate this influx were not determined here, APB has been shown to inhibit the conductivity of TRP (Clapham et al., 2001) or ORAI 1 (DeHaven et al., 2008; Schaff et al., 2010) channels, and it is therefore possible that one or both of these channels is mediating the influx caused by TPEN. In this regard, it is possible that the addition of TPEN may disturb, or reduce, intracellular levels of Mg^{2+}, an effect that has been shown to activate TRPM7 channels, which can conduct Ca^{2+} (Nadler et al.,
2001; Schmitz et al., 2003). In support of this possibility, our unpublished studies found that mouse oocytes express transcripts of this channel, although the presence of protein has not been tested. It is also worth noting that in the absence of extracellular Ca$^{2+}$, addition of TPEN induces a notable decrease in basal Ca$^{2+}$ levels in both GV oocytes and MII eggs, suggesting that indeed TPEN does affect Ca$^{2+}$ homeostasis. Whether this is due to directly chelating free Ca$^{2+}$ in the cytosol, which seems unlikely due to the low affinity of TPEN for Ca$^{2+}$, or due to the lowering of Ca$^{2+}$ in the ER, which then draws Ca$^{2+}$ from the cytosol to the ER lowering the cytosolic concentration of Ca$^{2+}$ is unknown. Regardless, a very low but persistent Ca$^{2+}$ influx associated with the addition of TPEN may be offsetting this chelation, as Ca$^{2+}$ basal levels are seemingly unaltered in MII eggs cultured in Ca$^{2+}$-containing media and in the presence of TPEN.

The effects of TPEN on [Ca$^{2+}$]$_{ER}$ levels and intracellular cell Ca$^{2+}$ were unexpected and reflect the uniqueness of the mouse oocyte as a model. Addition of TPEN either to GV or MII oocytes greatly decreased the amplitude of the Ca$^{2+}$ rise induced by addition of CPA, suggesting that TPEN may lower [Ca$^{2+}$]$_{ER}$, which is consistent with its possible role in chelating Ca$^{2+}$ in this organelle; this was especially so in MII eggs. Further, the findings that time to peak from baseline was significantly longer in TPEN-treated oocytes also seems to support this notion. Nevertheless, these results were obscured, at least in part, by the observation that the overall duration of the rise in TPEN-treated oocytes was longer. Nevertheless, TPEN has been suggested to inhibit SERCA in some studies involving somatic cells (Sztreye et al., 2009) and activate MAPK in others (Bernhardt et al., 2011; Kong et al., 2012), and these changes could alter Ca$^{2+}$ homeostasis masking some of the effects on TPEN on [Ca$^{2+}$]$_{ER}$. Remarkably,
following the addition of IO, while in GV stage oocytes TPEN seemingly increased 
$[\text{Ca}^{2+}]_{\text{ER}}$, it decreased it dramatically in MII eggs. Therefore, these results reflect great
differences in the mechanisms of Ca$^{2+}$ homeostasis between GV and MII eggs. Further,
the results demonstrate the likelihood of a certain degree of Ca$^{2+}$ chelation capacity of
TPEN in MII eggs, although given that the chelation is not absolute its use in mouse
oocytes for Ca$^{2+}$ studies seems limited.

Despite the ability to decrease $[\text{Ca}^{2+}]_{\text{ER}}$ in MII eggs, exposure to TPEN only
induced a minor Ca$^{2+}$ influx following CaCl$_2$ add back. In GV oocytes, while some
oocytes after TPEN exposure displayed a larger influx following CaCl$_2$ add back in GV
oocytes, a similar influx was also observed in DMSO treated oocytes, and this influx is
most likely associated with the more permeable GV stage than due to the treatment.
Overall, these results support our findings that, at the concentrations used, TPEN is not
able to fully deplete $[\text{Ca}^{2+}]_{\text{ER}}$, which might be required to induce substantial influx by
activating SOCE. Despite these mild effects on $[\text{Ca}^{2+}]_{\text{ER}}$, exposure to TPEN both in GV
and MII oocytes consistently inhibited oscillations induced by SrCl$_2$. In both stages,
none of the oocytes exposed to TPEN were able to mount Ca$^{2+}$ responses. SrCl$_2$ mediates
Ca$^{2+}$/SrCl$_2$ elevations from the ER and via IP$_3$R1. However, only optimal conditions
make possible oscillations by SrCl$_2$, as for instance older oocytes fail to oscillate.
Therefore, it is possible that the mild sequestration of Ca$^{2+}$/SrCl$_2$ in the ER disturbs IP$_3$R1
sensitivity compromising the ability to mount oscillations. The other possibility is that
TPEN may be chelating SrCl$_2$ as it enters the cell, effectively reducing cytoplasmic levels
of SrCl$_2$, which then fail to notably induce Ca$^{2+}$ responses. Additional studies are needed
to resolve this question. Additional studies should examine whether this effect of TPEN is reversible and also if it blocks PLCzeta-initiated oscillations.

The goal of this study was to identify the heavy metal chelator, TPEN, as an ER Ca\textsuperscript{2+} chelating tool in mouse/mammalian oocytes. Data collected from both GV and MII oocytes matured in different degrees of TPEN resulted in only minor ER Ca\textsuperscript{2+} chelation, as estimated by the Ca\textsuperscript{2+} release caused by addition of CPA; this effect was more significant in MII eggs than in GV oocytes. However, at the concentrations required to cause some of these effects, TPEN may be affecting the concentration of other ions and different molecules that also impact Ca\textsuperscript{2+} homeostasis, complicating the interpretations of its effects. Thus, while TPEN seems a useful tool to induce parthenogenetic development in mammalian oocytes, it’s possible application as a Ca\textsuperscript{2+} chelating tool is not promising.
CHAPTER 5

FIGURE LEGENDS

Table 1. TPEN induces activation of MII oocytes

MII oocytes were exposed to TPEN for 60 minutes in Ca\(^{2+}\)-containing CZB media supplemented with 0.1% PVA, after which, they were transferred to CZB media without TPEN. Of all the TPEN concentrations tested, 100µM TPEN consistently resulted in the highest development rates.

Figure 1. TPEN induces activation of MII oocytes

MII oocytes exposed to TPEN and SrCl\(_2\) through the PN stage underwent normal egg activation events without obvious morphological abnormalities; exposure to DMSO failed to induce egg activation. Both TPEN and SrCl\(_2\) treated oocytes displayed 2nd Polar Bodies and PN formations clearly.

Figure 2. Addition of TPEN to oocytes effects Ca\(^{2+}\) homeostasis.

The Ca\(^{2+}\) profiles of both GV and MII oocytes were monitored during addition of 100µM TPEN either in the presence or absence of Ca\(^{2+}\). The response observed immediately following the addition of TPEN and DMSO is enlarged in a panel to the right of each figure for clearer viewing. (A) TPEN added to MII oocytes in Ca\(^{2+}\)-containing CZB media and (B) to control MII oocytes in nominal Ca\(^{2+}\) free media. (C) TPEN added to GV oocytes in Ca\(^{2+}\)-containing media and to (D) GV oocytes in nominal Ca\(^{2+}\) free CZB media. (E) TPEN added to GV oocytes in Ca\(^{2+}\)-containing CZB media supplemented with 100µM 2-APB to block potential Ca\(^{2+}\) influx. Titles with (*) indicate that the graph
was plotted using the software Simple PCI (C-Imaging System, Cranberry Township, PA).

**Figure 3. Effects of CPA-induced Ca\(^{2+}\) release in MII and GV oocytes.**

MII and GV oocytes were pre-incubated for 30 minutes in Ca\(^{2+}\) containing CZB w/(0.1% PVA) supplemented with either 100 or 500µM TPEN followed by Ca\(^{2+}\) monitoring with the same TPEN concentration in nominal Ca\(^{2+}\) free TL-HEPES. A Ca\(^{2+}\) release was induced via 20µM CPA. (A) Resulting Ca\(^{2+}\) profiles from MII oocytes incubated in 100µM TPEN. (B) Resulting Ca\(^{2+}\) profiles from MII oocytes incubated in 500µM TPEN. (C) Graphical representation of the resulting Ca\(^{2+}\) profiles from GV oocytes incubated and exposed to 100µM TPEN. (D) Graphical representation of the resulting Ca\(^{2+}\) profiles from GV oocytes incubated and exposed to 5000µM TPEN. From the resulting calcium profiles, the amplitude of the release, duration of release, and the time from baseline to peak release were tabulated for both MII (E) and GV (F) oocytes. Bars with ** above them are statistically significant from those without them.

**Figure 4. Effects of TPEN on IO-induced Ca\(^{2+}\) release in MII and GV oocytes.**

MII and GV oocytes were pre-incubated in Ca\(^{2+}\) containing CZB w/(0.1% PVA) supplemented with 500µM TPEN for 30 minutes followed by Ca\(^{2+}\) monitoring with the same TPEN concentration in nominal Ca\(^{2+}\) free TL-HEPES. A Ca\(^{2+}\) release profile was induced via the addition of 2.5µM IO. (A) Resulting Ca\(^{2+}\) profile from MII oocytes incubated in 500µM TPEN. (B) Resulting Ca\(^{2+}\) profiles from MII oocytes incubated in 500µM TPEN. From the resulting Ca\(^{2+}\) profiles, the amplitude of the release, duration of
release, and the time from baseline to peak release were tabulated for both MII (C) and GV (D) oocytes. Bars with ** above them are statistically significant from those without them.

**Figure 5. TPEN exposure does not induce Ca\(^{2+}\) influx**

Both GV and MII oocytes were pre-incubated in Ca\(^{2+}\) containing CZB w/(0.1% PVA) supplemented with 500µM TPEN for 30 minutes followed by Ca\(^{2+}\) monitoring in the same TPEN concentration and in the absence of CaCl\(_2\) supplementation. 5mM CaCl\(_2\) was added to the oocytes with the intent of inducing a Ca\(^{2+}\) influx profile. (A) Resulting Ca\(^{2+}\) influx profile from GV oocytes incubated in 500µM TPEN. The response immediately following the addition of 5mM CaCl\(_2\) is enlarged and projected into a right panel for clearer viewing. (B) Resulting Ca\(^{2+}\) influx profile from MII oocytes incubated in 500µM TPEN. A representative Ca\(^{2+}\) trace for both the TPEN and DMSO conditions is extrapolated and displayed to the right for clearer viewing.

**Figure 6. SrCl\(_2\)-induced oscillations are blocked by incubation in TPEN**

Both GV and MII oocytes were pre-incubated in Ca\(^{2+}\) containing CZB w/(0.1% PVA) supplemented with 500µM TPEN for thirty minutes followed by Ca\(^{2+}\) monitoring in the same TPEN concentration. The monitoring dish was supplemented with 10mM SrCl\(_2\) and without the presence of CaCl\(_2\) from the start of monitoring with the intent of inducing oscillations immediately after the exposure to SrCl\(_2\). (A) Resulting Sr\(^{2+}\)/Ca\(^{2+}\) profiles from GV oocytes incubated in 500µM TPEN. (B) Resulting Sr\(^{2+}\)/Ca\(^{2+}\) profiles from MII oocytes incubated in 500µM TPEN.
### Table 1

<table>
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<th>Treatments</th>
<th># of samples</th>
<th>2(^\text{nd}) PB Formation</th>
<th>PN Formation</th>
<th>2-Cell</th>
<th>Morula</th>
<th>Blastocyst</th>
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<td>20 µM</td>
<td>22</td>
<td>86.4</td>
<td>72.7</td>
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<td>50 µM</td>
<td>76</td>
<td>91.3 ± 4.8</td>
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<td>69.5 ± 8.6</td>
<td>13.6 ± 4.5</td>
<td>8.4 ± 3</td>
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<tr>
<td>100 µM</td>
<td>78</td>
<td>93.5 ± 3.3</td>
<td>88.9 ± 5.6</td>
<td>77.3 ± 4.1</td>
<td>21.8 ± 6.4</td>
<td>11.6 ± 3.2</td>
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<tr>
<td>200 µM</td>
<td>57</td>
<td>75.6 ± 11.9</td>
<td>72 ± 11.4</td>
<td>63.6 ± 3</td>
<td>7.2 ± 1.1</td>
<td>3.6 ± 0.6</td>
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<tr>
<td>500 µM</td>
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<td>68.2</td>
<td>59.1</td>
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<td>0</td>
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<td>SrCl(_2)</td>
<td>68</td>
<td>24 ± 5</td>
<td>86.9 ± 1.6</td>
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<tr>
<td>DMSO</td>
<td>58</td>
<td>0</td>
<td>1.7 ± 1.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</table>
Figure 1

TPEN  
DMSO  
SrCl₂
Figure 2

A. MII 100µM TPEN

B. MII 100µM TPEN w/o Ca²⁺

C. GV 100µM TPEN

D. GV 100µM TPEN w/o Ca²⁺

E. GV 100µM TPEN

Legend:
- TPEN (n = 12)
- DMSO (n = 11)
- TPEN (n = 12)
- DMSO (n = 7)
- TPEN (n = 67)
- DMSO (n = 22)
- TPEN (n = 13)
- DMSO (n = 21)
- TPEN (n = 67)
- TPEN-APB (n = 36)
Figure 3

A  
MII  
100µM TPEN / DMSO  
CPA  
TPEN(n = 19)  
DMSO(n = 19)

B  
MII  
500µM TPEN / DMSO  
CPA  
TPEN(n = 15)  
DMSO(n = 14)

C  
GV  
100µM TPEN / DMSO  
CPA  
TPEN(n = 23)  
DMSO(n = 23)

D  
GV  
500µM TPEN / DMSO  
CPA  
TPEN(n = 22)  
DMSO(n = 27)

E  
Amplitude  
MII  
Duration  
TPEN  
DMSO

F  
Amplitude  
GV  
Duration  
TPEN  
DMSO

Time to Peak  
TPEN  
DMSO

GV  
TPEN  
DMSO
Figure 4

A  

MII

Time (min)

F340 / F380

0.0 0.5 1.0 1.5

500µM TPEN / DMSO

Ionomycin

TPEN(n=16)

DMSO(n=19)

B  

GV

Time (min)

F340 / F380

0.0 0.5 1.0 1.5

500µM TPEN / DMSO

Ionomycin

TPEN(n=28)

DMSO(n=27)

C  

Amplitude

MII

Duration

Time to Peak

D  

Amplitude

GV

Duration

Time to Peak

Amplitude

500TPEN

F340 / F380

0.0 0.2 0.4 0.6 0.8 1.0

(20)

(18)

Duration

500TPEN

Time (min)

0 10 20 30 40

(27)

(33)

Duration

500TPEN

Time (min)

0 5 10 15 20 25

(27)

(33)

Time to Peak

500TPEN

Time (min)

0 2 4 6 8 10

(20)

(18)

(33)

(27)
Figure 5

A

MII

500 µM TPEN / DMSO
5 mM Ca²⁺

F₃₄₀ / F₃₈₀

Time (min)

TPEN (n = 10)
DMSO (n = 13)

B

GV

500 µM TPEN / DMSO
5 mM Ca²⁺

F₃₄₀ / F₃₈₀

Time (min)

TPEN (n = 12/25)
DMSO (n = 14/23)
Figure 6

**MII**

**A**

<table>
<thead>
<tr>
<th>500μM TPEN / DMSO</th>
<th>10mM SrCl₂</th>
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</thead>
</table>

- TPEN(n = 0/40)
- DMSO(n = 17/34)

**GV**

**B**

<table>
<thead>
<tr>
<th>500μM TPEN / DMSO</th>
<th>10mM SrCl₂</th>
</tr>
</thead>
</table>

- TPEN(n = 0/45)
- DMSO(n = 28/52)
REFERENCES


