THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF BIOLOGY

THE ROLE OF HEME IN PLANT CHANNEL SKOR AND GORK VOLTAGE GATING

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ABSTRACT

Heme, an iron containing compound with a surrounding porphyrin ring, is important in biological processes. Heme can bind to a protein to have a large effect on its structure and activity. The biological role of heme extends to ion channel proteins, where the specific molecular mechanisms and sites by which heme affects the opening probability of an ion channel are unknown. GORK channels, or Guard cell outward rectifying K+ channels, are cyclic nucleotide binding domains that will be used to mechanistically understand this process. Guard cell regulation is important to understand because it greatly affects water use efficiency, photosynthesis, and stress tolerances. The mechanism by which heme can modulate *Arabidopsis thaliana* plant guard cell ion channels which are responsible for opening and closing stomata was investigated via voltage clamped electrophysiological recordings in *Xenopus Laevis* oocytes. The process by which redox factors provided by oxidizing environmental agents affect channel gating in relation to heme will also be similarly investigated. Additionally, the mechanism via which heme directly affects voltage gating will be investigated through voltage fluorometry. Voltage fluorometry allows us to directly measure voltage sensor movements independent of the channel opening by fluorescently tagging the voltage sensors and examining their movements during recordings with voltage changes as changes in fluorescence.

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Chapter 1: Introduction

Stomatal Regulation via K+ ion Channels

Plant ion channel gating affects a variety of plant functions, such as photosynthesis, respiration, and transpiration (Jegla, et al., 2009). K+ ion channels are responsible for controlling many of these functions via controlling stomatal aperture. Photosynthesis, where oxygen, sugars, and water are created from carbon dioxide and light energy, largely involves the stomata. The stomata in plants are responsible for controlling the intake of carbon dioxide and expulsion of water waste necessary for photosynthesis. The stomata also release oxygen created during photosynthesis into the environment (McAnish, et al., 1996). This gas exchange needs to occur because a lack of carbon dioxide or a buildup of water could inhibit the photosynthesis processes which provides the plant with sugars to break down during respiration. This gas exchange occurs through a pore found underneath the leaves of plants. The pore is opened and closed depending on the guard cells surrounding it. Guard cells pairs flank each stomata and when the guard cells are swollen, they swell larger, opening a pore between them where the stomata can found open. Open stomata allow for the uptake of carbon dioxide discussed earlier. When the guard cells are shriveled, the stomata are closed (Sierla, et al., 2016). Closed stomata prevent excess water loss.

Stomatal opening balances the need for carbon dioxide with the need for water. During extreme weather conditions such as a drought, the stomata may be pushed to stay closed, preserve any excess water, and avoid cellular dehydration (Leing and Giraudat, 1998). When the stomata are closed, gaseous exchange also decreases, causing a decrease in photosynthesis as

well (Singh, et al., 2019). During growth, where sugars and energy will be necessary, the stomata may be encouraged to open in order to intake more carbon dioxide for photosynthesis. It is the regulation of the guard cells, specifically the guard cell channels that cause for a turgid or shriveled state, that controls stomatal opening and closing (Sierla, et al., 2016). Further understanding on how these guard cell channels work helps increase understanding of stomatal impact on drought tolerances and general photosynthesis. Different stresses, such as drought, increased atmospheric carbon dioxide, or heat waves can all directly affect the gas exchange needs in a plant (Leing and Giraudat, 1998). More information of guard cell channel regulation can increase knowledge on adapting crops to the stresses of adverse climate conditions that the agricultural industry faces (Munemasa, et al., 2013). Therefore, understanding the mechanisms behind the gating of guard cell channels which control stomatal gas exchange in response to environmental pressures is valuable in practice.

CNBD Channels

Guard cell gating involves CNBD channels, or cyclic nucleotide binding domain channels. CNBD channels are a cation channel superfamily that are very distantly related to Shaker channels (Jegla et al., 2018). CNBD channels are in animals, plants, and prokaryotes. The CNBD channels relevant in guard cell regulation in this study include Guard cell outward rectifying K+ channel (GORK), Stelar K+ outward rectifying channel (SKOR), and KAT1/KAT2 channels. They are all transmembrane tetrameric channels with a voltage sensing domain and a pore domain (Ache, et al., 2000; Jegla et al., 2018). These voltage gated potassium

channels directly control stomatal aperture via controlling guard cell size. We will be investigating their functional gating patterns in order to gain information directly related to guard cell gating. The CNBD channel lineage has already been investigated in this lab, particularly the animal lineage. Using the heme modulated gating information found in animal CNBD channels, we hope to investigate and relate that information to these plant CNBD family channels.

GORK and SKOR are outward K+ rectifying channels, meaning when they are open there is an efflux of K+. The efflux of K+ drives osmotic water flux out as well, causing the guard cells controlled by GORK to shrink into the open pore, creating a closed stoma (Hosy et al., 2003). SKOR is not expressed in guard cells however it functions similarly to GORK. KAT1/KAT2 channels are inward K+ rectifying channels, meaning when they open there is an influx of K+ (Ache, et al., 2000). The K+ influx drives osmotic water flux in as well, swelling the guard cell pairs and causing the stomatal pore to open. This study investigates the regulation of these K+ channels in relation to stomatal opening and closing, which balances energy production with water loss.

SKOR channels are chosen during the experiment to be the channel of focus due to their strong expression. SKOR channels are expressed in the xylem parenchyma of the plant vascular system roots. SKOR can be activated via oxidation with an affected S3 cysteine residue, shared by GORK but not KAT1/KAT2 (Johansson, et al., 2006). As stated earlier, these CNBD family channels have a voltage sensing domain and a pore domain. The transmembrane domains are outlined in Figure 1. The voltage sensor domain involved the four transmembrane domains S1-S4 where S4 primarily senses the voltage gating charges. The pore domain involves the two transmembrane domains S5 and S6. The pore selectivity filter is formed between S5 and S6

(Jegla et al., 2018). These tetrameric CNBD family channels have with multiple possible heme binding sites to be investigated (Kalstrup and Blunck, 2017).

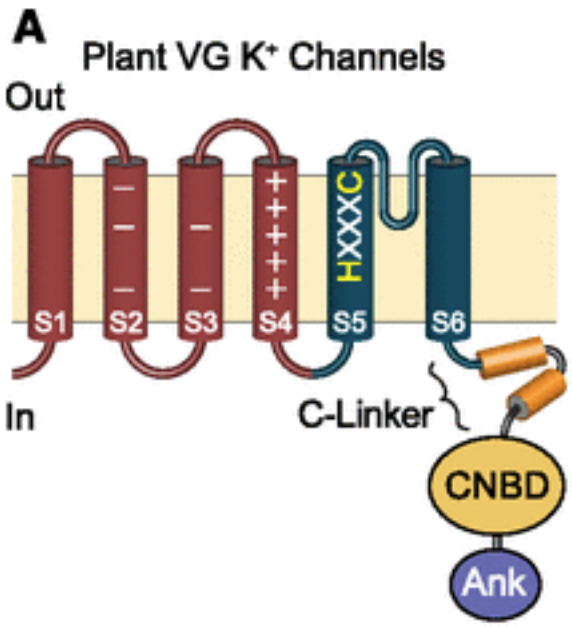

Figure 1: Channel schematic model of the plant CNBD voltage gated K+ channels (Jegla et al., 2018).

Heme

Heme is a compound consisting of a central iron ion coordinated to a surrounding porphyrin ring. Heme is present in all of the organisms containing CNBD channels in areas such as the electron transport chain, where it is a part of electron carriers such as cytochrome c (Hou, et al., 2006). In animals and plants, heme-ligand proteins are present throughout the cell (Burton, et al., 2016). For example, heme is well known as an oxygen carrier for hemoglobin in animals (Kosmachevskaya and Topunov, 2018). In plants, heme can fulfil other signaling roles as a redox regulator (Jaggar, et al., 2005; Singh, et al., 2019).

Heme can potentiate or inhibit channel voltage activation depending on the channel type (Hou, et al., 2006). In plants, heme can modulate the activity of plant channels via redox sensors. Heme is redox sensitive and binds environmentally found reactive oxygen species (ROS) gases (Jaggar, et al., 2005). Heme is known to bind to these ROS gases as either a means for direct channel regulation or as a redox sensor for these channels. Heme can alter channel voltage dependence via such redox sensitive actions. The direct mechanisms of which heme affects these CNBD channels is unknown, which this lab has a large interest in and will be investigated via voltage fluorometry.

Reactive oxygen species gases are known to promote stomatal closure and inhibit stomatal opening (Xie et al., 2014; Jaggar, et al., 2005). This means GORK would be potentiated and KAT1/KAT2 would be inhibited. This stomatal closure is influences by ROS because ROS causes an increase in cytosolic free Ca2+ via activating a Ca2+ channel in the guard cell plasma membrane, which is known to precede K+ efflux and further encourage stomatal closure (Munemasa, et al., 2013; Irving, Gehring, and Parish, 1992; McAnish, et al., 1996). However, the direct mechanisms through which the ROS promote stomatal closure are unclear. It is possible the heme could be an integrated sensor for these ROS and then the physiological effector in the binding pocket of the channel to affect its conductance.

Therefore, it is important to assess whether oxygen regulates these CNBD channel activities via heme. It is possible that heme could sense oxygen concentrations and assist in relaying the necessary functional messages to the guard cell channels or even act as the physiological effector itself (Burton, et al., 2016). Heme could therefore act as an environmental sensor and a physiological effector, which is different from other signal transduction cascade strategies (Jaggar, et al., 2005; Hou, et al., 2006). Heme would therefore allow for regulation of

stomata via K+ channel effectors, or the ROS environmental effectors. Heme would be a sensor that directly couples those environmental cues to cause changes in channel activity and affect stomatal opening. The mechanisms by which heme would regulate the K+ channels in CNBD channels is unclear and therefore needs to have its role as an integrated sensor for redox state or for gaseous signals investigated.

The most stable form of free heme is hemin, an oxidized form of heme. The hemin is a chloride salt which is dissolves in a base to make hematin, a hydroxide conjugated form used in the solutions during the voltage clamp experiments. Past research in this lab has been conducted relating heme with EAG family K+ channels, which are animal channels. Additional research in this lab was also conducted with these CNBD plant channels as well. This research demonstrated a hyperpolarizing effect on KAT1 K+ influx where the influx was inhibited by heme. Heme had a depolarizing effect on SKOR K+ efflux where the efflux was further activated by heme. The presence of heme therefore potentiated GORK channels and decreased KAT channels activation, which would favor closing the leaf pore. Using this prior knowledge of heme's effects on these channels, it is important to investigate whether heme maintains these effects with the presence of a ROS and the exact mechanisms by which heme does so.

Arabdiopsis as a model system and Xenopus as an oocyte system

The *Arabidopsis thaliana* plant was used as a model system in this experiment. The plant guard cell channel regulatory mechanisms that will be tested will be directly relevant to the functioning of these channels in plants, such as in this model system. Once the electrophysiological conclusions have been made, the experimental effects observed during

heme application could be investigate while the channels exist in the host plant as well and not simply in an oocyte. This would introduce other factors that have been isolated out when testing these isolated channels in *Xenopus* oocytes. *Arabidopsis* is a good choice because it is a standard model system with well established data (Leung and Giraudat, 1998). The genes encoding the CNBD guard cell K+ are already known and well documented, as well as ROS effects on them (Singh, et al., 2019).

The *Xenopus laevis* oocytes were used as the oocyte system for the electrophysiological recordings. Voltage clamped recordings could be carried out in the *Xenopus* oocytes by injecting the plant channel sequences into the eggs because these channels do not naturally express in *Xenopus*. Guard cell channels express at high rates in isolation, therefore using the *Xenopus* oocytes allows us to precisely hone in the direct effects of heme or other factors on the channel without any other confounding factors present (Vaid, et al., 2008; Kalstrup and Blunck, 2017; Singh, et al., 2019; Ache, et al., 2000). *Xenopus* oocytes are also a cost effective solution that already have suitable wild type expression vectors for the main three channel subunits used in this experiment. Essentially, the *Xenopus* oocytes allow us to effectively characterize site directed channel mutants via electrophysiology.

Experimental Aims and Hypotheses

The aim of this study is to determine whether or how heme regulates plant guard cell K+ channels, particularly with the channel responding to interacting ROS and heme. Heme application to these channels will affect current magnitude and voltage activation, both of which will be measured during voltage clamping procedures. The hypothesis is that oxidized heme will

potentiate GORK, considering heme has already been shown to potentiate SKOR and inhibit KAT1/KAT2 within this lab. GORK is also expressed in the guard cells while SKOR is not, therefore it is important to investigate GORK for the interest of this thesis. We will also investigate whether heme sensitizes GORK to gaseous messengers by applying a ROS to the oocyte expressed channels with prior heme application. The secondary hypothesis is therefore that the ROS effects on channel functions will be enhanced with prior heme application.

To investigate the mechanisms by which heme changes the gating of the voltage sensor in these channels, a voltage fluorometry set up will be required. Voltage fluorometry, or VCF, allows us to directly measure voltage sensor movements independent of the channel opening by fluorescently tagging the voltage sensors and examining their movements during voltage changes as changes in fluorescence (Kalstrup, et al., 2017). Heme affects the voltage sensor through one of three proposed mechanisms that we will investigate: heme either directly affects the four voltage sensor movements in the channel, stabilizes the already open channel after the voltage sensor movements are already activated, or acts independently of voltage gating entirely which is not what our previously collected functional data suggests. We can investigate these hypotheses through voltage fluorometry.

The fluorophore presence on the cysteine mutation in the voltage sensors will allow us to directly see any voltage sensor movement in and out of the channel. If heme is altering the kinetics or voltage range of delta F during channel activation, then heme is facilitating forward movement of the voltage sensors directly. If the fluorescence slows upon the return of the voltage sensors at the end of the voltage step, it means the heme is modulating the final stabilization of the channel. If heme doesn't change any of the fluorescence, then heme is independent of the voltage gating entirely. This will tell us about the mechanistic modulation of

heme on these channels, which we can continue to apply to our growing knowledge on how heme provides a pathway for different redox factors to also regulate guard cell channels.

His and Cys are the target residues at the voltage sensing site for the fluorophore to bind to in order to report movement as controlled and heme modulated gating commences (Zakany, et al., 2019; Es-Salah-Lamoureuz, et al., 2010). The aim towards the end of this project was to first create clones of the CNBD channels with the target His or Cys mutation incorporated in and then effectively characterize them with heme. The fluorophore will be binding to that incorporated Cysteine in order to report information the movement of the channel, therefore the mutation must be incorporated into the gene without affecting channel function. The voltage clamping electrophysiology methods that will be incorporating the mutated channels created in this experiment will measure the channel function in controlled and heme present cellular environments. The mutated channels must be proven to be functioning adequately, where they have similar controlled and heme modulated voltage gating as compared to their wild type counterparts. Once the similar gating modulation is confirmed, they can proceed the voltage fluorometry experiments in the future where similar voltage clamping electrophysiology will occur, all while concurrently observing the fluorophore movements.

Chapter 2 : Methods

Molecular Cloning

Molecular cloning procedures were used to generate the CNBD K+ channel expression vectors to put into the *Xenopus* oocytes. The GORK, SKOR, and KAT1/2 constructs had already been created and the past and were continually used and refilled as necessary. New mutant channel expression vectors had to be created for the VCF portion of the experiment. Five different mutations were introduced into the SKOR WT gene. One of the five vectors was L189C, a leucine mutated to a cysteine at amino acid number 189 in SKOR. The other vectors included G179C, V184C, L187C, and L188C.

The cloning procedure used the SKOR WT template from *Arabidopsis thaliana*. Primers were designed and ordered containing the desired mutation at the potential target heme interaction sites. The mutated vectors were introduced to the template sequence via PCR. The PCR was purified and then digested with DPN1 and Cutsmart 10X. The digests were introduced to Stellar competent cells via a heat shock method. The cells were plated and incubated for 18 hours at 37 ºC. The colonies were then picked, introduced to culture media, and miniprepped to extract the DNA from the cells. The DNA was digested again with XBAL, ECOR1-HF, and Custmart 10X. The sequences were then run on gels to confirm the size of the targeted mutants and make sure the primers attached where was desired. After the gels were confirmed, sequences were sent to Huck Life Science's Genomics Core Facility for verification. Figure 2 details the SKOR WT and L189C successful mutation site.

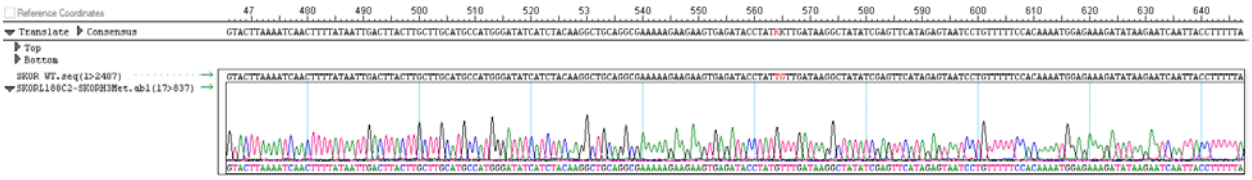

Figure 2: L189C mutation from SKOR wild type genome molecular cloning results

Electrophysiology

Voltage clamp recordings were used with both the GORK and VCF experimental components. In order to record from the *Xenopus* oocytes, their eggs had to be harvested from delivered frog ovaries. The follicle layer was digested with collagenase in Ca^{2+} free media in order to properly inject the eggs. The plasmid DNA from the molecular cloning stages was linearized and transcribed into mRNA for injection. 1 µL of mRNA was combined with RNAse inhibitor and then injected into frog oocytes, 50.6 nL at a time. Roughly 30 eggs are injected per mRNA sequence in order to get proper recordings from eight to ten of them, which is the minimum required for a data set. The injected oocytes were then incubated at 18 ºC for two to three days, depending on the specific channel type because they require varying lengths of time to properly express in the oocytes. They were incubated in a culture solution consisting of 98 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES, 2.5 mM Na-Pyruvate, 10 mL penicillin/streptomycin solution at pH 7.2.

The voltage clamp recordings involved controlling voltage and measuring current via two standard electrodes that impaled the injected oocytes. The voltage clamp recordings collected data via particular protocols that were specific to the channel type. Controlling the voltage allowed for the determination of the probability of channel activation, measured via the current

of the selected ion passing through. Therefore, the voltage dependence of these channels could be measured.

The recording electrodes were half filled with 3 M KCl. Particular solutions constantly flowed over the oocyte during the recordings in order to simulate the environment the channel would actually experience in the model organism. The solution used for GORK had 2 mM KCl, 5 Hepes, 98 mM NaCl, 1 mM CaCl2, at ph7. The solution used for the VCF SKOR recordings was 5 mM KCl, 2 mM NaCl, 93 mM NaOH, 1 mM $CaCl_2$, 5 HEPES, all at ph7. These base solutions could then have hemin or ROS agents added to simulate additional environmental factors to the oocyte. The heme solution added to the base solution was created using 35 µL of 3.5 mg of hemin dissolved in .5 mL DMSO. The H_2O_2 solution was added to the base solution using 35 µL of 30% H_2O_2.

The GORK recordings involved the control, heme, and H_2O_2 solutions. During recordings, the oocytes flowed under a series of the three solutions. The three different series of solution flows included switching from control to heme to control solutions, control to heme to H_2O_2 solutions, and control to control to H_2O_2 solutions. The solutions were switched on in a stepped fashion, where multiple minutes were given to observe the different solution changing.

The VCF SKOR recordings involved the control and heme solutions. Similar to the GORK recordings, the solutions had a few minutes to properly switch flows before subsequent recordings commenced.

Data Analysis

All of the recordings were analyzed using the pCLAMP and Origin8.1 programs. G/V curves were fit to the Boltzmann distribution in order to understand the voltage-gated properties behind the potential of the channels being open. The Boltzmann distribution, $f(V) = (A_1 + A_2)/(1 + e\ (^{V-V\ 50\ /s}) + A_2$, which tells us about the shift in voltage of activation in a GV curves, also gives us parameters for our data values. A_1 and A_2 are asymptotes in the Boltzmann plot. The parameter x0 presents as v50 in the Boltzmann distribution, which is the midpoint of a single Boltzmann fit presented in mV. The parameter dx presents as s, which is the slope of the Boltzmann fit in mV. These values for the recordings were found and tabled in order to present the original data prior analysis.

An example of the Boltzmann distribution analysis is included in Figures 3 and 4, where a voltage stepped trace from a controlled solution WT SKOR oocyte is used. Figure 3 depicts how measurements are made in the tail current in comparison to the ionic current prior to the tail current via the positioning of the labeled cursors. Figure 4 shows a Boltzmann distribution created from the G/V data gathered from the trace in Figure 3. The further data analysis in the GORK results, including the G/V data analysis and subsequent 2 tailed t-tests, was conducted using Microsoft Excel (Microsoft, 2016).

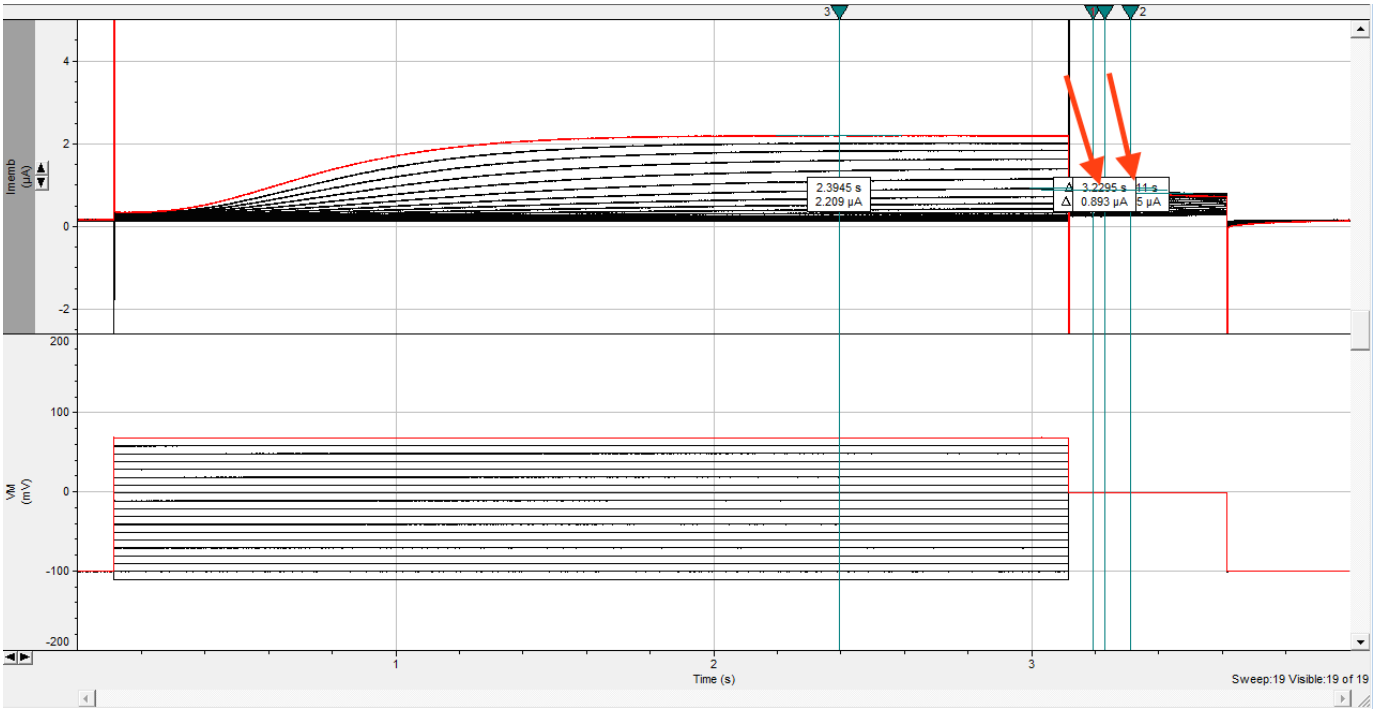

Figure 3: Boltzmann recording example of a G/V analysis where cursors 1 and 2 are labeled with red arrows and placed in consistent locations in the tail current, while cursor 3 precedes the tail current. These are chosen to track the shift in voltage activation.

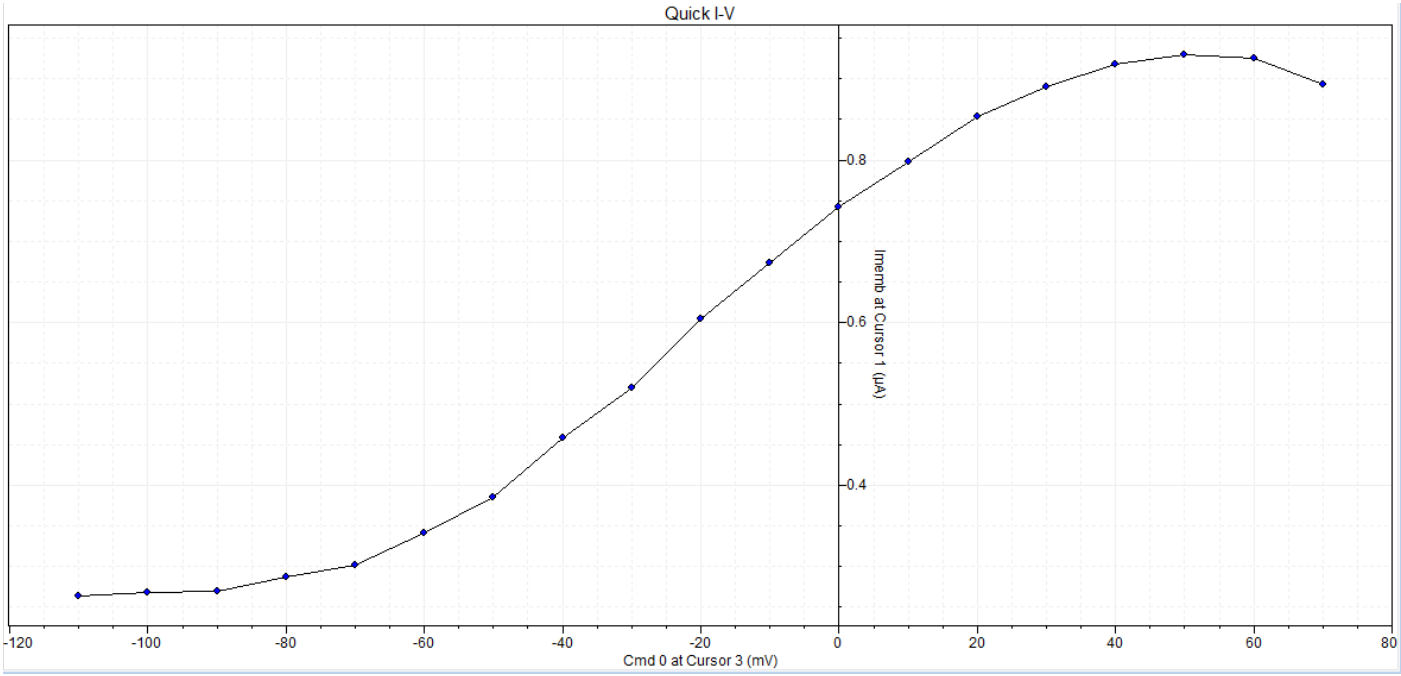

Figure 4: Boltzmann distribution example of a G/V analysis, plotted from the data selected in Figure 3.

Chapter 3: Results

GORK

GORK recordings were done for the eggs flowing on the control to heme to control, control to heme to H_2O_2, or control to control to H_2O_2 solutions. An example of what each of the control to heme to H_2O_2 recordings looked like is included below.

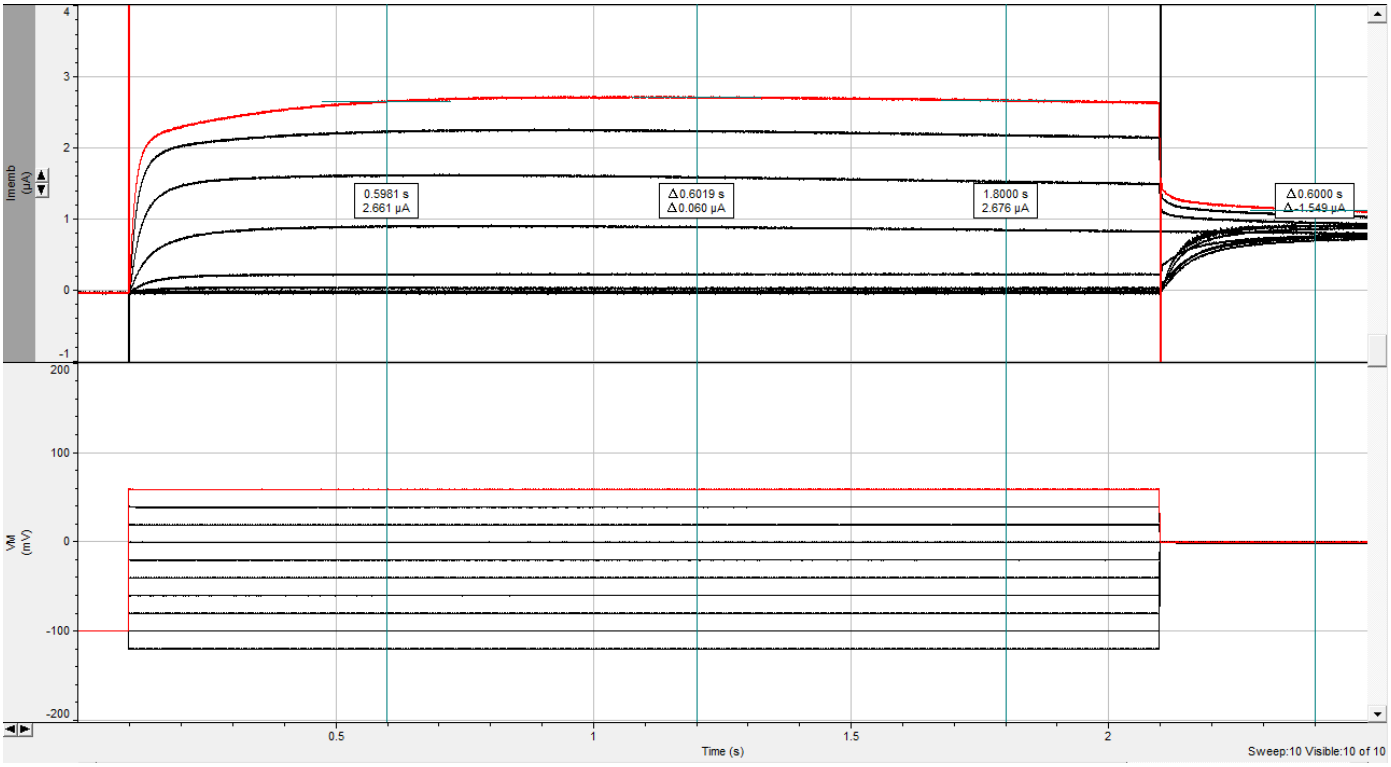

Figure 5: GORK controlled conditions.

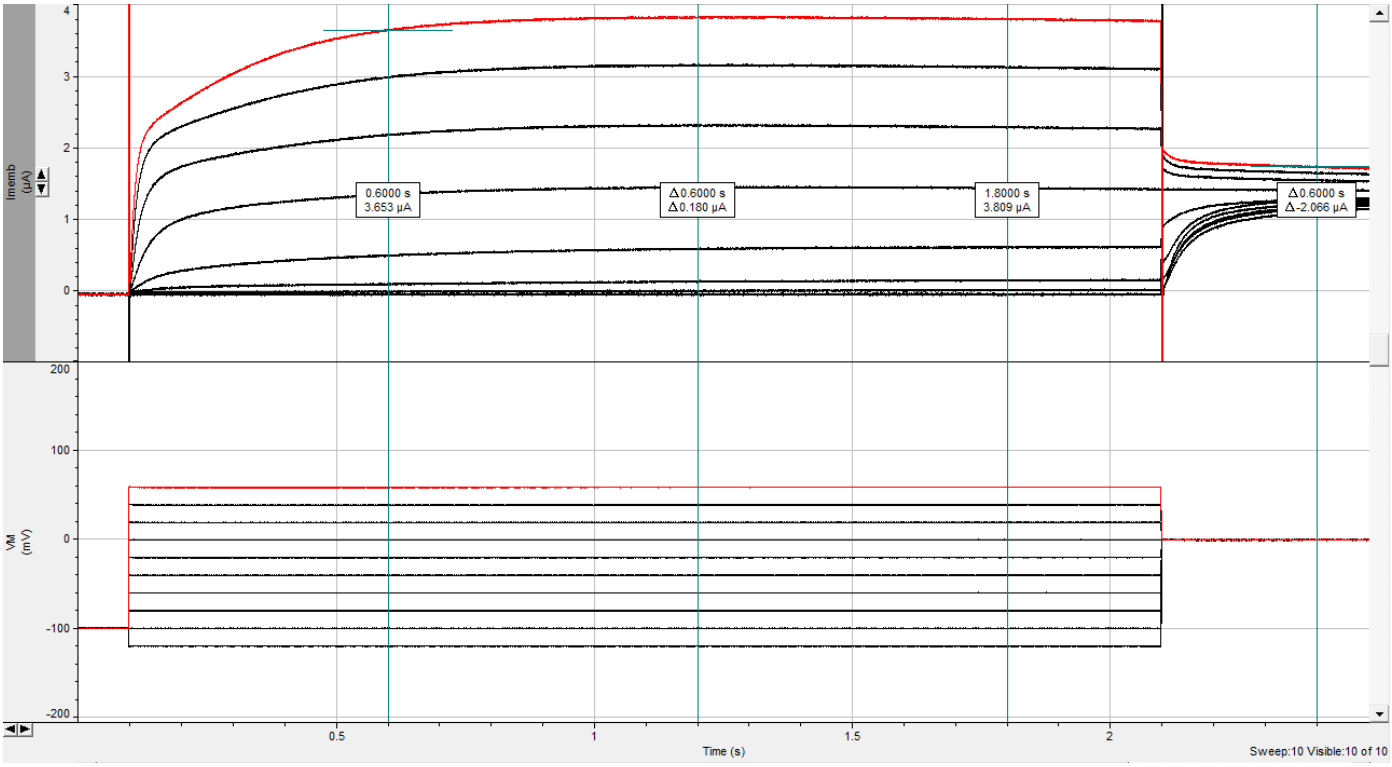

Figure 6: GORK stepped into a heme solution from the controlled solution in Figure 2. An observable increase in current size compared to the current size in Figure 2 is notable.

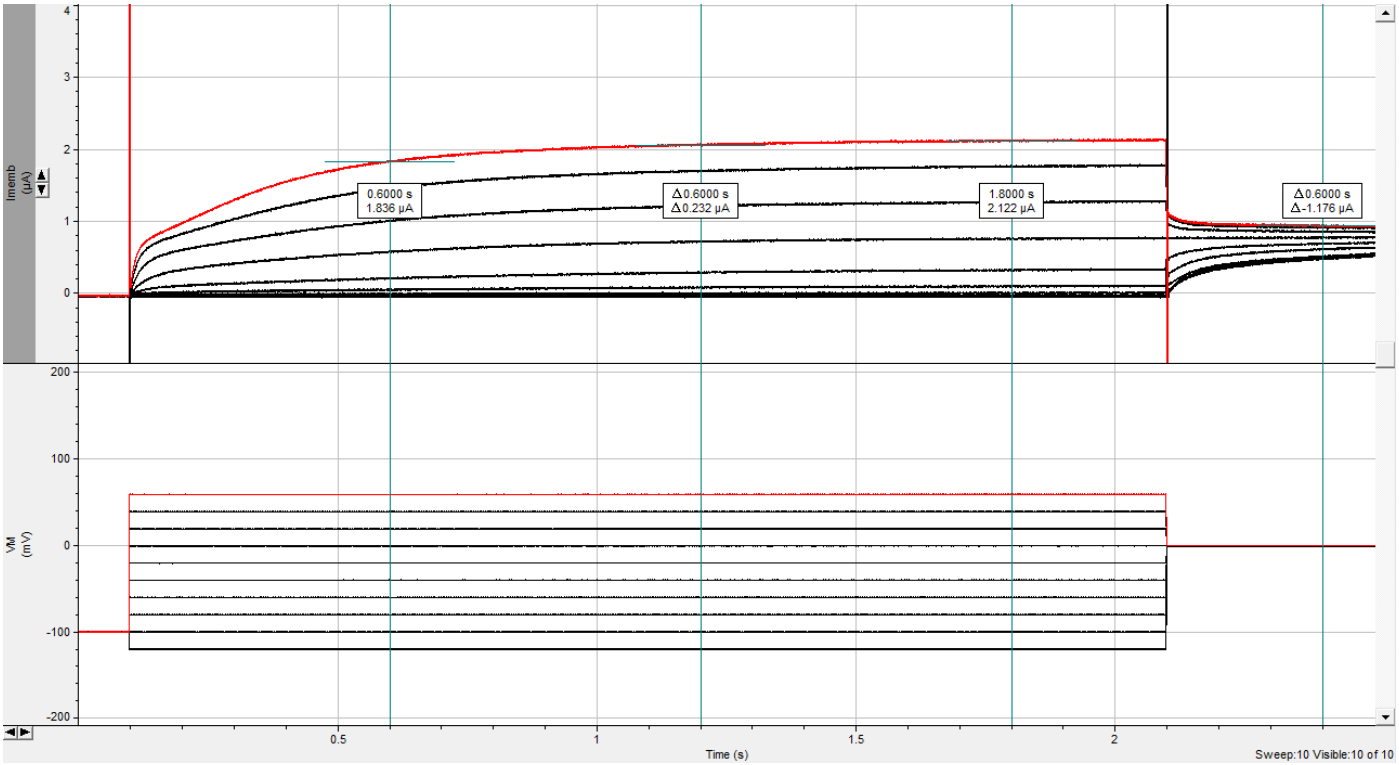

Figure 7: GORK stepped into an H202 solution from the heme solution in Figure 3: There is an observable decrease in current compared to the current expressed by heme in Figure 3.

The figures depict how upon adding heme, current size increases, as seen in the max gating current between Figure 5 and Figure 6. After removing the heme and adding the H_2O_2 oxidizing agent seen in Figure 7, the current decreases. The tail currents following the voltage application tell us how many channels open without voltage influence in the selected environmental condition. These recording depictions were fairly standard among all of these control to heme and heme to H_2O_2 switches.

Full data sets for each of the combinations of flowing solutions were collected and analyzed into the tables below.

Table 1: GORK recording averaged dX (V50) and x0 (s) values.

Egg Experiment and *Condition (n)*	Average dX	Average S.E.	Average x0	Average S.E.
Control1-heme-control2 *Control1 (10)*	24.033535	2.838067	-19.659163	2.907894
Control1-heme-control2 *Heme (10)*	27.290991	2.99454	-45.77869	2.999235
Control1-heme-control2 *Control2 (10)*	26.759821	1.941691	-39.140329	2.003025
Control-heme-H_2O_2 *Control (8)*	19.8213375	3.99702875	-7.916615	4.46468375
Control-heme-H_2O_2 *Heme (8)*	23.98178375	5.974565	-31.53665375	6.70131625
Control-heme-H_2O_2 *H_2O_2 (8)*	23.98178375	5.974565	-31.53665375	6.70131625
Control1-control2-H_2O_2 *Control1 (11)*	23.93276	1.78164	-28.17972	1.71206
Control1-control2-H_2O_2 *Control2 (11)*	24.22629	1.97841	-31.7832	1.89084
Control1-control2-H_2O_2 *H_2O_2 (11)*	23.96413	1.89152	-26.91977	1.81874

Table 2: 2 tailed T-tests with assumed unequal variance conducted on the data from Table 1. Statistically significant results ($p<0.05$) marked with asterisk.

Test	T-Test Measured	P-value (** if <.05)
Control to heme to control (n=10)	Control to heme	0.000734416**
	Heme to control	0.480348223
Control to heme to h202 (n=8)	Control to heme	0.075632174
	Heme to H202	1.15611E-05**
Control to control to heme (n=11)	Control to control	0.80737625
	Control to H202	0.09493761

Areas to note with table two include the statistically significant values. Control to heme solution t-tests generally demonstrated statistically significant or nearly statistically significant results. The maximal current generally doubled or tripled when shifting from flowing under a control solution to a heme solution. Moving from heme to H_2O_2 led to a statistically significant result, where the current sizes decreased upon switching to the oxidizing agent. The control to H_2O_2 result was not statistically significant but demonstrated a general slight increase in current size upon transition. The largest p value is seen for the control to control switch, where the solution flow stayed the same in order to act as a control for the experiment.

VCF

The VCF mutants were successfully cloned and sequenced. The sequence for one of the mutants, L189C, is available in Appendix A with the mutation compared to the SKOR WT gene highlighted.

The VCF mutants proceeded to be characterized via voltage clamping experiments. The SKOR WT gene and the mutated genes were tested under controlled and heme solutions. An example of the SKOR WT recordings vs the L189C recordings is demonstrated below.

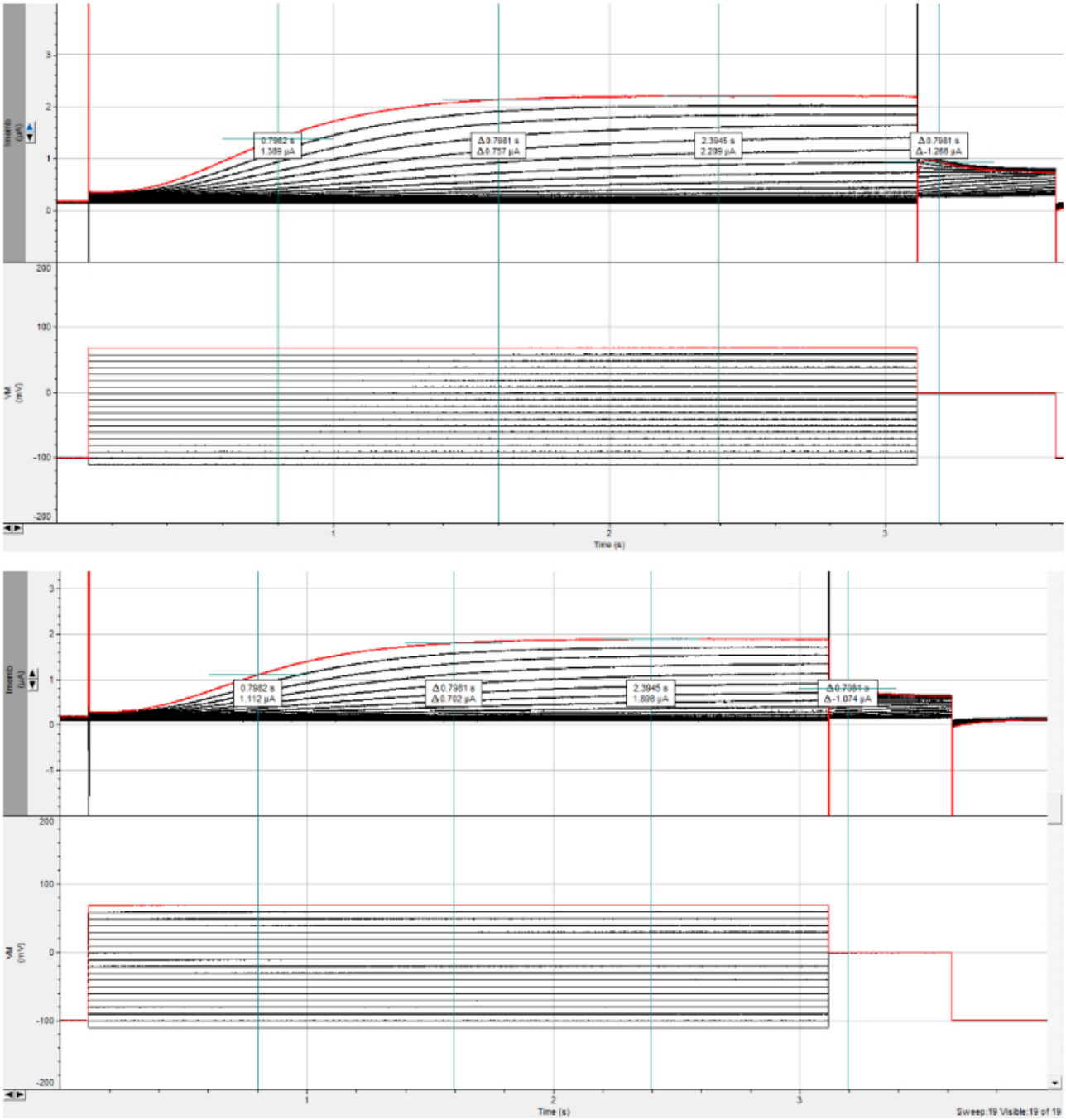

Figure 8: SKOR WT (top) and SKOR L189C (bottom) oocytes characterized under control conditions.

Figure 8 shows how the SKOR WT and SKOR mutated L189C gene seem to express at similar levels under controlled solution conditions. The basic characterization of these genes is therefore similar. Similar results were found in other the seven eggs tested.

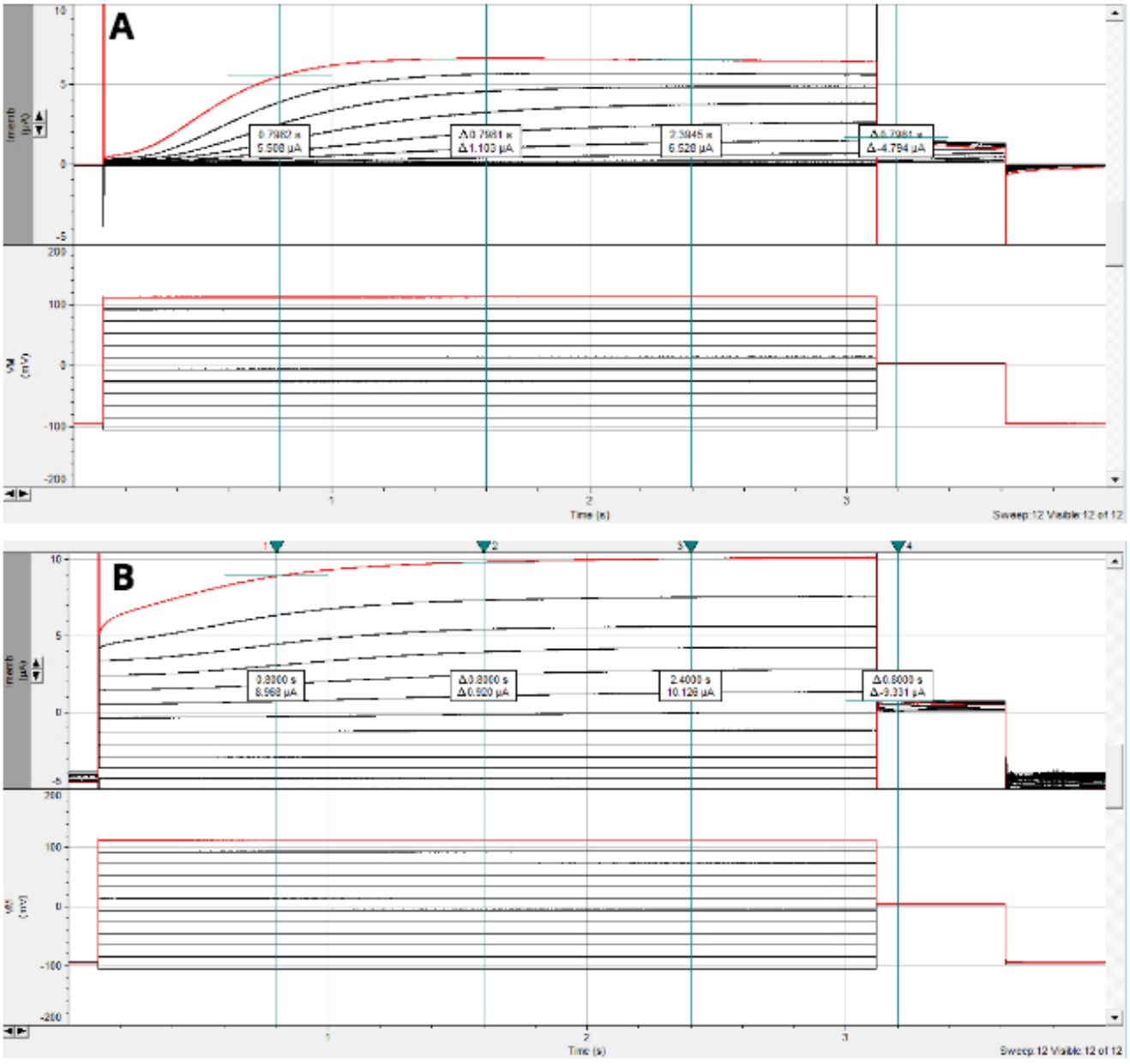

Figure 9: SKOR WT oocytes characterized in controlled (A) and heme (B) solutions.

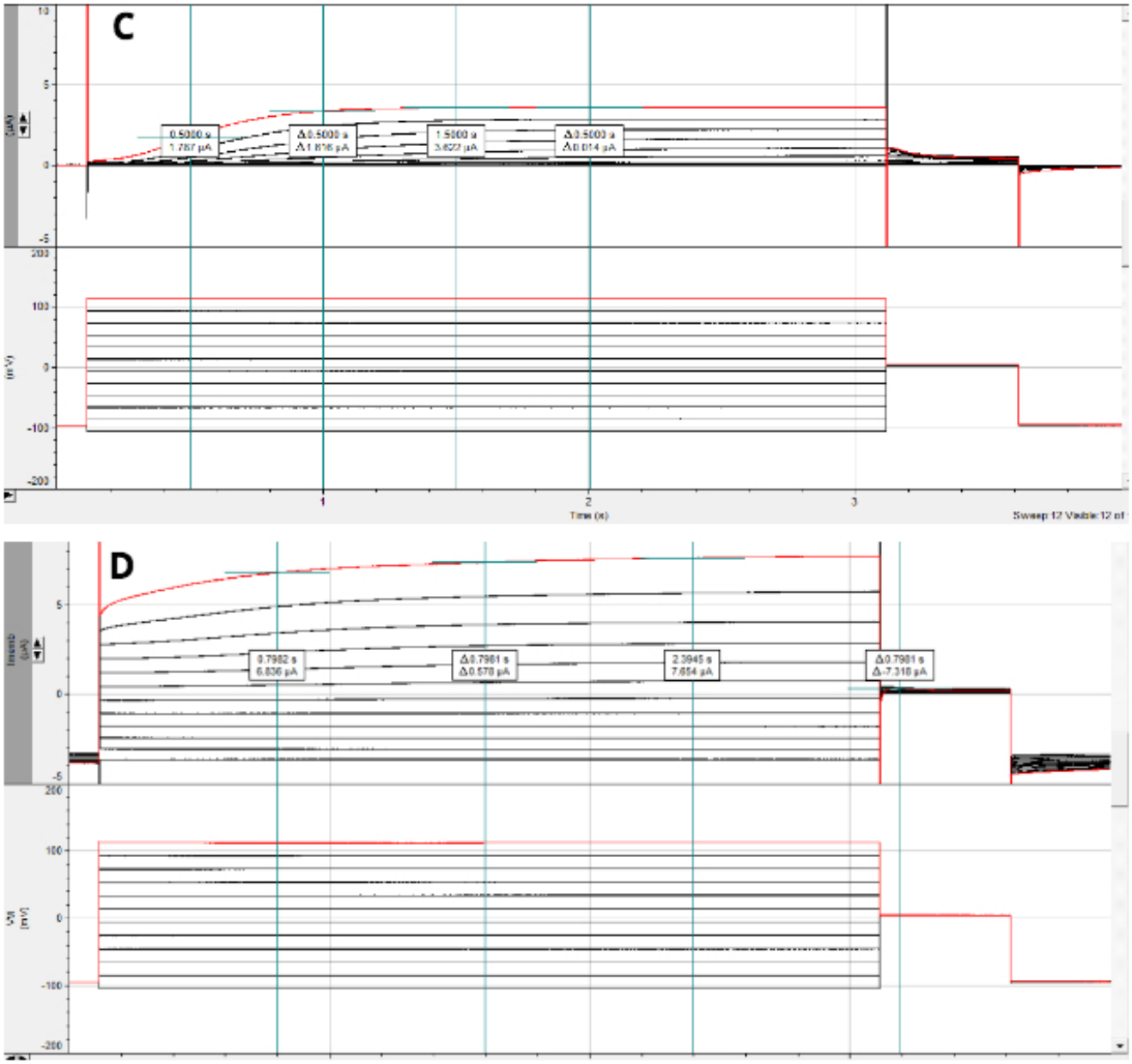

Figure 10: SKOR L189C oocytes characterized in controlled (C) and heme (D) solutions

Figures 9 and 10 depict the SKOR WT oocyte and the SKOR mutated L189C oocyte recordings from controlled and heme flowing solutions. Figure 9.A and Figure 9.B are from the same SKOR WT egg with A depicting controlled condition solution flow and B depicting heme

solution flow. Figure 10.C and Figure 10.D depict the SKOR mutated L189C gene with C depicting controlled condition solution flow and D depicting heme solution flow. The change from controlled solution flow to heme flow results in roughly double the size of ionic currents when stepped at the same voltages. Similar results were found in the other four eggs tested.

Results from the other VCF experiment eggs are summarized in Table 3.

Table 3: SKOR WT and SKOR L189C mutant recordings averaged dX (V50) and x0 (s) values.

Egg Experiment and *Condition (n)*	Average dX	Average S.E.	Average x0	Average S.E.
WT *Control (5)*	19.38237	0.918823333	-18.63439667	0.945306667
Mutant *Control (4)*	17.271905	0.9168475	-19.484145	0.9854075
WT Control-Heme *Control (3)*	19.19468333	1.709963333	-8.122585	1.868936667
WT Control-Heme *Heme (3)*	15.98805	2.946633333	-18.83388667	3.317543333
Mutant Control-Heme *Control (3)*	25.14350667	1.394683333	2.14921	1.415056667
Mutant Control-Heme *Heme (3)*	16.77852667	3.133533333	-18.70158333	3.51025

Chapter 4: Discussion

GORK

From the GORK recordings, only two of the paired t-tests seen in Table 1 were statistically significant. One of the control to heme flows was statistically significant, where the other one was nearly statistically significant. The current sizes nearly doubled to tripled upon application of the heme solution from the control solution. This confirms the first hypothesis made that heme would potentiate GORK. This corresponds to previous data collected studying heme's effects on SKOR, another outward K+ rectifying CNBD channel (Johansson, et al., 2006). Heme therefore likely has a similar interaction with the voltage gating mechanisms in GORK as in SKOR.

The other statistically significant result was the shift from heme to H_2O_2. The current size would observably decrease switching to the H_2O_2 oxidizing agent from the heme solution. H_2O_2 is known to induce stomatal closure (Singh, et al., 2019; Munemasa, et al., 2013). H_2O_2 application should therefore induce larger ionic currents in GORK or other outward potassium rectifying cells in order to shrink the guard cells and close the stomata. While literature suggests that H_2O_2 would increase the current, this is not what is observed moving from heme to H_2O_2 but it was generally observed moving from the control solution to H_2O_2 (Singh, et al., 2019; Munemasa, et al., 2013; Irving, et al., 1992). However, the latter observation was not statistically significant. These observation rejects our initial hypothesis that ROS effects on channel function would be enhanced by prior heme application considering the current permeability decreased upon shifting from a heme solution to an oxidizing solution. Heme may therefore block the oxidative increase in current, concurrent with the intended stomatal closure, with an antagonistic process. The heme

to H_2O_2 shift, while lower in ionic currents, was still slightly larger than the current would have been with H_2O_2 without heme application prior. Therefore, it should be investigated whether heme sensitizes GORK to gaseous messengers specifically or if there were lingering effects from the heme application that were independent of the gaseous messenger. Experimental flaws might be at play here as well, where a few minutes might not be enough time to properly step between solutions. Heme lingering in the H_2O_2 flow would cause the H_2O_2 currents to appear larger than they would be. Furthermore, when switching between eggs completely, we simply waited a couple minutes before proceeding because there was no protocol run to make sure the last solution had completely flowed through in favor on the control solution. Changing the solution stepping time and ensuring the solutions have changed when switching out the eggs could reduce some possible experimental error. More data is necessary to investigate this, along with more data on the control to H_2O_2 shift, possibly with reducing agents present as well.

Heme to control solution flows results in small decreases in current size. The currents would be smaller than the heme expressed currents but would not return back to the original control solution control size. This result was not statistically significant, likely because of the small intermediary change. The least significant t-test result was with the control to control solution pairing. The solution flow stayed consistent, as well as the three-minute solution stepping time, to function as a control. The current sizes should not have changed if the solution flowing on stayed the same. The high p-value proves there was no statistically significant change however there was still a small decrease observed in current between the recordings. This change was insignificant however those small observed trends may mask some of the heme-induced increases in this experiment.

The GORK controlled conditions recordings generally express only a couple mA of current. Compared to SKOR, the GORK expression is very low. Only two of the six paired T-Tests were statistically significant. During recordings, there were also numerous inconsistencies seen, partially because the current expression is so small that small discrepancies suddenly become very significant. These recordings gave us a general confirmation that heme affects GORK similarly to how it affects SKOR, however more experiments are necessary in order to make any strong conclusions on how heme's influential presence was affected by oxidizing agents. More accurate results would be needed with the control to H_2O_2 shift as well considering it was nearly statistically significant, and literature shows that it should be statistically significant (Singh, et al., 2019), calling in to question why this data set was less usable. The current data obtained highlighted subtle changes with heme and ROS application however the smaller currents don't allow for effective analysis of these trends. Having bigger current sizes to evaluate would allow for more detailed comparisons and determinations of regulatory relationships. Therefore, this identical experimental set up should be reproduced with SKOR, focusing on the effects of the ROS. Considering SKOR's current expression is much larger, doing the initial mass of work identifying the subtler relationships between heme and any oxidizing or reducing agents with SKOR is more effective. However, considering SKOR is not expressed in guard cells, the experiment will have to be refocused back to GORK once specific relationships to be investigated have been established. With this information, the specific relationships identified or theorized can then be tested with GORK. This will happen in order to streamline some efficiency into GORK's recordings which will need more focus and care in order to be accurate due to the much smaller expression levels.

VCF

The mutations introduced, changing Leucine, Glycine, and Valine into Cysteines, via molecular cloning were successful. The mutations changed the amino acid site in order to allow the fluorophore required for voltage fluorometry to bind a location involved with heme interaction that would not affect the channel gating. Once the mutations were successfully introduced to the SKOR WT gene, voltage clamp recordings were done in order to confirm similar gating patterns with the mutation introduced. The voltage gating results must not be affected by the mutation otherwise it would betray the whole point of the experiment, where the gating pathways of the voltage sensor in response to heme is being investigated.

The voltage gating results were similar in the WT controlled recordings and the VCF mutants controlled recordings. The basic characterizations of the channels, observed in Figure 6, was similar in nature. That means the addition of the mutant did not cause a change in channel function that would be apparent in these recordings (Vaid, et al., 2008). The gating currents were similar between the two WT and mutated eggs and among all the eggs checked, however there were slight variations in gating current. This occurs due to egg to egg variability in channel expression.

When heme was applied after the control solution, as in Figure 9B and 10D, there was an observable doubling in gating current from their respective currents at the controlled solution flow. With this information, it is evident that the VCF mutants were created and integrated into the SKOR WT genes properly in that the channels react similarly to the same environmental conditions.

When collecting full data sets for final results, the data is collected similarly to the GORK results. However, considering that these recordings were simply meant to assess that the

mutation had not altered channel functioning or reaction to heme, the mutations are deemed to be successful. A full data set will not be necessary for all of the mutants yet. During VCF, when the further successful mutants are identified via the affected fluorophore results, a completed data set would be necessary for those specific mutants when publishing a paper.

Considering that the VCF mutant gene gates similarly the WT gene in both controlled and heme conditions, the experiment can proceed to the next step. Now that the mutant genes characterizations have been confirmed, the actual voltage fluorometry experiments can occur. At this time, the set up for the recording rig required for analyzing the fluorometry changes took longer than expected to set up and is not yet finished in this laboratory. In the future, when the technology has been established in the lab, the VCF experiments can continue with the confirmed mutant functionality already approved.

Future Directions

Aside from the next steps detailed in the voltage fluorometry discussion section, there are many future directions in which to take this research. Creating charge mutants to investigate a specific heme interaction site could provide important insight into how heme modulates these channels.

There are many charge clusters present in the pore voltage sensor section of the CNBD channels discussed in this thesis (Jegla et al., 2018). The charged mutant's original sites have negative charge residues in the pocket of the voltage sensor in order to modulate its position. Heme may possibly have a redox interaction with these negative charge sites as a proton shuttle, impacting the gating and therefore open state of the channel (Jaggar, et alk. 2005). There are

three candidate sites for interaction with heme. If these sites are mutated, the channel could be tested via basic control and heme characterizations as conducted in this thesis to see if there is a difference in heme sensitivity.

The charge mutants must be physically created to start this experiment. They will be incorporated into SKOR genes, where one of three three sites will be mutated to change its charge to positive. The negative charge residues are thought to be stabilized by heme therefore changing the charge cluster interaction would affect heme interaction with the channel. The WT sequence of three sites is DND, the mutants created and tested will include AND, DDD, DNA, NND, DNN, and DAD. The created mutants will have their activity checked via voltage clamp experiments with controlled solutions in order to make sure the channel still gates properly. After that is confirmed, heme can be incorporated into the solutions in order to see heme's affected interaction with this altered charge cluster site.

From unpublished ELK animal channel results in this lab, it has been seen that charge clusters are critical for heme effect. There was reduced heme sensitivity in charged mutants in animal channels, therefore we want to investigate and see if that mechanism is the same in plant channels. In plants, not all of the charge clusters are negative. Those that are not negative have H binding residues because the extracellular fluid in plants is very acidic providing more hydrogens. If all the charge clusters were negative in plants, then the pKa of the system would go up and they would likely be protonated. Through choosing particular mutations to create in the three candidate sites in the S4 region of the channels, mutants can be created and integrated into the WT sequences. The mutant's basic characterizations in a controlled solution can then be confirmed and then heme application experiments can follow. From those results, there will be a

better understanding about the location and mechanism behind the heme interaction site in these channels.

Appendix A

VCF Nucleotide Sequence

SKOR MUTANT SEQ L189C:
CTTATCGTAGCGGCGGCGAATCAGACGTGGAATTAGAGGATTACGAGGTTGATGTA
TTTCAGAGATGGGATTGTAGAATCGCGAGGAAACAGATTCAATCCTCTCACCAATTT
CTTAGGGTTAGACTTCGCCGGCGGTAGCGGTGGAAAGTTCACCGTCATTAATGGAAT
CAGAGATATCTCCAGAGGCTCCATTGTTCATCCCGATAACCGGTGGTACAAGGCGTG
GACGATGTTTATATTGATATGGGCACTTTATTCTTCCTTCTTCACTCCATTGGAATTC
GGATTCTTCAGGGGATTACCAGAGAATCTGTTCATCCTCGATATCGCTGGCCAAATC
GCTTTCTTAGTAGATATTGTCTTGACATTCTTCGTTGCTTATCGTGATAGCCGAACTT
ATAGAATGATCTATAAACGCAGCTCAATTGCTTTACGGTACTTAAAATCAACTTTTA
TAATTGACTTACTTGCTTGCATGCCATGGGATATCATCTACAAGGCTGCAGGCGAAA
AAGAAGAAGTGAGATACCTAT**GT**TTGATAAGGCTATATCGAGTTCATAGAGTAATC
CTGTTTTTCCACAAAATGGAGAAAGATATAAGAATCAATTACCTTTTTACAAGAATC
GTCAAGCTTATATTCGTCGAGCTTTATTGCACTCACACCGCAGCTTGTATCTTCTATT
ACTTGGCCACGACGCTTCCTGCTTCTCAAGAAGGGTACACTTGGATTGGAAGCTTGA
AGTTAGGAGATTACAGTTACTCGAAGTTTAGAGAGATCGATCTCTGGACTCGATACA
CTACTTCTATGTACTTTGCAGT

CORRESPONDING SKOR WILD TYPE SEQ:
CTTATCGTAGCGGCGGCGAATCAGACGTGGAATTAGAGGATTACGAGGTTGATGTA
TTTCAGAGATGGGATTGTAGAATCGCGAGGAAACAGATTCAATCCTCTCACCAATTT
CTTAGGGTTAGACTTCGCCGGCGGTAGCGGTGGAAAGTTCACCGTCATTAATGGAAT
CAGAGATATCTCCAGAGGCTCCATTGTTCATCCCGATAACCGGTGGTACAAGGCGTG
GACGATGTTTATATTGATATGGGCACTTTATTCTTCCTTCTTCACTCCATTGGAATTC
GGATTCTTCAGGGGATTACCAGAGAATCTGTTCATCCTCGATATCGCTGGCCAAATC
GCTTTCTTAGTAGATATTGTCTTGACATTCTTCGTTGCTTATCGTGATAGCCGAACTT
ATAGAATGATCTATAAACGCAGCTCAATTGCTTTACGGTACTTAAAATCAACTTTTA
TAATTGACTTACTTGCTTGCATGCCATGGGATATCATCTACAAGGCTGCAGGCGAAA
AAGAAGAAGTGAGATACCTAT**TG**TTGATAAGGCTATATCGAGTTCATAGAGTAATC
CTGTTTTTCCACAAAATGGAGAAAGATATAAGAATCAATTACCTTTTTACAAGAATC
GTCAAGCTTATATTCGTCGAGCTTTATTGCACTCACACCGCAGCTTGTATCTTCTATT
ACTTGGCCACGACGCTTCCTGCTTCTCAAGAAGGGTACACTTGGATTGGAAGCTTGA
AGTTAGGAGATTACAGTTACTCGAAGTTTAGAGAGATCGATCTCTGGACTCGATACA
CTACTTCTATGTACTTTGCAGT

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