INVESTIGATION OF KcsA ACTIVATION USING ION CHANNEL RECORDING TECHNIQUES

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree

Master of Science

Department of Biochemistry and Biomedical Sciences

McMaster University

Master of Science (2006) McMaster University

(Biochemistry and Biomedical Sciences) Hamilton, Ontario

Title: Investigation of KcsA activation using ion channel recording techniques

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Number of pages: IX, 78

ABSTRACT

Potassium channels allow selective flow of K^+ ions across impermeable membranes. They switch between closed and open states in response to stimulus. The closed state of KcsA, a potassium channel from the bacteria *streptomyces lividans*, has been crystallized and studied. However, attempts to obtain a structural description of gating transition of the channel have been hampered because the open state is transiently occupied. In this study, we investigated changes in gating functions of KcsA caused by variations in environmental parameters such as pH, voltage and also mutation in KcsA using planar bilayer and patch clamp channel recording techniques to improve our understanding on the functional aspect of gate activation of KcsA.

ACKNOWLEDGEMENTS

Many sincere thanks go to my supervisor, Dr.S.C.Yang, for his scientific guidance and his spiritual support. I would especially like to express my appreciation for his provision of an environment for growth. I would also like to thank my supervisory committees, including Dr. Boris Zhorov, Dr. Murray Junop and Dr. Joaquin Ortega. I call myself a lucky person because I had a committee that allowed me to work freely and always stimulated intellectual thinking throughout my project. Thanks are also extended to Dr. Chris Miller (University of Brandies, USA). He patiently answered my questions and taught me a lot on the details in planar bilayer technique.

My work would not have been as enjoyable without the friendship and help of my labmates, both past and present. Victor Pau whom I consider to be a brilliant person, Michael Yuchi with whom I shared the starting time in Yang's lab and Dr. Quyen Hoang who still shows much concern for me even after he has left the lab, gave me a lot of ideas for my project. Those good times we shared will be remembered forever.

Here at the University I have also met some other wonderful people, for whom I will always be thankful. I would like to thank Dr. Epand Richard M.and his wife Mrs. Epand. They taught me a lot of knowledge about liposome. I also would like to thank Dr. Min Zhang (biology department) and Dr. Yaohui Zhu (medicine department), who have taught me a lot of skills about patch clamping. Without their help my patch clamping work would not have been possible. Dr. Tommy Tsui (biology department), who is a very nice and talented person, has been very helpful and I have gained much from our

discussions. William Chiuman, who is an excellent example of enthusiastic scientific researcher, has been helpful in my every committee report.

Finally, I would like to thank enormously my dear husband Zhijun, who has been with me all along. He has been there in my failures and triumphs, always backing me up, giving me comfort, love and support. Special thanks for his effort in helping me to set up my system bench. I also want to thank my two lovely sons Alan and Peter, who make me laugh and enjoy life together with my husband. They gave me the strength to continue my study.

I dedicate this thesis work to all the above people, for they gave me the opportunity to be the best of me. This degree is an achievement of all of you, without you this could not have been possible. Thanks God!!!

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ABBREVIATIONS

CHAPS	3-[3-Cholamidoprophl)-dimethylammonio]-1-propanesulfonate
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
KcsA	potassium channel from *Streptomices lividans*
MthK	prokaryotic Calcium gated K^+ channel
N/E channel	nystatin-ergosterol channel
POPE	palmitovl-Oleovl-Phosphatidyl-Ethanolamine
POPG	palmitovl-Oleovl-Phosphatidyl-Glycerol
RMS	root mean squared
SDS PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
20K4	20mM KCl, pH 4
100K7	100mM KCl, pH 7

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CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVE

Potassium channels are found in almost every organism. They are integral membrane proteins that allow K^+ ion to flow across membranes in response to signals, such as pH changes, membrane voltage changes and the presence of ligands with exquisite selectivity and high efficiency. The functional behavior of ion channels can be described as two fundamental processes: permeation (how ions flow selectively and effectively through the pore) and gating (how the pore gate, or open, in response to appropriate stimulus).

The knowledge of ion channel structure and function has advanced dramatically since the discovery of KcsA, a small potassium channel from the bacteria *streptomyces lividans*.

The KcsA crystal structure from residue 23 to 119, which formed the pore of the channel, was determined in 1998 (Doyle et al, 1998). This finding has led to an improvement of our understanding of the ion permeation and selectivity mechanism on a physical basis (Figure 1) (Doyle et al., 1998; Jiang and MacKinnon, 2000).

However, attempts to obtain a structural description of the gating mechanism of the channel have been hampered because the open state is transiently occupied and so far only crystals of closed-state KcsA have been obtained. Furthermore, the C-terminal of KcsA, which is thought to be involved in the gating process, has not been resolved in any

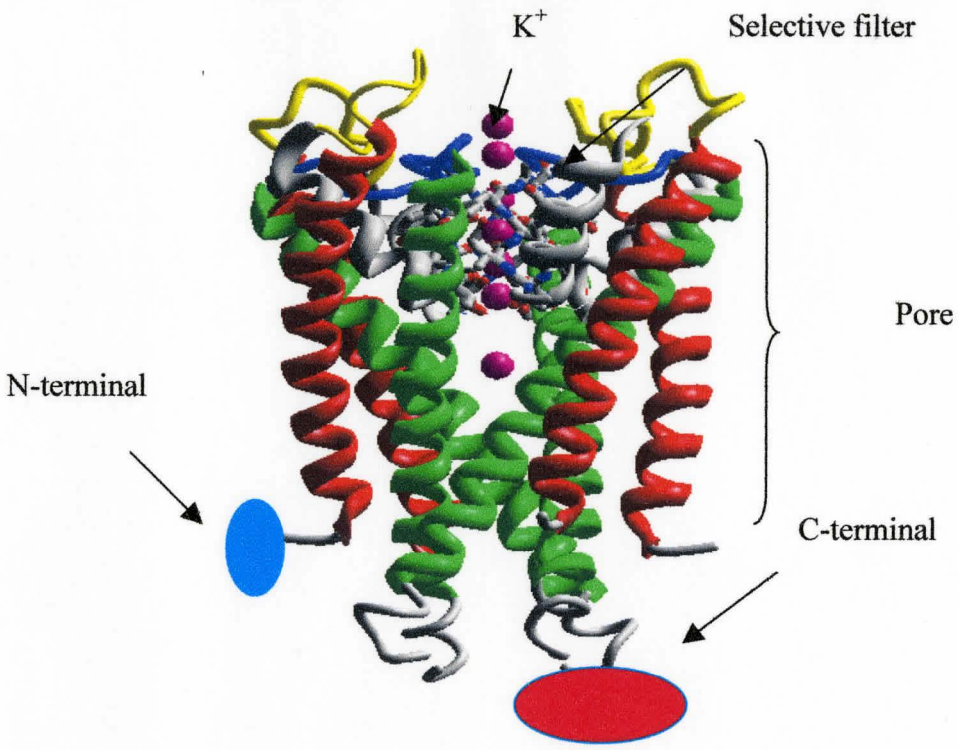
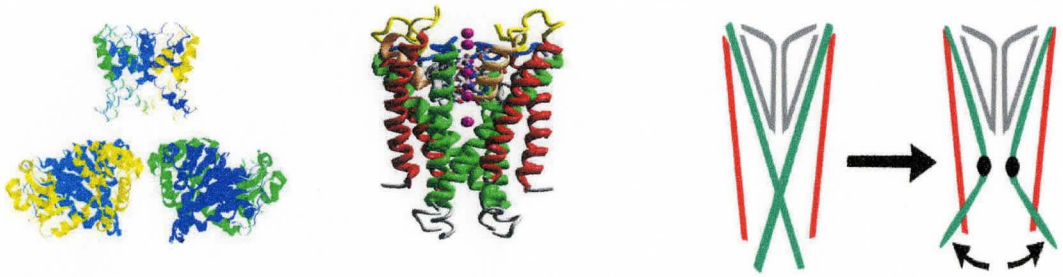


Figure 1: KcsA structure. The transmembrane portion (inner helix, green, residue 86-118; outer helix, red, residue 24-51) and pore helix (gray, residue 61-73) of the KcsA channel are shown with an ellipse diagram. The selectivity filter (residue 75-79) is shown with ball-and-stick diagram (MacKinnon et al., 1998; Stacey et al., 2002). The C-terminal and N-terminal structure are still not available. The N-terminal domain is suggested to act as an anchor at the membrane-water interface (Cortes et al., 2001). The figure was generated from PDB file KcsA using ZMM-mvm modeling program (Zhorov et al., 2004)

of the previous x-ray structures of KcsA. There are two fundamental questions concerning gating in ion channels: 1) The location of the gate and pH sensor, 2) The physical mechanism for pH-dependent opening and closing of KcsA (Cuello et al., 1998; Perozo et al., 1999)

Recent studies have led to a model for channels undergoing conformational change during gating (Figure 2) (Stacey et al., 2002). This gating model was derived from comparison between the crystal structures of KcsA and MthK. MthK, a potassium channel from the bacteria *Methanobacterium thermoautotrophicum*, has a membrane topology similar to that of KcsA. The KcsA and MthK structures are thought to reflect the closed and open states, respectively, of potassium channels. A conserved Glycine residue on the 2nd transmembrane helix has been proposed to be critical for the gating transition acting as a hinge for gating opening and closing. The Mthk-derived opening model suggests that an outward hinge-bending movement of the inner helices is the main conformational change during gating event. The conformational change is restricted to the intracellular half of the membrane-spanning region, leaving the extracellular half basically unchanged.

KcsA lacks a voltage sensor like the other two-transmembrane helices K^+ channels (Heginbotham et al., 1999). One of the reliable ways to open the KcsA channel is by lowering the pH (Cuello et al., 1998; Perozo et al., 1998) on the intracellular side (Heginbotham et al., 1999). Functional analysis of C-terminal deletion mutants suggests that the C-terminal domain exerts a modulator role in the pH-dependent gating



a b c

Figure 2: The hinge-bending model. a. Mthk crystal structure. Open form state. b. KcsA crystal structure. Closed form state. c. The hinge-bending model. Cartoon of KcsA depicts two of the four subunits. The inner helices are shown in green, the outer helices are shown in red and the selectivity filter are shown in gray. In this model, the inner helix splays outward with a hinge (circles) near the middle of the membrane. This model does not perturb the structure near the selectivity filter. (Ref: Brent et al., 2003)

mechanism (Perozc et al., 1999; Cortes et al., 2001). Cortes et al. suggested that the charge cluster (e.g. Glu, His, Lys & Arg) in the cytoplasmic domain was a prime candidate for the pH sensor. Our lab further suggests a hypothesis: At pH 7, the positive charges on the side chains of lysines and arginines in the cluster are neutralized by the negative charges of the glutamates, thus stabilizing the closed form. The charges on the histidine residues however may change the balance of charges in the cluster at different pHs. The net charge in the cluster is likely to be close to zero at pH 7. As pH decreases to 6, half of the histidine residues become positively charged. The net charge in this region becomes positive and the C-terminals repel each other, destabilizing the closed form and favoring transition to the open form.

For the gating mechanism of KcsA, although several gating models have been proposed by comparing the structures of different potassium channels, obtaining a further structural description of the gating mechanism is still a difficult task due to the lack of C-terminal domain structure and open state structure. However, previous planar bilayer single channel studies on KcsA demonstrated a pH dependent current change (Figure 3) and the sensor domain of KcsA have been proposed by Rb^+ flux studies (Heginbotham et al., 1999; Perozo et al., 1999; Cortes et al., 2001). These suggest that functional description may offer another opportunity to further investigate the mechanism of gating and sensor-gate coupling/interactions. In the present study, we set up the planar bilayer and patch clamp channel recording system in order to obtain more information on the gating of KcsA. To facilitate such studies, we have tested the system with wild type

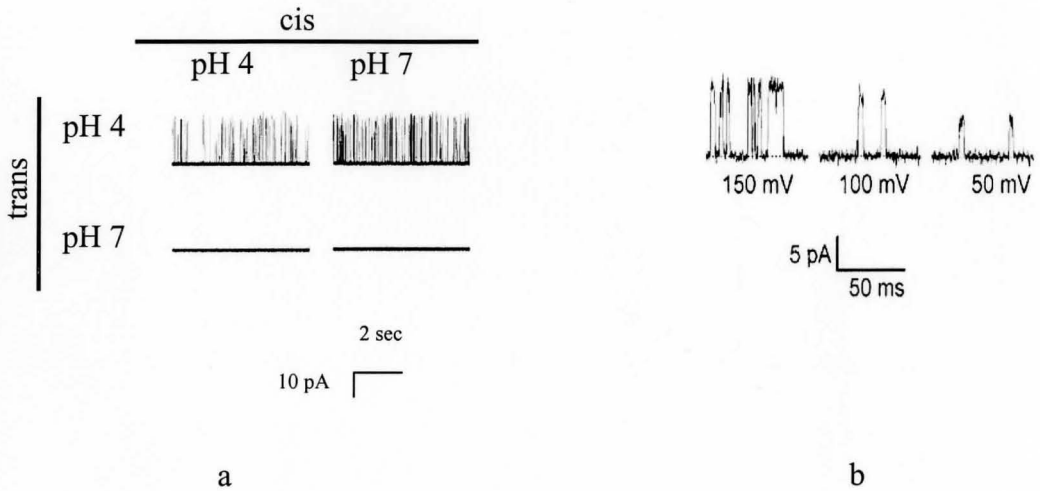


Figure 3: Current through single KcsA channel. a. pH dependence of KcsA. Currents were recorded at 200 mV in symmetrical 100 mM K^+ solutions, with pH variation on cis and trans sides. Channels were first recorded with pH 4 on both sides. The cis chamber was brought to pH 7, and more records were collected. After perfusion to pH 7 trans, where no openings were observed, the trans chamber was returned to pH 4 to confirm reversibility. b. Representative channel openings at different voltages. (Taken from: Heginbotham et al., 1999)

KcsA to reproduce the published results. Then we compared the wild type KcsA with mutant of KcsA at different environmental parameters such as pH and voltage to determine how mutation affects the gating of KcsA. The final results may improve our understanding on the gating of KcsA.

CHAPTER 2

EXPERIEMTNTAL MEASURMENT PRACTICE WITH WILD TYPE KcsA

2.1 Introduction of two electrical single channel recording methods

Ionic channels are integral membrane proteins that, upon activation, allow passive flow of specific ions down chemical and electrical gradients established between both sides of the membrane. Thus, assessing the functionality of isolated molecular components comprising these channels requires their reconstitution into model membranes in which translocation of ions may be measured (Miller et al., 1983, 1984). However, monitoring ionic channels responses is difficult since channels activation promotes very rapid changes in the permeability of the membrane to certain ions. Previously, ion fluxes were mostly monitored by using radioactive ion trace assays with poor time resolution. Electrical methods, having higher sensitivity and time resolution, are better alternatives to quantify flow of ions. Thus, two basic systems have been developed to combine membrane reconstitution of ionic channels and electrical monitoring of ion flow: planar bilayer and patch clamping liposomes (Miller et al., 1984).

The planar bilayer channel recoding technique, in which ion channels are incorporated into an artificial planar lipid bilayer by fusion of vesicles containing channels with the bilayer, provides an unique approach for studying single ion channels, enabling experimental design that are impractical or impossible using standard patch-clamp methods (Figure 4). For example, the effects of membrane composition on channel

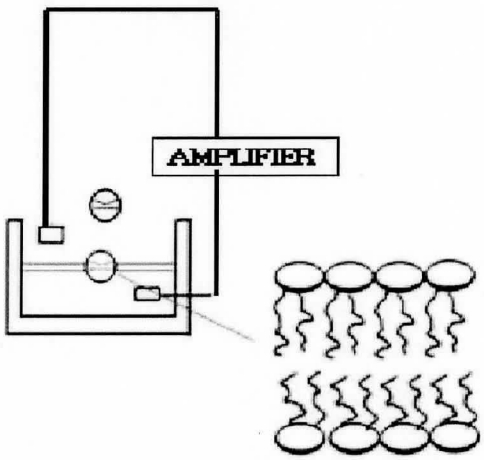


Figure 4: Experimental design of the horizontal planar channel recording. The chamber was separated by film with hole. The planar bilayer bedded with channel were formed on the hole.

function can be rigorously studied; changes in solution compositions on either side of the membrane can be made easily (Wonderlin et al., 1990; Wonderlin et al., 1991). The utility of the planar bilayer method for studying single ion channel, however, is often limited by a low recording bandwidth, primarily due to the high background current noise, and poor resolution of voltage activation step applied to the bilayer (The Axon Guide, 1993). However, with several modifications on the equipment and technique that partially overcome these limitations and improve the quality of planar recordings, recording of single channel activity with low background noise (<0.35 pA rms at 5 kHz) and rapid resolution (<<1ms) of currents after a voltage step can be done. For more detailed discussion of the equipment and methods, please see Ref. The Axon guide.

The single-channel patch clamp is a special case of the voltage-clamp technique. This technique permits the direct measurement of the dynamic activity of individual membrane-bound channel proteins (Figure 5). Like the whole-cell patch technique, a blunt pipette is sealed onto a patch of membrane. If single-channel recordings are intended, the membrane at the tip of the pipette is preserved. The current recorded is then simply the current that flows through the membrane at the tip of the pipette. Since this membrane area is very small, there is a good chance that just one or a few ion channels are located in the patched membrane. Individual ion-channel currents can thus be recorded.

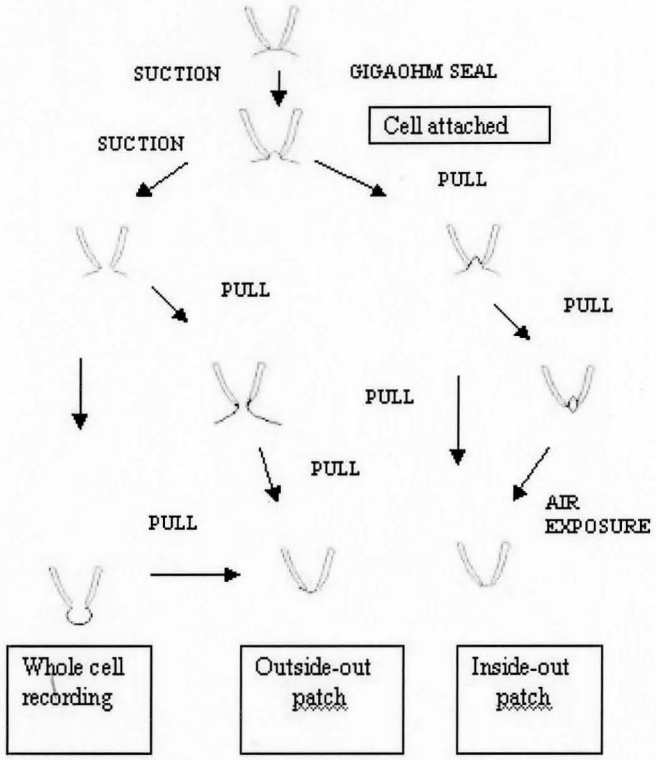


Figure 5: Schematic representation of the procedures that lead to recording configurations. The four recording configurations are "cell-attached", "whole-cell recording", "outside-out patch", and "inside-out patch". The upper most frames is the configuration of a pipette in simple mechanical contact with a cell, upon slight suction the seal between membrane and pipette increases in resistance, forming what we call a "cell--attached patch". With the manipulations to isolate membrane patches which lead to two different cell-free recording configurations (the "outside-out patch" and "inside-out patch". Alternatively, voltage clamp currents from whole cells can be recorded after disruption of the patch membrane. The manipulations include withdrawal of the pipette from the cell, pull, short exposure of the pipette tip to air and short pulses of suction or voltage applied to the pipette interior while cell attached (Taken from: Bert and Erwin, 1995).

2.2 Planar bilayer channel recoding

Single channel measurement using planar bilayer generally involves four steps: 1) Forming the artificial bilayer in the measurement chamber. 2) Reconstituting detergent-solubilized and purified channels into liposomes. 3) Fusing or incorporating liposomes containing channels of interest into the planar bilayer. 4) Measuring the activity of channels under desired conditions.

2.2.1 Methods and materials

2.2.1.1 Micelle solution preparation

Phospholipids (7.5mg/ml POPE, 2.5 mg/ml POPG) was hydrated with 34 mM CHAPS in reconstitution buffer (450 mM KCl, 10 mM HEPES, 4 mM N-methylgucamine, pH 7.0) (Heginbotham et al., 1998).

2.2.1.2 KcsA mutagenesis and expression

The following KcsA constructs were used in the present study: 1) wild type KcsA; (2) mutants with His to Ala mutation on C-terminal. They have been made and supplied by Victor Pau (Department of Biochemistry, McMaster University).

2.2.1.3 KcsA purification and reconstitution

KcsA wild type and mutant H124A were purified by Victor Pau according to previous described procedures (Heginbotham et al., 1997; MacKinnon et al., 1998). Purified KcsA tetramers were added to lipid micelle solution with different concentrations, according to the number of channels per liposome desired. After 30 minutes incubation, the mixture was dialyzed overnight.

2.2.1.4 Preparation of liposomes with N/E channel

Protein-free liposomes containing N/E (nystatin /ergosterol) channel were formed from 10 mg lipid in 1 ml chloroform: methanol (2:1 v/v) to which 10 µl of nystatin stock solution (5mg/ml) had been added. The final nystatin concentration was 50 µg/ml. Lipid composition was (mol percent): 60, POPE; 20, POPG; 20, ergosterol. After evaporating the solvent under a stream of nitrogen, reconstitution buffer was added. The mixture was vortexed for ~5 min and sonicated in a bath-type sonicator at room temperature for ~2 min. The mixture was cloudy after vortexing and then became translucent after sonication. Liposomes were frozen at –80°C.

For liposomes containing both N/E channel and KcsA channel, the freeze-thaw-sonication (FTS) reconstitution procedure was used. Liposomes carrying KcsA channel from dialysis were mixed with protein-free liposomes containing N/E channel at appropriate ratio. After two cycles of FTS, the KcsA would disperse into the liposomes containing N/E channel.

2.2.1.5 Liposome fusion and single channel recording in lipid bilayers

Single channel recordings were performed in a horizontal planar lipid bilayer setup (Heginbotham et al., 1999). Briefly, partition, made from overhead transparency film with a hole of roughly 90 µm in diameter by the melt-and-shave method (Wonderlin et al., 1990), was pretreated by application of 0.5 µl of lipid solution (15mg/ml POPE, 5 mg/ml POPG in decane) to the hole, followed by 30 min of drying in air. The cis chamber (liposomes application side) was then filled with 100K7 (100mM KCl, pH 7) solution and the trans chamber, 20K4 (20mM KCl, pH 4) solution. Bilayer was then

formed by painting the film with a glass rod dipped in the lipid solution. After 5-30 minutes, 1 µl liposome solution (containing channels embedded in liposomes) was added to the cis chamber using pipette. These steps were monitored optically and electrically. The system was electrically connected to the recording system through Ag/AgCl electrodes and agar bridges filled with 200mM KCl. Fusion events were detected as sudden jumps in bilayer conductance. A holding voltage of 100 mV was used to measure the corresponding current. Upon appearance of single KcsA channel current, solutions in both chambers were changed to experimental solutions and excess liposomes were removed. For analysis, currents were measured with holding voltages ranging from – 100 mV to +100 mV. The current was sampled at 2 kHz with Dagon 8100 single electrode system (Dagan corporation, USA) or Model 2400 patch clamp amplifier (A-M systems, Inc, USA) and data were acquired with a Labjack U12 device (Labjack corporation, USA) and analyzed using Clampfit 9.0 software (Axon instrument, Inc) on a PC.

2.2.2 Results and discussion

2.2.2.1 Measurement system

Since the currents we were measuring were in the magnitude of picoamperes, reducing noise in measurement was a challenge in our measurement. The majority of background system noise in a planar bilayer recording was a result of "voltage clamp" across the bilayer capacitor (Wonderlin et al., 1990). For the purpose of making small, low capacitance bilayer, we have adapted the melt-and-shave method (Wonderlin et al.,

1990) with slight modifications. Briefly, a tiny pin was warmed and pressed against one surface of an overhead transparency film. After the pin removal, the surface on the other side was shaved away by a razor blade with rapid rotation of the film. With this method, a film with a 95 μm diameter hole was obtained.

Other noises came from the access resistance, which was the sum of several resistances in series, including the electrode/electrolyte, the salt bridges and the recording solution (Wonderlin et al., 1990). Salt bridges were essential for isolating the Ag/AgCl electrodes from the bath solution and providing a constant, optimal environment for the electrode/electrolyte interface, minimizing the junction potential. We constructed Ag/AgCl electrode with salt bridges (2% agar, 200mM KCl) and the access resistance with different solutions was tested. In our system, the access resistance was in always below 500 kΩ (Figure 6).

A chamber was custom built for this study and shown in Figure 7. The whole system with this chamber was tested using model cell to determine the noise level and used as a test bed for all the noise reduction modifications. Plots of Currents vs. Voltages were used to determine resistance of the system. Our result showed that the noise level was in the range of 1 pA and therefore was acceptable for our measurement purposes (Figure 8).

2.2.2.2 Bilayer formation

The formation of bilayer was examined optically and electrically. It was visually examined under microscope. Before sample application, the hole on an overhead

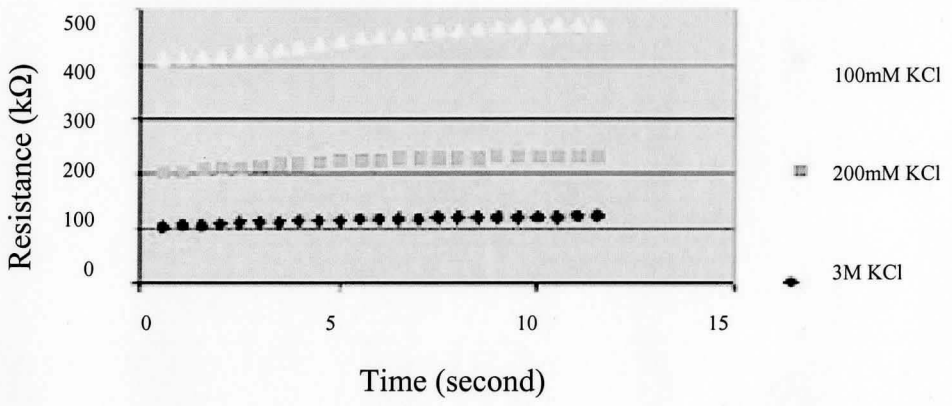


Figure 6: Resistance with different solutions in chamber. Ag/AgCl electrodes with salt bridge (2% agar, 200mM KCl) were connecting the system.

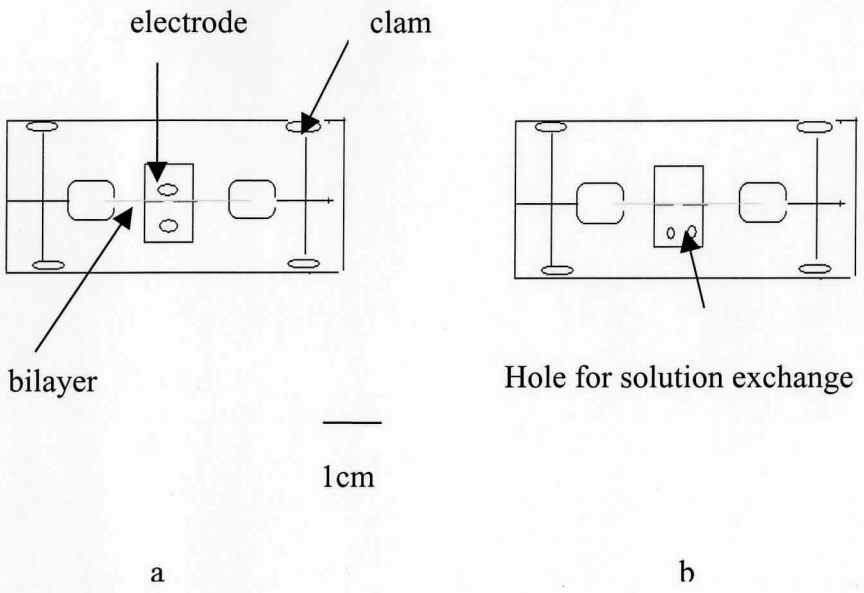
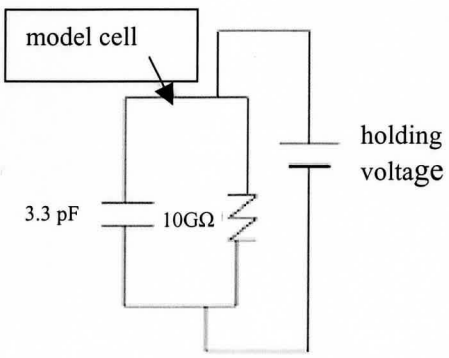
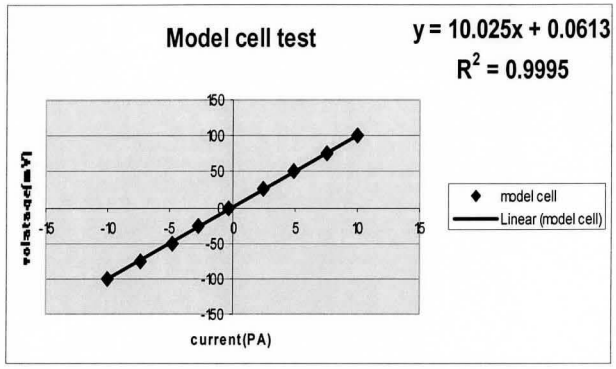


Figure 7: An illustration of the custom built chamber for bilayer studies. a. Front. b. Back.





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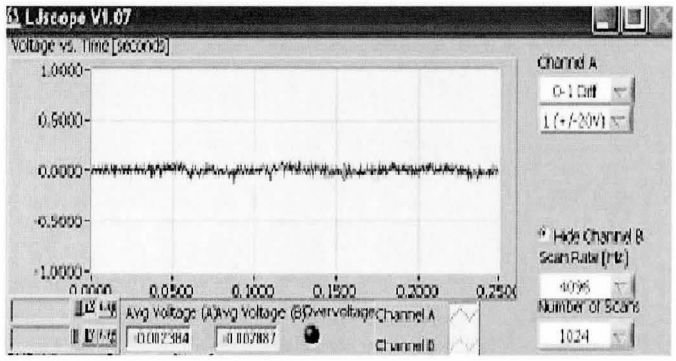


c

Figure 8: Results of system test under -100~100 mV holding clamp voltage. a. Model cell. b. Plots of Currents vs. Voltages (System connect to model cell. Resistance was 10.025 GΩ). c. System connected to model cell (noise level is shown at 1 pA).

transparency film for bilayer mounting was empty and transparent. After it was pretreated with ~0.5 μl of phospholipid solution (15mg/ml POPE, 5mg/ml POPG in n-decane) and was allowed to be air-dried for about 30 minutes, the edge of the hole was covered by a dried layer of POPE/POPG in decane. This pretreatment facilitated bilayer formation by reducing the surface tension and providing a good foundation or attachment site for later single bilayer formation. When the bilayer forming solution, POPE/POPG in decane, was subsequently painted to the pretreated hole, the solution spreaded across the hole to form a layer of POPE/POPG aggregate. It became transparent when it was allowed to stay at room temperature for a few minutes, or when decane was painted again. Observation of the transparent layer indicated the formation of lipid single bilayer (Figure 9).

The bilayer formation was also analysed by electrial method by measuring the resistance and capacitance of the bilayer. When 50mV voltage was applied across an solution-filled chamber with an empty hole, a 50 nA current was detected. On the other hand, an observable decrease in current was measured when the hole was filled with the bilayer (Figure 10a). When the holding voltage was varied from –100 mV to 100 mV, the measured currents were always within 1 pA range (Figure 10b). Since the sensitivity level of our system was also about 1 pA, these data indicated that the bilayer resistance was at least 100 GΩ, which suggested that the bilayer was of good quality.

Single bilayer could be further verified by measuring its capacitance (Figure 11). The capacitance of the membrane was determined by applying an alternating voltage across the membrane and measuring the current generated due to the capacitance. The

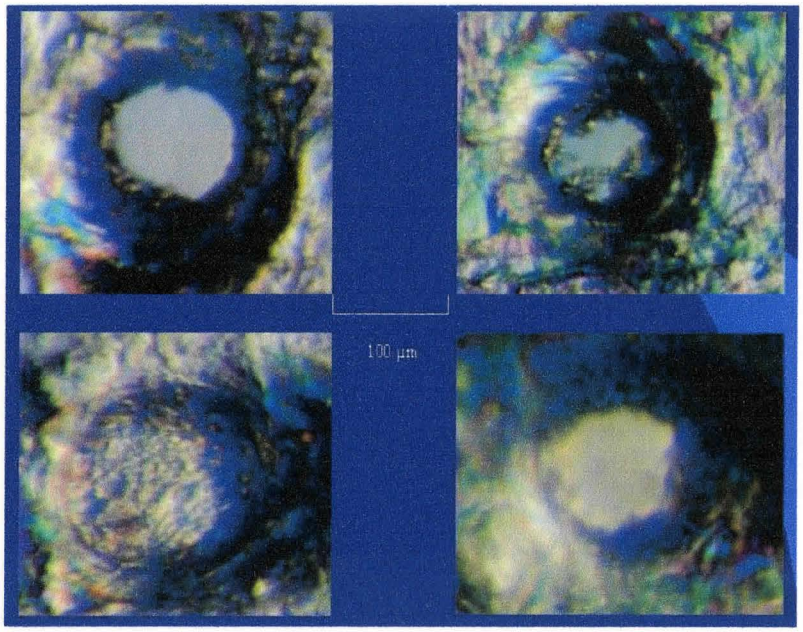
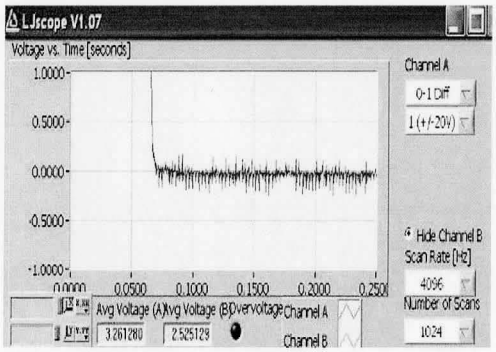


Figure 9: Bilayer formation observation under microscope. a. The empty hole. b. The hole was pretreated with ~0.5 µl of phospholipid solution (15mg/ml POPE, 5 mg/ml POPG in n-decane) and air-dried ~30 minutes. c. The trans chamber was filled with 20K4 solution and the cis with 200K7 solution. Then bilayer was spread on the hole with a pipette wetted with phospholipid solution kept at room temperature. Shows POPE/POPG aggregate. d. After using decane paint on the bilayer.



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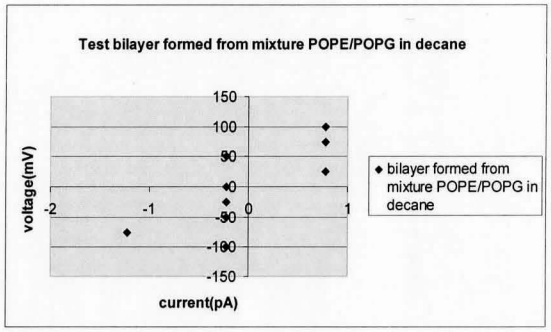

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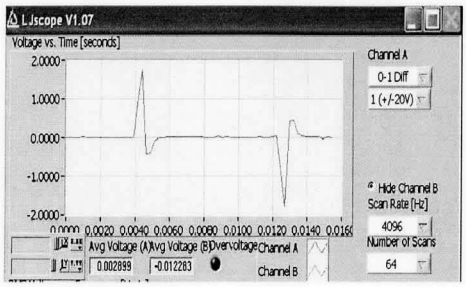
Figure 10: Bilayer formation tested electrically. a. Current drop to almost 0 when bilayer was formed. b. Plots of Currents vs. Voltages. Current showed random display.



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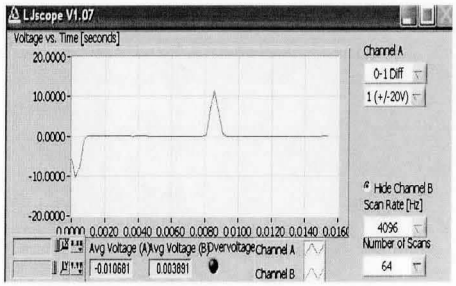

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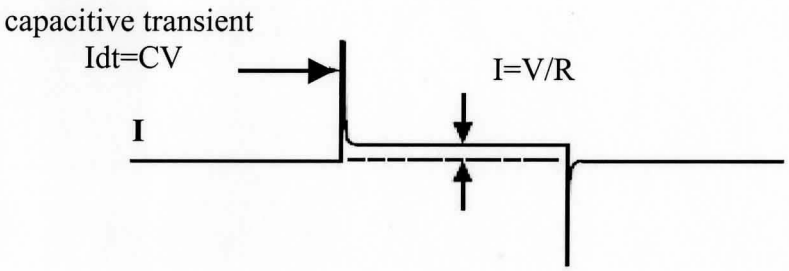

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Figure 11: Transients at the frequency of the clamping signal. a. Electrodes connected to model cell. b. Electrodes connected to bilayer. c. Typical capacitive transient. (Take from The Axon Guide, 1993)

alternating voltage was obtained by using the internal signal a low frequency (60 Hz), low amplitude (10mV peak to peak) square wave from the patch-clamp device. Before the capacitance of the bilayer was measured, a model cell with a known resistance and capacitance of 10 GΩ and 3.3 pF, respectively, was used as a positive control. When the applying voltage was constant, the resulting current was close to zero since the resistance of the model cell was high. During the voltage alternation, a sharp transient peak could be observed. The frequency of the clamping signal was in sync with that of the transient peak. The height of the peak was dependent upon the rise time of the square wave and the capacitance of the membrane (The Axon guide, 1993). The capacitance of the bilayer or model cell was equal to the area under the peak (Idt) divided by the value of the applied voltage. From our measured data, the capacitance of the model cell was around 3.9 pF, which was very close to the value given by manufacturer. After this method had been demonstrated to be able to measure the capacitance of a model system, it was applied to the formed bilayer. We observed that the thickness of the bilayer was inversely proportional to its capacitance. The capacitance and the diameter of a painted bilayer were about 39 pF and 90 μm, respectively. It had been suggested that capacitance per area between 0.4~0.8 $\mu f/cm^2$ was optimal for single channel measurement (Alan, 1994). The capacitance per area of our bilayer was 0.48 $\mu f/cm^2$, which was in the range of the suggested value.

2.2.2.3Protein reconstitution into liposome

As mentioned before, detergent-solubilized KcsA had to be first reconstituted into liposomes before any measurement took place. In the reconstitution process, two issues

had to be addressed 1) Verifying the identity of the protein of interested. 2) Confirming the reconstitution event.

The KcsA was purified and was reconstituted into liposomes. Liposomes were obtained by dialysing detergent-solubilized KcsA and lipid micelle overnight. The dialysed sample was centrifuged at 10,000 g for 1 minute. After centrifugation, two phases, supernatant and pellet were observed. The pellet corresponded to liposomes while the supernatant corresponded to detergent-solubilized lipid and KcsA that were not incorporated into liposomes (Figure12 a).

The identity and folding after reconstitution of KcsA could be verified by boiling test (Figure 12b). Unlike other water-soluble proteins, KcsA tetramer was very stable in harsh detergent such as SDS without extensive boiling. It was denatured and dissociated irreversibly into monomers after boiling for 5 minutes in the present of SDS. This observation was used as the basis to analyse the folding of KcsA on SDS PAGE (Cortes et al., 1997). In our experiment, KcsA remained as tetramer when it was analysed on SDS PAGE without boiling step. It was dissociated into monomer after boiling. The sizes of the monomer and tetramer were consistent with the expected value. This observation implied that the folding of reconstituted KcsA was correct.

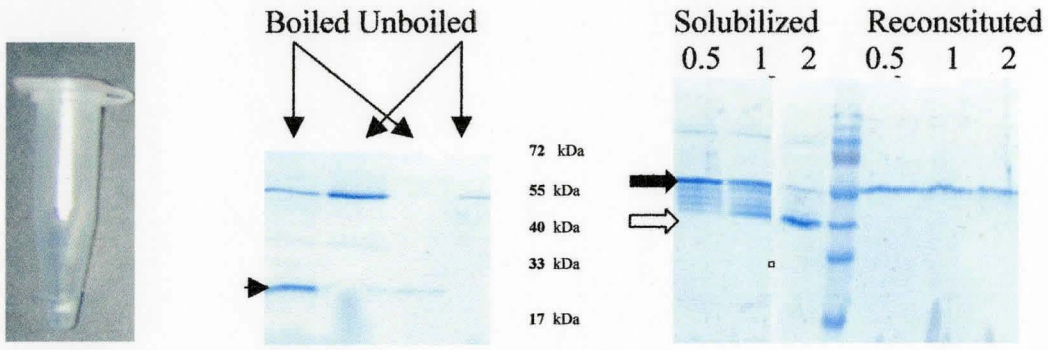
Reconstitution of KcsA into liposomes was further confirmed by trypsin digestion. It had been demonstrated that KcsA inserted into liposomes were protected and were not digested by trypsin. In our experiment, reconstituted KcsA and detergent-solubilized KcsA were subjected to trypsin digestion for 0.5h, 1h, and 2 h at room temperature. The samples were analysed on 15% SDS PAGE afterward without boiling

treatment (Figure 12c). The apparent size of detergent-solubilized KcsA decreased over time after trypsin digestion and a few bands with the size of around 45kDa were observed. The reconstituted KcsA remained intact after 2h of incubation with trypsin. These results strongly indicated that KcsA was successfully incorporated into liposomes.

2.2.2.4 Incorporation of ion channels into planar phospholipid bilayers

Following the formation of bilayer and the reconstitution of protein into liposomes, the channel-containing liposomes were fused into the pre-formed planar lipid bilayers. Fusion was preceded first by the development of a pre-fusion state in which liposomes became closely associated with, or bound to, the planar bilayer. Since we used the planar bilayer and liposomes that contained negatively charged phospholipids (POPG), the occurrence of the pre-fusion state was encouraged by the addition of millimolar concentrations of divalent cations such as Ca^{2+} (Gregor, 1987). In experiment, we were also suggested by Dr Miller to add 1M KCl into the cis chamber just above the liposomes. This resulted in a high local salt concentration, which caused dehydration of liposomes and bilayer (Gregor, 1987) and hence encouraged the occurrence of the pre-fusion state.

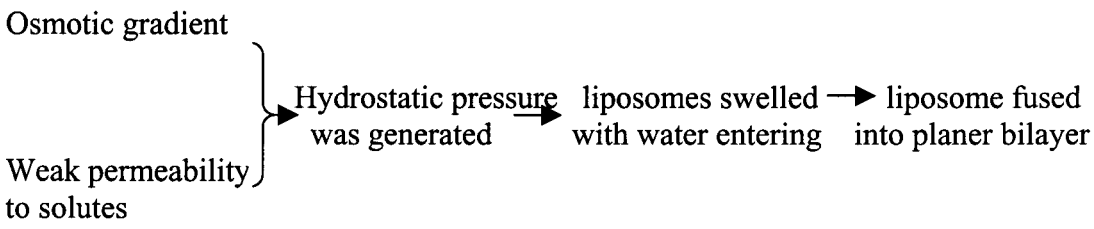
Liposomes in pre-fusion state associated with a planar bilayer would fuse with the bilayer only if they were induced to swell. Osmotic gradients with salt solutions would induce hydrostatic pressure if the liposome contained channel permeable to one or both of the ions. In experiment, the osmotic pressure of the solution in the chamber to which liposomes were added (cis) was greater than that of the solution on the other side of the



a b c

Figure 12: Determination of the correct fold KcsA inserted into the liposome. a. After centrifugation, two phases, supernatant and pellet were observed. b. Boiling test to further verify the correct folding of reconstituted KcsA(~64kDa). ▶ Shows monomer of KcsA (~21kDa). c. Time course of trypsin proteolysis for solubilized and reconstituted KcsA. Shown are KcsA (1mg/ml) incubated with trypsin (5 µg/ml) at 0.5-, 1-, 2h at room temperature. The reaction was stopped by addition of protease inhibitors (PMSF, 0.7 mg/ml). The filled arrow points to the intact KcsA (~64 kDa); the open arrow to the main cleavage product (~45kDa).

bilayer (trans). And the osmotic pressure of the solution in liposomes is greater than that in cis side. Due to the hydrostatic pressure, some of the water will flow from the trans chamber to cis chamber; some of this water will enter liposomes bound to the bilayer in the pre-fusion state. These liposomes would swell and some would burst, leading to fusion with the bilayer (Figure 13). The causal chain was thus:



In summary, liposomes should fuse into bilayer in the presence of an osmotic gradient if they contained a permeability pathway for solutes (Alan, 1994). However, when liposomes with closed KcsA channel were used, we still observed fusions (confirmed by later single channel current data) at a very low rate. This might be due to leakages in liposomes. A possible reason for leaky bilayer in liposomes was that detergents were not completely removed by dialysis. It was very likely that there were residual detergent remained in the liposome after dialysis. In our study, two kinds of detergents were used: 1) CHAPS from lipid-CHAPS micelle and 2) LADAO from protein-LADAO micelle.

To improve the fusion efficiency, we incorporated nystatin into liposomes in the presence of the ergosterol (Dixon et al., 1990). Nystatin-ergosterol channel (N/E channel) provided a weakly ion permeability pathway so that liposomes would readily fuse in the

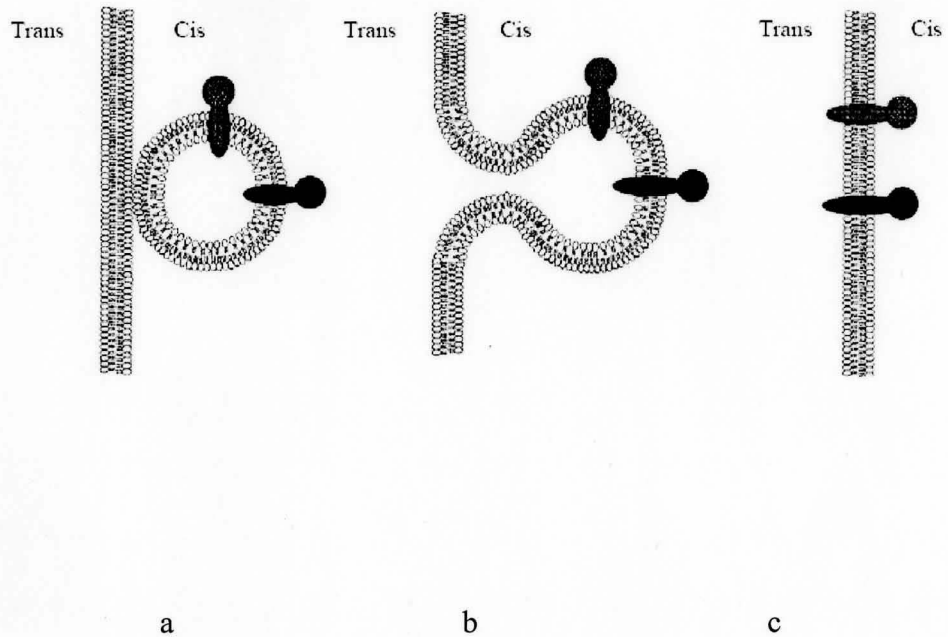


Figure 13: Fusion of a liposome with planar bilayer. a. The pre-fusion state. b. c. Establishment of an osmotic gradient across the bilayer induces the liposome to swell, burst and incorporated. (Taken from Alan, 1994)

presence of a salt gradient. N/E channel consists of about 10 nystatin monomers held together by egrosterol (Dixon et al., 1999). With this method, the probability of the fusion events increased to 50% in our experiment.

Studies of channel gating kinetics required the presence of a single channel in the bilayer and so it was important that fusion rate was controlled and stopped at an appropriate point. In practice, this could be achieved by changing both the cis and trans solutions with the same KCl concentration and thus dissipating the osmotic gradient to stop further fusion event. This method had an additional advantage that non-fused liposomes were removed from the solutions.

2.2.2.5 Current analysis

Current recordings at various clamped-voltage (-150mV~150mV) were measured for different cases: 1) KcsA single channel recording, 2) N/E channel recording and 3) KcsA mixed with N/E channel recording.

KcsA single channel recording

Although liposomes with closed KcsA had very low fusion efficiency, we were able to make about 10 recordings of the KcsA single channel (protein to lipid (v/v) ratio was equal to 1:4 in liposomes). Figure 14 shows that KcsA was a proton-activated channel. As described by Cuello et al., 1998, the channel opened significantly in planar bilayer at pH 4. We also compared KcsA channel activity at opposite voltage polarities. At high positive voltage (100mV), channel activity was marked by frequent openings throughout the duration of the recording. And the direction of the current was changed

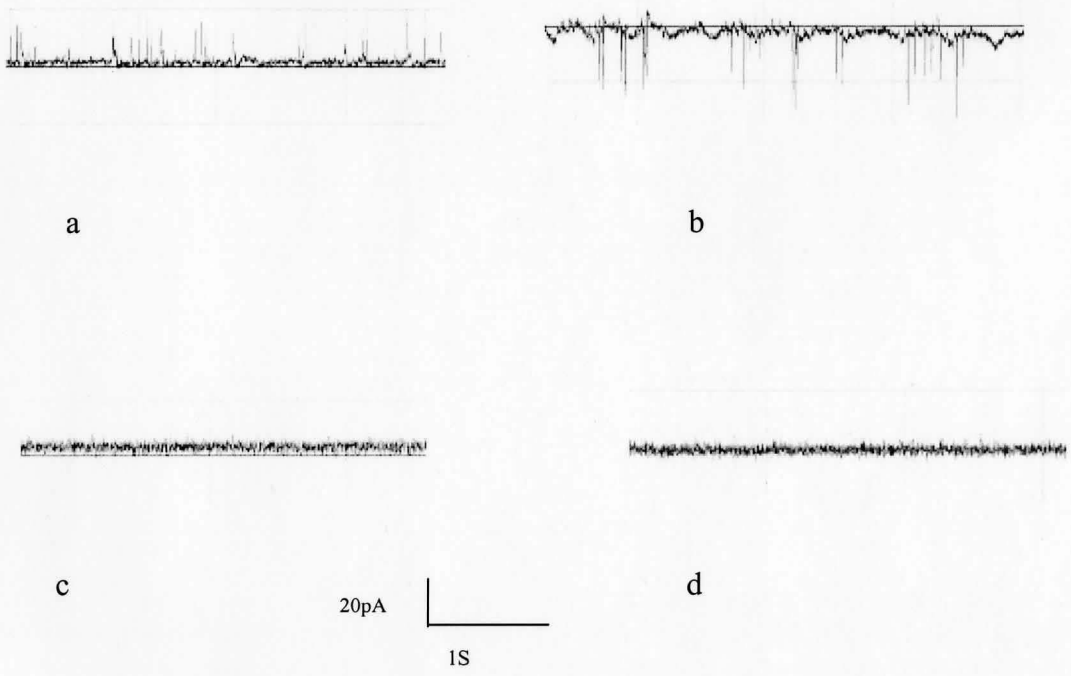


Figure 14: KcsA planar bilayer single channel recording. a. cis 100k7 trans 100k4 holding voltage 150mV. b. cis 100k7 trans 100k4 holding voltage -150mV. c. cis 100k7 trans 100k7 holding voltage 150mV . d. cis 100k7 trans 100k4 holding voltage 150mV, add 5 μl 1M Ba^{2+} in cis chamber.

when the voltage was reversed in polarity. We also found that Ba^{2+} inhibited KcsA conductance. Ba^{2+} has been viewed as a "divalent analogue" of dehydrated K^+ that prevents K^+ conduction by binding tightly within the pore (Heginbotham et al., 1998).

N/E channel recording

To develop conditions under which liposomes could be induced to fuse reliably and finally build an N/E-KcsA liposome system, which could be easily fused into planar bilayer, N/E channel recording was carried out. Figure 15a shows the effect on bilayer conductance of adding nystatin to the aqueous solution bathing an ergosterol-containing planar bilayer. With addition of nystatin to the aqueous phase, the conductance steadily increased, which suggested that N/E was spontaneously inserted. Control trace showed that the conductance of an ergosterol-free planar bilayer remained unaffected by nystatin (Figure 15b), even at 5-fold higher concentration of nystatin.

Figure 16a shows a N/E channel –induced fusion of liposomes into planar bilayer. Here, both liposomes and planar bilayer contained 20-mol % ergosterol, so that N/E channel could be formed in liposomes and in planar bilayer. With a transbilayer osmotic gradient, addition of N/E–containing liposomes resulted in discrete step increases of the bilayer conductance. The appearance of these steps was a result of independent fusion events (Dixon et al., 1990). In Figure16b, liposomes were made with N/E channel, but in this case the planar bilayer was ergosterol-free. Under this condition, the N/E channel raised bilayer conductance at the moment of a fusion event, but the conductance returned to baseline within tens of seconds. The spike amplitudes were similar to the step-sizes

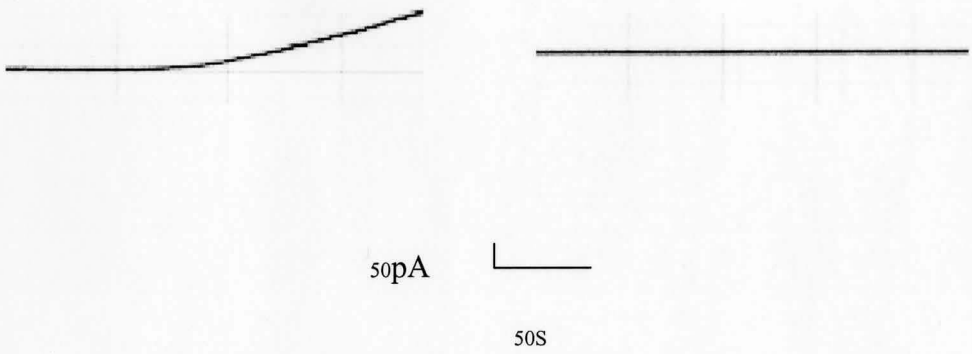


Figure 15: Ergosterol-dependence of nystatin-induced conductance. a. The effect on bilayer conductance of adding nystatin to the aqueous solution bathing an ergosterol-containing planar bilayer. 100k7 in cis 20k4 in trans, 0 mV holding voltage. bilayer form with 20 mol% ergosterol, nystatin with final concentration 50μg/ml was added in cis chamber. b. The effect on bilayer conductance of adding nystatin to the aqueous solution bathing an ergosterol-free planar bilayer. Other conditions were the same as (a).

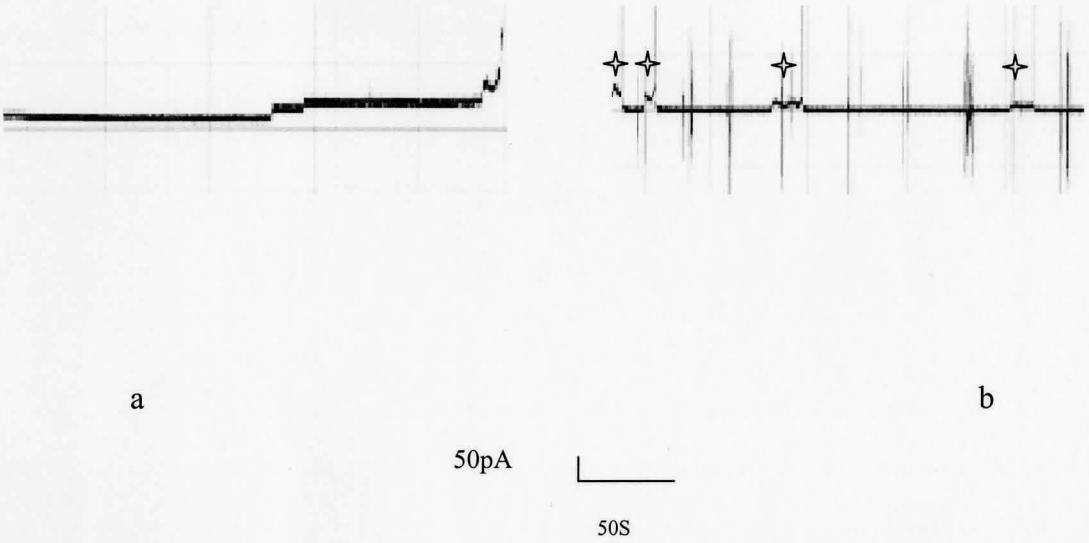


Figure 16: Fusion into bilayers of N/E containing liposome. a. Planar bilayer containing 20-mol% ergosterol separated solution with cis 100k7, trans 20k4. Just before the trace, 2 μl liposomes containing N/E (50 μg/ml nystatin, 20 mol % ergosterol) channels were added to the cis side. 100mV holding voltage. b. Fusion spikes in ergosterol-free planar bilayer. Other conditions were the same as (a). Fusion events are marked by stars. Other spikes indicated environment noise.

seen in ergosterol-containing bilayer (compare with Figure 15a). The appearance of these "spikes" could be explained as follow: as soon as the ergosterol-rich liposomes fused into the ergosterol-free bilayer, the nystatin-associated ergosterol dissociated from the channel complex and diffused away into the planar bilayer (Dixon et al., 1990), and, as a result, the channel fell apart and ceased to conduct ions.

KcsA mixed with N/E channel recording

The above results suggested a practical way to transfer reconstituted KcsA into planar bilayer. Despite diligent efforts over the past year, reconstituted KcsA showed very low rate of insertion into planar bilayer. Possibly, this failure was a consequence of a lack of permeability pathway to both anions and cations, which was required for liposome-bilayer fusion. Thus, N/E channel-containing liposomes carrying KcsA channels may offer a key to solve the fusion problem.

N/E channel-containing liposomes carrying KcsA channels (N/E-KcsA channel) were prepared by the freeze-thaw-sonication (FTS) reconstitution procedure. N/E channel-containing liposomes and KcsA-containing liposomes (protein to lipid (v/v) ratio was equal to 1:4) were mixed. During freeze-thaw, individual liposome ruptured and fused together to form large liposomes. Sonication created vibration oscillates within the solution that broke up large liposomes (Dixon, 1999). Sonicating in a bath-type sonicator for ~ 20 s yields mostly unilamellar liposomes ~ 250 nm in diameter (Dixon, 1999). After at least two cycles of FTS, N/E channel-containing liposomes carrying KcsA channels were obtained.

In Figure 17a, when N/E-KcsA liposomes were added to an ergosterol –free planar bilayer in the presence of an osmotic gradient, spikes were observed, indicating fusion events. And these spikes were similar to those seen when KcsA-free liposomes were used (Figure16b). However, apparent noise was observed after these spikes (Figure 17a). These "noise" actually represented KcsA channel activities, because KcsA channel had been inserted along with N/E-KcsA liposome fusions. After solutions in cis and trans chambers were changed to solutions with same KCl concentration, fusions stopped and only KcsA activity was monitored under the voltage clamp condition (Figure 17b).

When compared with data collected using KcsA liposomes, N/E-KcsA liposomes method produced data with more fluctuations and very short channel activity life time (KcsA channel activity stopped very soon). This might be due to effects of nystatin or ergosterol on membrane or on KcsA channel itself. Further investigations are needed to clarify the issue.

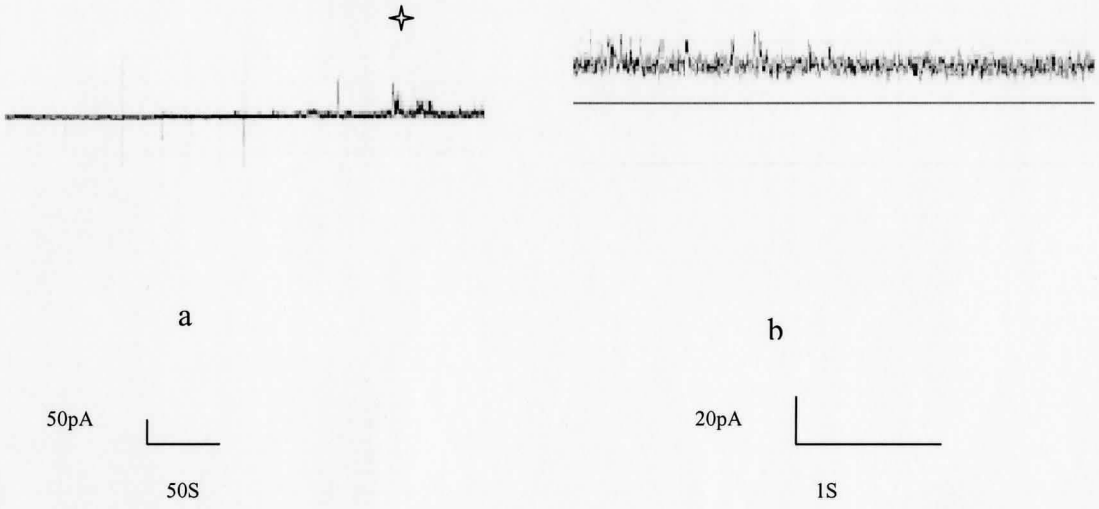


Figure 17: Fusion of N/E channel-containing liposome carrying KcsA channel into ergosterol-free planar bilayer. a. N/E channel-containing liposome carrying KcsA channel prepared as method, liposome with N/E channel/ liposome with KcsA channel ratio 2:1.Chamber separated solution with cis 100k7, trans 20k4. Just before the experiment, 2 ųl liposomes containing N/E (50 ųg/ml nystatin, 20 mol % ergosterol) channels were added to the cis side. 100mV holding voltage. The fusion events were marked by star. b. High time resolution of the fusion of a KcsA channel containing liposome. Recording begins after change the solution with 100k4 in cis and 100k7 in trans side once the noisy fusion event appears. 100mV holding voltage.

2.3 Patch clamp liposome channel recording

Single channel measurement using patch clamp generally involves 2 steps: 1) Making giant liposomes containing reconstituted purified channel suitable for patch clamp. 2) Measuring the activity of the channel under desired conditions.

2.3.1 Methods and materials

The two basic approaches, which involved the incorporation of either native or purified channel proteins into small unilamellar liposomes and the transformation of these liposomes into structures suitable for conventional patch clamp analysis, were 1) freeze-thaw and 2) dehydration and rehydration.

2.3.1.1Formation of giant liposome

Freeze and thaw

The unilamellar liposome formation procedure was the same as discussed above in the bilayer planar section, except that, after dialysis, the freeze-thaw procedure (Kasahara et al., 1977) was carried out 5-6 times by immersing <0.5 mL of unilamellar liposome suspension in a glass culture tube in liquid nitrogen for 2 min followed by thawing at room temperature. The previously clear suspension then became highly turbid.

Dehydration and rehydration

The procedure used in these studies was modified from a method previously described by Criado and Keller (1987), Anne (1989), Gloria (1990). Asolectin lipids were suspended in 10 ml of distilled water to ~100mg/ml, followed by sonication until clarity. Then, micelle solution containing 0.02mg/ml protein was added. The mixture was

dialysed three times to remove detergent. 1ml of the dialysed mixture was centrifuged at 45000g for 1hr (TLA-100 ultracentrifuge rotor) at 4^{O}C. The pellet was then resuspended in 100 µl of 10 mM HEPES buffer (pH 7.4), containing 5% (v/v) ethylene glycol. 20 µl of this suspension was then placed on a clean glass microscope slides. After dehydration for 4h at 4^{O}C in a desiccator containing $CaCl_2$, the sample was rehydrated by placing 20ul of rehydration buffer (100mM KCl, 10 mM HEPES) on top of the sample overnight at 4 ^{O}C inside a large petri dish containing a wet paper pad. Liposomes were used only on the day they were prepared.

2.3.1.2 Patch clamp recording of ion channels

Aliquots (5 µl) of giant liposomes were deposited onto 3.5 cm petri dishes with 200-300 µl buffer (10 mM HEPES, 100mM KCl, 0.1 mM $CaCl_2$, pH 7.4) and incubated for 20 min at room temperature. This treatment anchored the liposomes to the bottom of the dish, thus allowing extensive washing with buffers of choice for electrical recording (bath solution) while leaving a high number of liposomes remaining in the dish. Single channel recordings were obtained by using patch-clamp techniques as described by Hamill et al. (1981) and Cortes et al. (2001). Gigaseals (1-10GΩ) were formed on giant liposomes with regular patch pipette microelectrodes (10-20MΩ resistance for the open electrode) by apposing the pipette tip to the exposed liposome surface with the help of a micromanipulator. After the pipette was sealed, "cell attached" single channel could be recorded. Careful withdrawal of the pipette from the liposome surface and, if necessary, quickly passing the tip through the air-/water surface interface resulted in an excised patch ("inside out") configuration. In most experiments, both the patch pipette and bath

solutions were identical containing 100 mM KCl, 0.1 mM $CaCl_2$, but with different pH values in bath. The patch pipette solutions were keep 3.0. The holding potential was applied to the interior of the patch pipette, and the bath electrode was maintained at virtual ground. All experiments were conducted at room temperature. The recordings were filtered at 2 KHz and digitized by Clampit 9.0, at sampling interval ranging from 1ms to 1s. Routinely, for each holding potential, current amplitude values of channel opening events were plotted to produce histogram (current vs. number of events) and used to determine the mean amplitude values of open channel states and the open probability of channel opening under those conditions. Mean amplitude values at the different holding potentials were used to calculate the open- channel conductance from the slope of current amplitude vs voltage plots.

2.3.2 Results and discussions

2.3.2.1 Formation of large/giant liposome

Reconstituted liposomes could be formed in bulk quantities and analyzed biochemically (Miller et al., 1984). Nevertheless, even the largest reconstituted liposomes, produced by the commonly employed detergent elimination procedures, were too small to be used in electrical monitoring of incorporated ionic channels. The average size was 200 nm in diameter (Philip, 1993). Alternatively, small liposomes could be fused into large liposomes by freezing and thawing. And the formation of cell-sized, giant liposomes using dehydration/rehydration procedure presenting active, reconstituted ion channels had been reported (Criado & keller, 1987).

Comparing these two methods, we observed that liposomes formed by dehydration–rehydration method were larger than those formed by freeze-thaw method. Using dehydration-rehydration procedure resulted in the formation of cell-sized, reconstituted giant liposomes. These liposomes formed rapidly upon rehydration of the dehydrated sample, and their appearance could be monitored under an inverted microscope. Experimental variables such as lengths of dehydration and rehydration periods did not seem to affect the formation of giant liposomes. In fact, we have obtained giant liposomes by dehydrating samples from 2 to 5 h or rehydration from 4 to 24 h. However, when POPE/POPG, instead of asolectin, was used, the sizes of the resulting liposomes were greatly decreased. Giant liposome formation, therefore, was a complex process that may be affected by many variables, each of which needs further investigation. Nevertheless, when defined experimental conditions such as those described under materials and methods were used, giant liposomes were easily and reliably formed.

When liposomes were prepared using the freeze-thaw method, the size of liposomes usually ranged from 2 to 5 μm and they are mostly unilamellar (checked under inverted microscope) (Figure 18). On the other hand, when liposomes were prepared using the dehydration-rehydration method, the size of liposomes ranged from 20 to 100 μm and they were mostly multilamellar. Since unilamellar liposomes were needed for "cell-attached" patch clamp experiment, we used the freeze-thaw method for all our subsequent preparations.

2.3.2.2 Protein reconstitution into liposomes

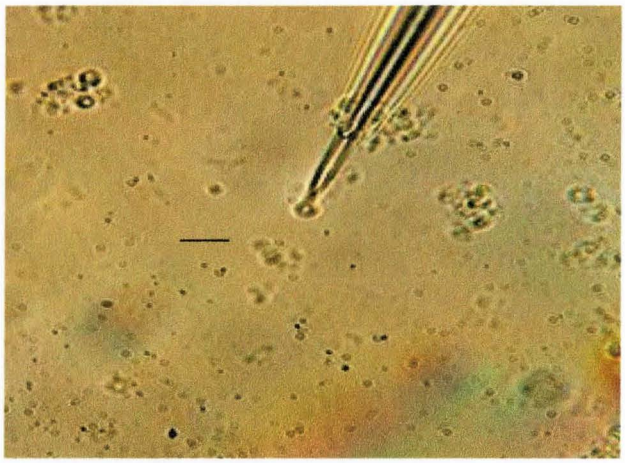


Figure 18: Formation of large liposomes by freeze- thaw .Bar shows 10 μm.

Same as bilayer planar channel recording, in the reconstitution process, two issues had to be addressed: 1) Verification of the identity of the protein of interest 2) Confirmation of the reconstitution event. These issues have been addressed above in the planar bilayer section. However, there was another question: Was KcsA reconstituted into liposomes vectorially?

Results from Rb^+ uptake and planar bilayer experiments suggested that KcsA reconstituted vectorially (Cuello et al., 1998, Heginbotham et al., 1999). Cuello et al. tried to determine the orientation of the reconstituted KcsA by performing proteolysis experiments on solubilized and reconstituted KcsA. They found that trypsination did not digest reconstituted KcsA. Since majority of trypsin cleavage sites were located at C-terminal (where the pH sensor is located) of the channel, they proposed that C- terminal segment of KcsA were located within the liposome. On the contrary, By using the conclusion from Heginbotham et al. in 1999, we deduce that majority of the pH sensor of KcsA was facing outside of the liposome. They found that mutation of Y82, a residue located on the external side of liposomes, resulted in changes in tetraethylammonium (TEA) affinity exclusively from the cis side. and thus they reported the pH sensor was facing cytoplasmic compartment. We think that Cuello group was misled by their proteolysis-controlled experiment. C-terminal segment might be protected by the liposome membrane from trypsin cleavage so that the proteolysis method could not be used to determine the vectoriality of the KcsA channel. From these two groups' research, we conclude that KcsA was reconstituted vectorially after dialysis, with pH sensor facing outward in unilamellar liposomes.

2.3.2.3 Gigaohm seal formation

When a heat-polished patch pipette is pressed against a cell membrane it may adhere tightly. The interior of the pipette is then isolated from the extracellular solution by the seal that is formed. If the resistance of this seal is infinite, no current can leak across it. High-resistance seals are often called "gigaseals".

Current leakage through the seal resistance has a crucial bearing on the quality of the patch current recording. Depending on the size of the seal resistance, a fraction of the current passing through the membrane patch will leak out through the seal and will not be measured. Thus, a high seal resistance is a prerequisite of low-noise recording.

In our experiment, upon submergence of pipette in solution, the current recorded ranged from 5 to 10 nA (depending on the composition of solution) when the voltage was held at 100mV. The corresponding resistance was thus between 10 to 20 MΩ. After formation of gigaseal, the base current was 1-10 pA, which indicated that the resistance was 1-10 GΩ. These features are consistent with the literature report.

The first difficulty in our experiment was to form stable and good quality gigaseals effectively. We found that physical stability of the working station as well as vigorous removal of detergent using bio-beads greatly improved gigaseal formation efficiency.

The second difficulty in our experiment was that when we tried to pull out the membrane from liposome, the gigaseal was easily lost. And therefore "inside-out" and "outside-out" patch clamp were not possible. We tried to make larger liposomes and hoped that membrane would be pulled out more easily. However, in our hands, the

successful rate in pulling membrane out from liposomes was still very low. This might be due to inexperience or inherent efficiency limitation. Thus, most experiments in this study utilized the "cell-attached" patching technique.

2.3.2.4 Patch clamp recording using "cell-attached" method

In our experiment, stable patches could be formed on KcsA-containing large liposomes. With pH 3.0 buffer in both patch pipette and bath, ion channel activity could be detected in 30% of trials. The main features of this ion channel were that it fluctuated between close and open states, but with a different opening probability at different pH values. As the pipette potential was made increasingly positive, the probability of channel opening increased; and the opening probability decreased at negative voltage potential (Figure 19). These features, including the characteristic pH gating, and high opening probability at positively holding voltages were similar to those reported by Miller group (Heginbotham et al., 1998) and Perozo group (Cortes et al., 1997, 2001; Cordero-Morales et al., 2006).

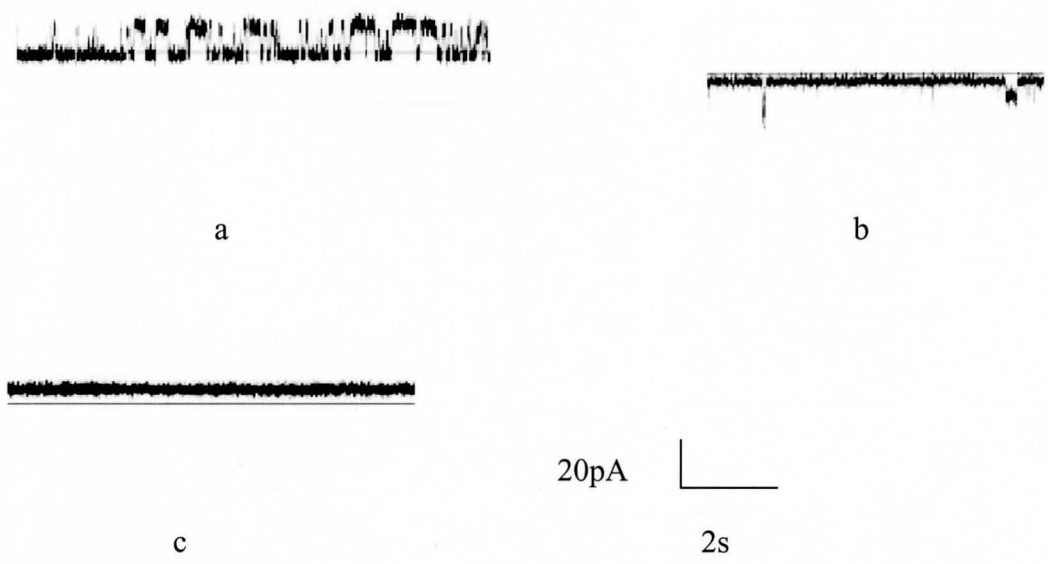


Figure 19: KcsA patch clamp liposome single channel recording. a. patch pipette 100k3 bath 100k3.8 holding voltage 150mV. b. patch pipette 100k3 bath 100k3.8 holding voltage -150mV. c. patch pipette 100k3 bath 100k7 holding voltage 150mV

2.4 CONCLUSION

Clearly, there are a number of methods available to researchers who wish to investigate the properties of an ion channel in a reconstituted system. Before embarking on a study using any of these approaches, it is worth spending some time considering which is the most appropriate for the task. For our KcsA study, two factors were considered:

Ease of use and reliability

Planar bilayers have been extensively used since they can be voltage-clamped and provided easy access to both sides of the membrane. In our experience, painted bilayers were the easiest to make and will provide a mechanically and electrically stable environment for KcsA. However, this approach may not be suitable for our application. Firstly, we had little control on the insertion of proteins into bilayer (Miller et al., 1983). Secondly, when we employed N/E channel liposomes, the bilayers contained some solvents, which might affect the channel performance.

A disadvantage of patch clamping giant liposomes when compared to using planar bilayer was that, because of the limitations of the use of "inside-out" and "outside-out" patch clamp, monitoring KcsA single channel activation with different pH was not easy. For "cell attached" patch application, monitoring KcsA activation in large unilamellar liposome was complicated by desensitization caused by other channels in the same liposome.

Resolution

The usefulness of a particular system of ion channel reconstitution is limited by the signal-to-noise ratio. For planar bilayer system, large membrane area was necessary for high liposome fusion rate. However, large membrane area also led to large noise (The Axon guide, 1993). On the other hand, patch clamping had a much higher signal-to-noise ratio.

In conclusion, although each method has its own advantages and disadvantages, we have decided to use the patch clamp method for our subsequent study. This was because it gave us a good signal-to-noise ratio, which allowed us to perform meaningful statistical analysis. More importantly, the rate of liposome fusion in the planar bilayer method was, despite our effort for improvement, so low that our work progress would have been greatly impeded.

CHAPTER 3

FUNCTIONAL CHARACTERIZATION OF PROTON ACTIVATED POTASSIUM CHANNEL-KcsA

3.1 Introduction

The activity of ion channels is governed by mechanisms that allow the selective conduction of ions across the membrane (selection) and control the onset and duration of the conductive state (gating) (Julio et al., 2006). Reported studies and our recent data on wild type KcsA have shown a pH-dependent current change. We suggest that this characteristic is due to histidine acting in concert with the sensor located in the inner helix bundle. At the single channel level, gating is characterized by transitions between conductive and nonconductive conformations via structural rearrangements along the permeation path (Mackinnon, 2004). Quantitative analysis of measurable variables (e.g. the channel open amplitude, the channel-open duration) and comparison of these quantities with theoretical distributions could be performed in order to infer biological mechanisms. Thus, by comparing wild type with Histidine mutant KcsA using single-channel measurements, we can deal with the key issue regarding the molecular basis of activation gating in KcsA channels: define the roles of histidine in gating and establishing the structural basis that underlies gating.

The first part of this chapter will discuss some of the fitting and statistic method for analysing channel opening event in "cell-attached" patching case. In the second part

of this chapter, data were analysed and deduction on mechanisms on pH-dependent opening of KcsA in the molecular level was attempted.

3.2 Statistical analysis of single-channel recording

The analysis procedures we describe below involve: 1) Identification of channel events. 2) Analysis of channel current amplitudes. 3) Analysis of channel open probability.

3.2.1 Identification of channel events

The problem in identifying channel events in an experimental record is how to distinguish real channel activity from noise. Electronic filter is often utilized for identification. However, filtering might also result in loss or distortion of real channel activities.

In order to find a suitable filter, we used a model developed by Bert et al. (1995). In this model (Figure 20), the unfiltered current signal x(t) is represented as the sum of real channel activity s(t), and noise n(t). y(t) is the output from filter, which is detected by a threshold detector Φ. Therefore, a certain channel activity can be detected only if y(t) > Φ at time t.

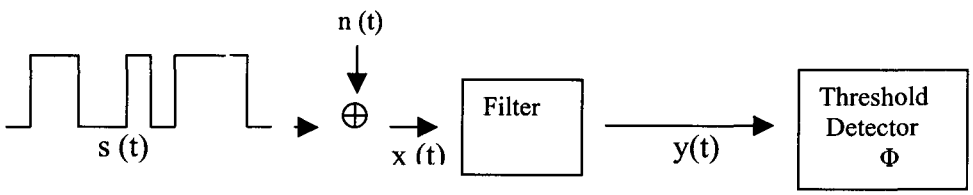


Figure 20. Model of single-channel event detection.

The following steps were used to identify a suitable filter: 1) Choose a filter value that is as large as possible. 2) Choose a threshold level Φ based on amplitudes of channel activities. 3) Measure noise n(t) with a RMS voltmeter (Figure 21) and check if n(t) is lower than Φ. 4) If not, decrease the filter value.

Throughout this section, we have assumed the base-line level to be zero. In experimental records the base-line current level was nonzero and typically a slow drift with time was observed. The most reliable correction technique was to fit the base line manually (Figure 22).

In our study, a filter value of 2 kHz was chosen. With this filter, we could detect current larger than 1 pA. An example of our recording is illustrated in Figure 23.

3.2.2 Analysis of channel current amplitudes

3.2.2.1 Characterization of current amplitudes

In theory, single-channel current events should appear as rectangular pulses of fixed amplitude, except in the case of multiple conductance levels (Bert et al., 1995). However, we did observe variation in current amplitude in our experiments. These variations could arise from two sources.

The first source of the observed variation could be related to the filter bandwidth. Each filter has a particular rise time T, which is inversely proportional to the filter bandwidth. T is the time needed for the filter to give a full-amplitude response when a square step is applied (Bert et al., 1995). Events that are shorter than T will never reach full amplitude (Figure 24). In our experiments, a 2-kHz Bessel filter with a rise time of

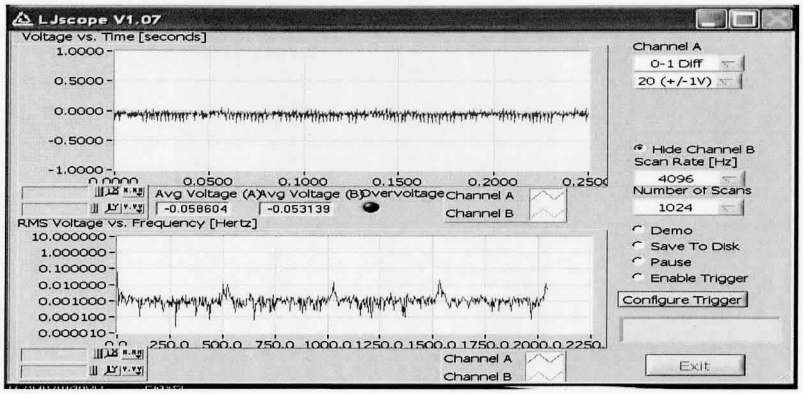


Figure 21: Noise level showed by RMS voltage. 0.01v is corresponding to 1pA

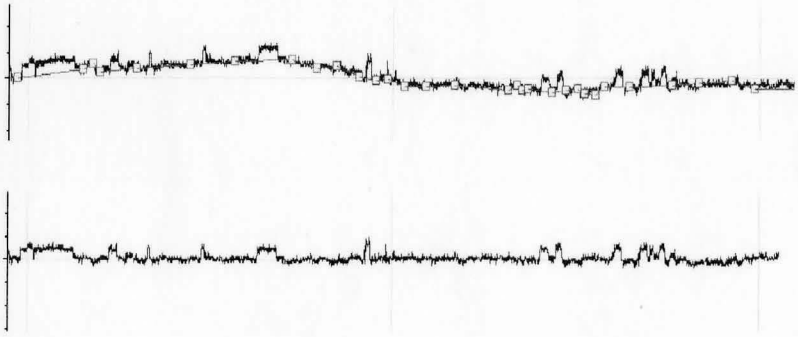


Figure 22. Manually adjust baseline.

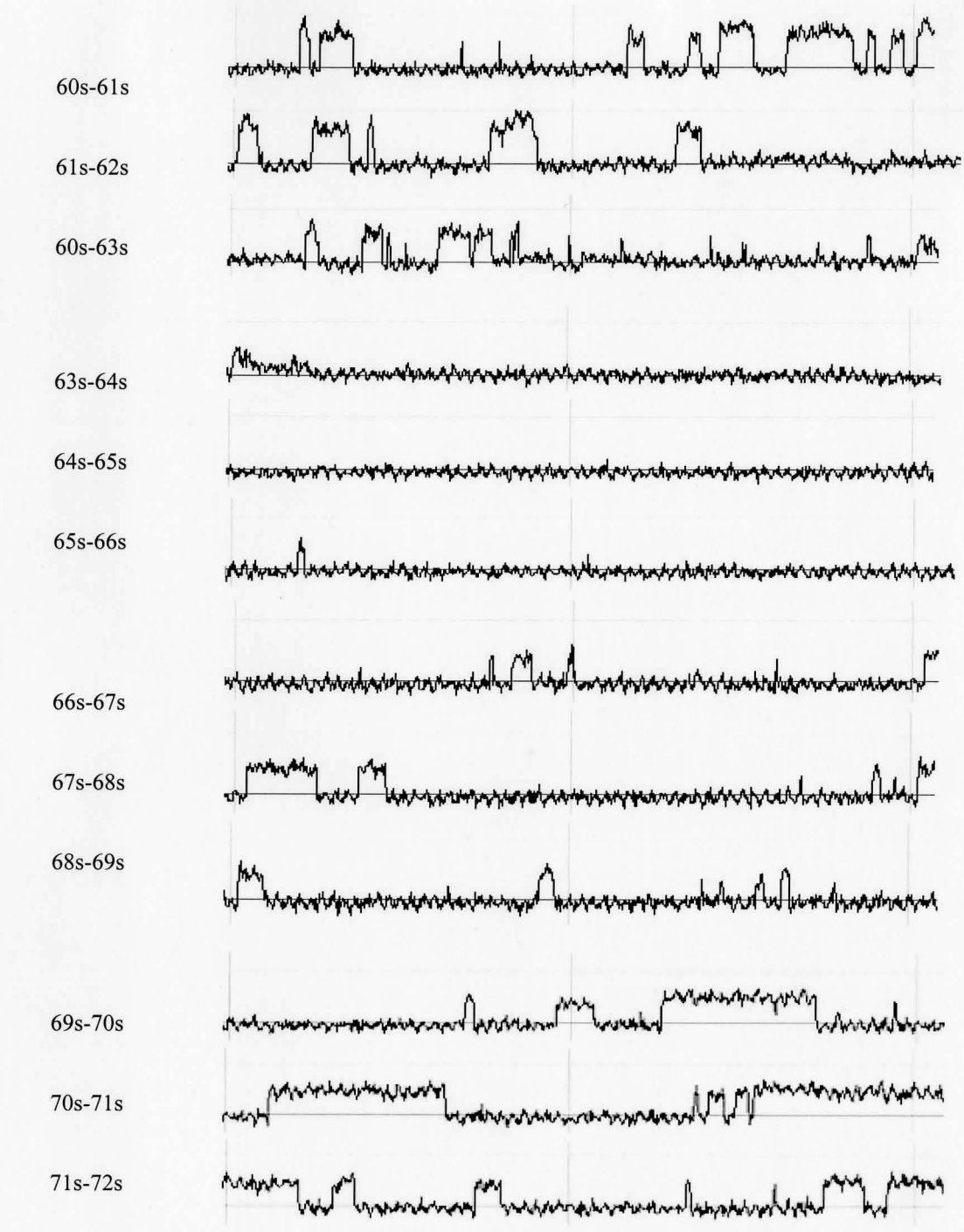


Figure 23. A continuous 12-sec segment of the recording on wild type KcsA. 150mV holding voltage, pH 3 buffer in both pipette and bath.

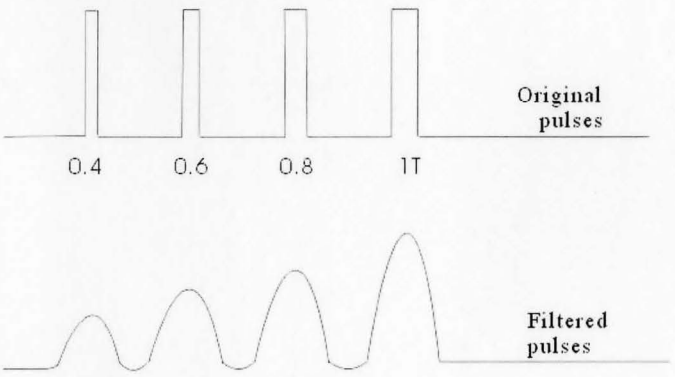


Figure 24: Relationship between true amplitudes and filtered amplitudes. Note that when the duration is shorter than T, the filtered amplitude will be distorted. (Bert et al., 1995)

about 165 μsec (Bert et al., 1995) was used, and thus events shorter than 165 μsec would appear shorter than their real amplitudes.

The second source of variation in amplitudes could be a result of the fact that we were measuring the sum current of many individual channels on the liposome. "Cell-attached" configuration was used in our experiments (Figure 25). The sum current I was equal to:

$$I=v/[R1+1/(1/R2+1/R3\ldots+1/Rn)]$$

where v = applied voltage,

R1 = resistance of channel within patch pipette,

R2, R3...Rn = resistances of other channels on liposome.

The number of opened channels varies over time because opening of channel is a stochastic event. Therefore, the resistance of the liposome and thus the current amplitude we measured also varied over time.

3.2.2.2 Amplitude distributions and fitting of KcsA and its mutant

In order to extract more information from the variation in the measured current amplitude, we counted (using the software Clampfit 9.0) the number of event of each measured amplitude and created histograms (Figure 26). From these histograms, we observed normal distributions of current amplitudes.

The individual normal distributions were then fitted into a Gaussian function to determine the mean conductance of the channel:

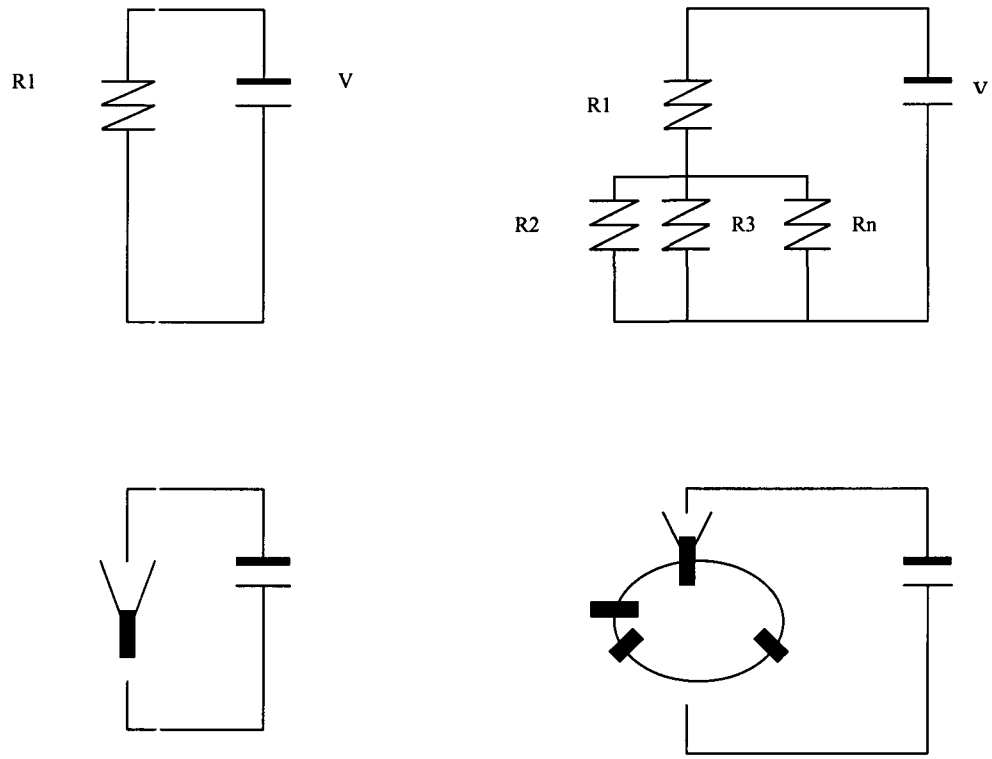
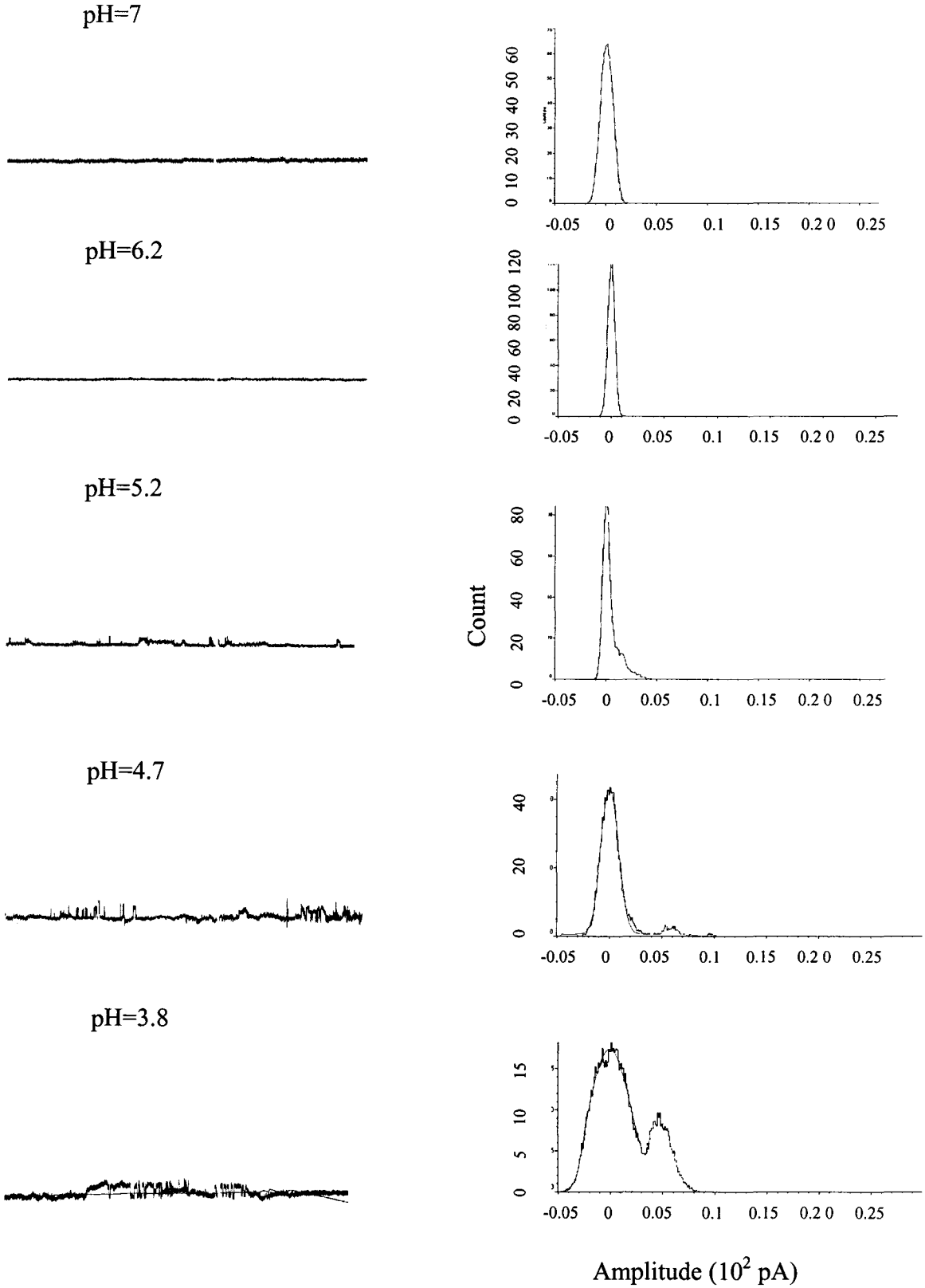


Figure 25: Patch clamp illustrations. Upper panel: Circuit diagrams. Lower panel: Corresponding patch clamp configurations. a. Excised patch with one channel. b. Cell-attached configuration.


pH=7
pH=6.2
pH=5.2
pH=4.7
pH=3.8
Count
0 10 20 30 40 50 60
0 20 40 60 80 100 120
0 20 40 60 80
0 20 40
0 5 10 15
-0.05 0 0.05 0.1 0.15 0.2 0 0.25
Amplitude (10^2 pA)

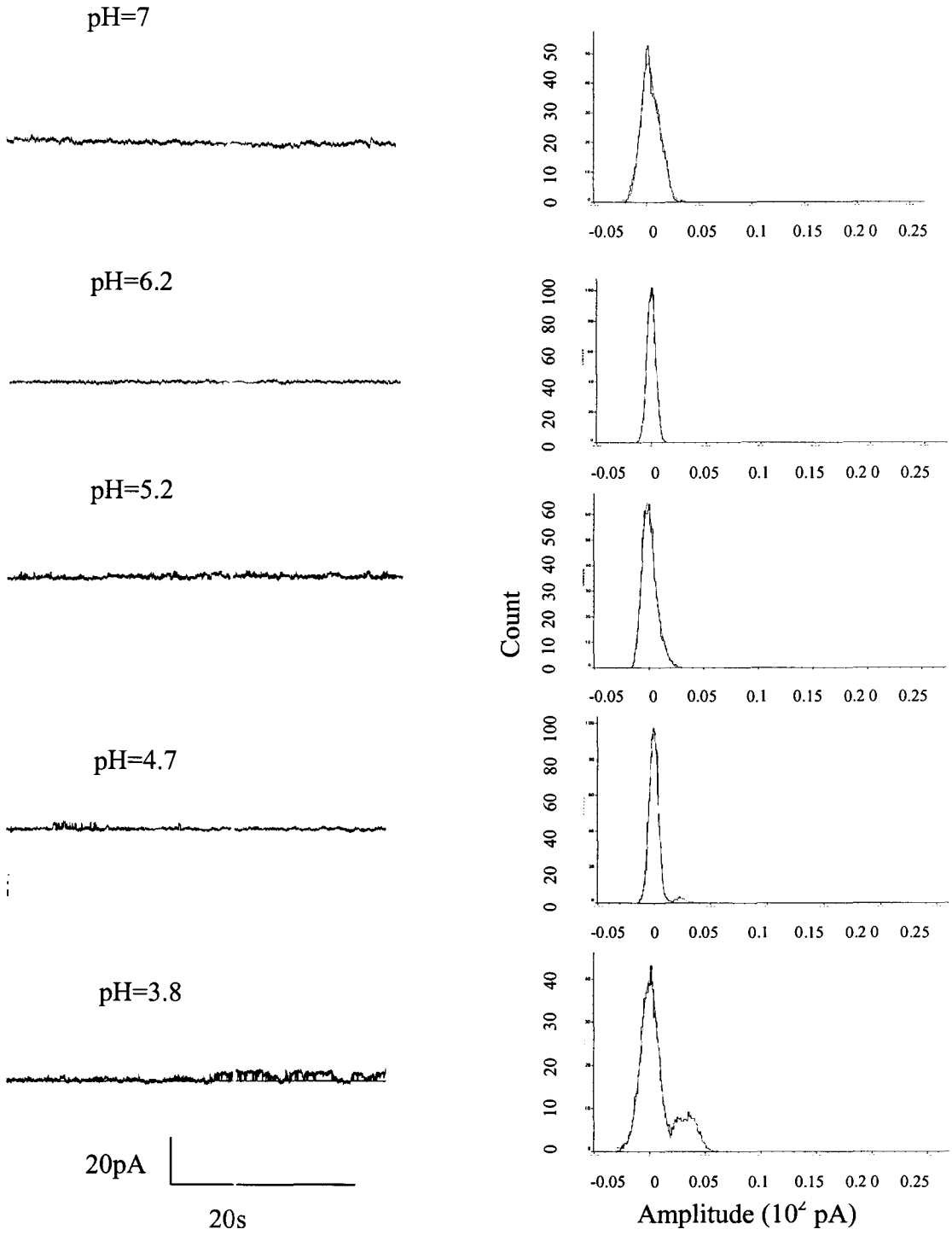


Figure 26: Amplitude histogram. Histogram of all points' amplitude. the abscissa is the step amplitude in picoamperes; the ordinate is events per bin. Bin width of 0.001mV (0.1pA). Superimposed lines indicate a Gaussian function with mean value shown in peak. a. Wild type's current and amplitude histogram with different pH buffer, b. Mutant type's current and amplitude histogram with different pH buffer. The entire recording holding voltage is 100mV.

$$f(x) = \sum_{i=1}^{n} A_i \frac{e^{-(x-\mu_i)^2/2\sigma_i^2}}{\sigma_i \sqrt{2\pi}} + C$$

where A_i = number of count,

μ_i =Gaussian mean,

σ_i =Gaussian standard deviation

C =constant y-offset.

For wild type KcsA, when the holding voltage was 100 mV, there was only one single peak in the histogram at pH 7.0 and pH 6.2 (Figure 26). As the pH decreased, another peak appeared (Figure 26). The number of peaks equalled to the number of conducting states of the channels. Thus, there were 2 states in KcsA. The Gaussian means and standard deviations of channel currents measured at different pHs and different voltages were summarized in Table 1a. These results indicated that when pH was 6.2 or above, majority of the channels were in closed state (state 1). More channels were in open state (state 2) as the pH decreased to 5.2 or below.

For the H124A mutant of KcsA, normal distributions were also obtained. However, one important difference was that only one peak was observed at pH 5.2 (Table 1b).

The significance of above results will be discussed in section 3.3.

Table 1a. Current amplitude of wild type in different pH bath buffer

	pH=3.8		pH=4.7		pH=5.2		pH=6.2		pH=7.0	
	State1	State2	State1	State2	State1	State2	State1	State2	State1	State2
-150mV	-	-	-	-	-	-	-	-	-	-
-100mV	-0.31(+/-0.50)	-4.43(+/-0.15)	-	-	-	-	-	-	-	-
-50mV	-0.03(+/-0.05)	N.D.	-	-	-	-	-	-	-	-
0mV	-0.04(+/-0.06)	N.D.	-	-	-	-	-	-	-	-
50mV	0.03(+/-0.68)	N.D.	-	-	-	-	-	-	-	-
100mV	-0.07(+/-0.23)	4.65(+/-1.20)	0.07(+/-0.81)	5.33(+/-0..21)	0.01(+/-0.03)	1.70(+/-0.18)	0.33(+/-0.01)	N.D.	-0.02(+/-0.75)	N.D.
150mV	-0.03(+/-0.01)	9.18(+/-0.55)	-	-	-	-	-	-	-	-

N.D. = Not Detectable

*All data recorded with pH 3.0 buffer in patch pipette. Number shown in the table without and with blanket are corresponding to Gaussian mean current and its standard deviation, respectively.

Table 1b. Current amplitude of mutant type in different pH bath buffer

	pH=3.8		pH=4.7		pH=5.2		pH=6.2		pH=7.0	
	State1	State2	State1	State2	State1	State 2	State 1	State2	State1	State2
-150mV	-	-	-	-	-	-	-	-	-	-
-100mV	0.09(+/-0.01)	-1.8(+/-0.21)	-	-	-	-	-	-	-	-
-50mV	-0.06(+/-0.01)	N.D.	-	-	-	-	-	-	-	-
0mV	0.02(+/-0.33)	N.D.	-	-	-	-	-	-	-	-
50mV	0.15(+/0.21)	N.D.	-	-	-	-	-	-	-	-
100mV	0.55(+/0.01)	3.20(+/-0.62)	0.29(+/-0.02)	2.30(+/-0.49)	0.61(+/-0.73)	N.D.	0.05(+/-0.42)	N.D.	0.09(+/-0.03)	N.D.
150mV	0.47(+/-0.12)	6.30(+/-0.41)	-	-	-	-	-	-	-	-

N.D. = Not Detectable

*All data recorded with pH 3.0 buffer in patch pipette. Number shown in the table without and with blanket are corresponding to Gaussian mean current and its standard deviation, respectively.

3.2.3 Analysis of channel current open probability

3.2.3.1 Characterization of current open probablity

We used the concept of open probability to quantitatively analyse current open durations. We observe that variation of open probability with different pH value in both wild type KcsA and mutant (Figure 26). Since the measured current was the sum current of many individual channels in "cell-attached" case, the open probability of measured activity were reflections of the states (open or closed) of all channels on the liposome.

Throughout the whole experiment, pH of buffer in pipette remains constant as 3 and pH of the bath buffer were changed (from 3.8, 4.7, 5.2, 6.2 to 7). When the pH sensor domain of channel was facing the pipette (see section 2.3.2.2), the open probability of channel (within patch pipette) were expected to be constant if and only if the total of other channels on the liposome $[(N-1)p_o] >= 1$,

where N=number of channels in the lipsome.

p_o= individual channel open probability.

The concentration of KcsA and lipid were known, the number of channel inserted into a liposome could be estimated, Up to 22 channels could be inserted into one liposome after reconstitution (See Appendix for full calculation). However, since channel might not be completely functional ((Dixon et al., 1999) and individual open probability of KcsA was very low in high pH value buffer (Heginbotham et al., 1999). The $(n-1)p_o$ might be less 1, which prevented us from observing the constant of patched channel open probability.

3.2.3.2 Determination of open probability of KcsA and its mutant

To determine the total open probability NP_o of channels on the liposome, the following equation was used:

$$NP_o = [T_o/(T_o+T_c)] \text{ X } 100\%$$

where T_c = total closed time,

T_o = total open time.

Table 2 summarized NP_o of channels measured at different pHs at 100 mV holding voltage. It could be seen that NP_o increased as the bath pH decreased. See the discussion in section 3.3.

Table 2. Open probability (NPo) of wild type and mutant KcsA in different pH bath buffers.

	PH=3.8	PH=4.7	PH=5.2	PH=6.2	PH=7.2
Wild	35.15%	18.98%	9.46%	0%	0%
Mutant	28.36%	8.09%	0%	0%	0%

3.3 Molecular basic analysis of pH sensitivity in KcsA

The small potassium channel KcsA has become a popular biochemical preparation for studying structural aspects of ion channels. Currently, there is a considerable amount of high-resolution structural information on its close conformation, which facilitated a better understanding of ion permeation at the molecular level (Doyle et al., 1998; Zhou et al., 2001). We now know that KcsA channel is activated by protons (Cuello et al., 1998), proton seems to act at the intracellular side of the protein (Heginbotham et al., 1999) and proton produce channel activation by controlling the activating gate (Perozo et al., 1998, 1999). Although it is quite possible that this protonation effect may not be KcsA primary gating mechanism, it might still play an important modulator role (Heginbotham et al., 1999). In addition, these findings have opened the possibility of using electrical single channel recording to probe the functional behaviour of purified KcsA under a wide range of conditions.

Paradoxically although all these functional studies have been done, the molecular basis of KcsA pH sensitivity has remained highly elusive. In addition, several scientific reports have attributed KcsA pH-sensitivity to different factors, which include the presence of negative lipids on the membrane (Heginbotham et al., 1998), and voltage-dependent inactivation at the KcsA selective filter (Julio et al., 2006). However, the central aim of the studies described in this chapter was to identify KcsA structural elements responsible of pH sensitivity. In order to address this question, we attempted to identify these elements based on published literature.

KcsA can be divided into three regions: N-terminal helix, transmembrane domain and C-terminal domain (Cortes et al., 2001). The N-terminal helix spans residue 1-20 and is thought to be sitting along the membrane-cytosol boundary in a titled fashion (Cortes et al., 2001). Although the N-terminal helix was intact when KcsA was crystallized, its structure could not be resolved. The transmembrane domain, residue 23 –119, forms the pore of the potassium channel and its structure was first reported in 1998 (Doyle et al., 1998). The remaining residues, 120 to 160, are the c-terminal domains and the majority of it was removed by protease before crystallization; therefore, the reported KcsA structure only contains the region between residues 23 –125. The crystal structure of the cytoplasmic domain (residues 126- 160) has yet to be determined (Zhou et al., 2001).

Previous studies showed that KcsA conducts potassium across the membrane when the pH on the cytoplasmic side becomes acidic (Heginbotham et al., 1999). This suggests that the sensors of KcsA are located in the structural elements (N-terminal helix and C-terminal domain) facing cytoplasmic compartment. It has been demonstrated that deletion of residue 120-160 changes the profile of pH-dependent opening of the channel (Cortes et al., 2001). This observation suggests that residues 120-160 are involved in the pH-dependent opening of KcsA. On the other hand, Deletion of N-terminal helix did not change pH sensitivity, hence ruling out its possible role as part of KcsA pH sensor (Perozo et al., 1999, Cortes et al., 2001). From these results, pH sensor is likely to be located on the C-terminal domain of the protein.

The KcsA C-terminal domain contains a high density of charged amino acids (about 50% of the residues in this region are charged). At neutral pH, the theoretical

charge in this region is slightly positive, about +2. This cluster of charged residues very likely stabilizes the closed conformation of KcsA through electrostatic interaction. When the pH in the cytoplasmic compartment becomes acidic, this region will become more positively charged due to protonation of the residues; and the repulsion of the positively charged residue shift the closed-open equilibrium to the open conformation (Cortes et al., 2001). This charged cluster is located at the end of the inner helices and right after the convergence point of the internal helices or 'gate'. Thus, this region is a good candidate for the pH sensor.

In 2001, Cortes et al. demonstrated that the opening/closing transition (50% open probability) pH of KcsA was around 6.5. The theoretical pKa of the side-chain of the histidine is also close to this transition pH (Figure 27). There are three histidines (H124, H128, H145) in the C-terminal domain and they may be the pH sensors. Since removal of residue 140 –160 of the C-terminal domain did not affect the opening/closing transition pH profile of the channel, H145 is very unlikely to be part of the pH sensor (Cortes et al., 2001). Therefore, in the present study, we would like to determine if H124 is part of the sensor. H124A, a full-length mutant of KcsA, was generated and its activity was compared with the wild type at different pH.

Our wild type KcsA data showed that its current conductance and opening probability were pH-dependent. The channel was in open state when the pH was between 3.8 and 5.2; however, both the current conductance and opening probability of the channel decreased when the pH of the bath solution increased (Table1A and Table 2). For

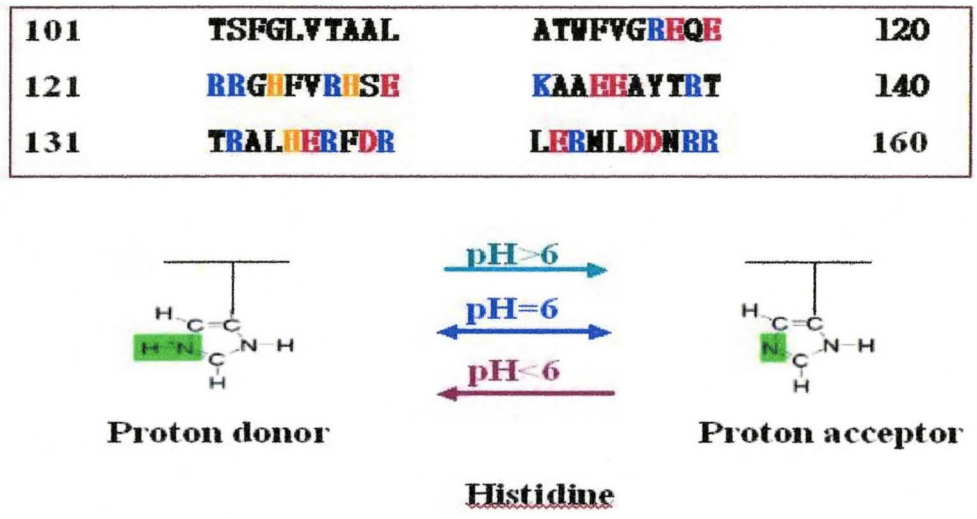


Figure 27: The role of histidine. Upper: Show the charge of c-terminal. Lower: show the role of histindine with pH dependence.

H124A mutant, its current conductance and opening probability were lower than that of the wild type at the same pH. Indeed, wild type KcsA was still slightly opened at pH 5.2, but H124A mutant was closed (Table 1B and Table 2).

It would be easier for us to determine the effect of H124A mutation on the activity of the channel if we could directly compare the mean conductance and open probability of the wild type with that of the mutant at various pHs. Since the number of channels incorporated into liposomes in each experiment might be different, normalization of the measured mean current amplitude and NP_o had to be performed in order to facilitate direct comparison between wild type KcsA and its mutant. The measured mean current amplitude and NP_o were normalized to the corresponding values obtained at pH 3.8, because both channels were assumed to be fully opened at this pH (Figure 28). It appeared that the mutation caused a reduction in transition pH. However, the transition pH derived from using normalized amplitudes and that from normalized NP_o were different (compare Figure 28a and 28b). We think that transition pHs derived from normalized amplitudes would be more accurate because measurement of amplitudes included many individual channel events whereas calculation of NP_o treated all channel events as one single entity.

Our data showed that the opening/closing transition pH for wild type was around 5 (Figure 28a), which was different from the transition pH reported by Cortes et. al. in 2001. It is very likely that full protonation of histidines is not sufficient to cause channel opening. The lower transition pH indicated that other charged residues besides histidines

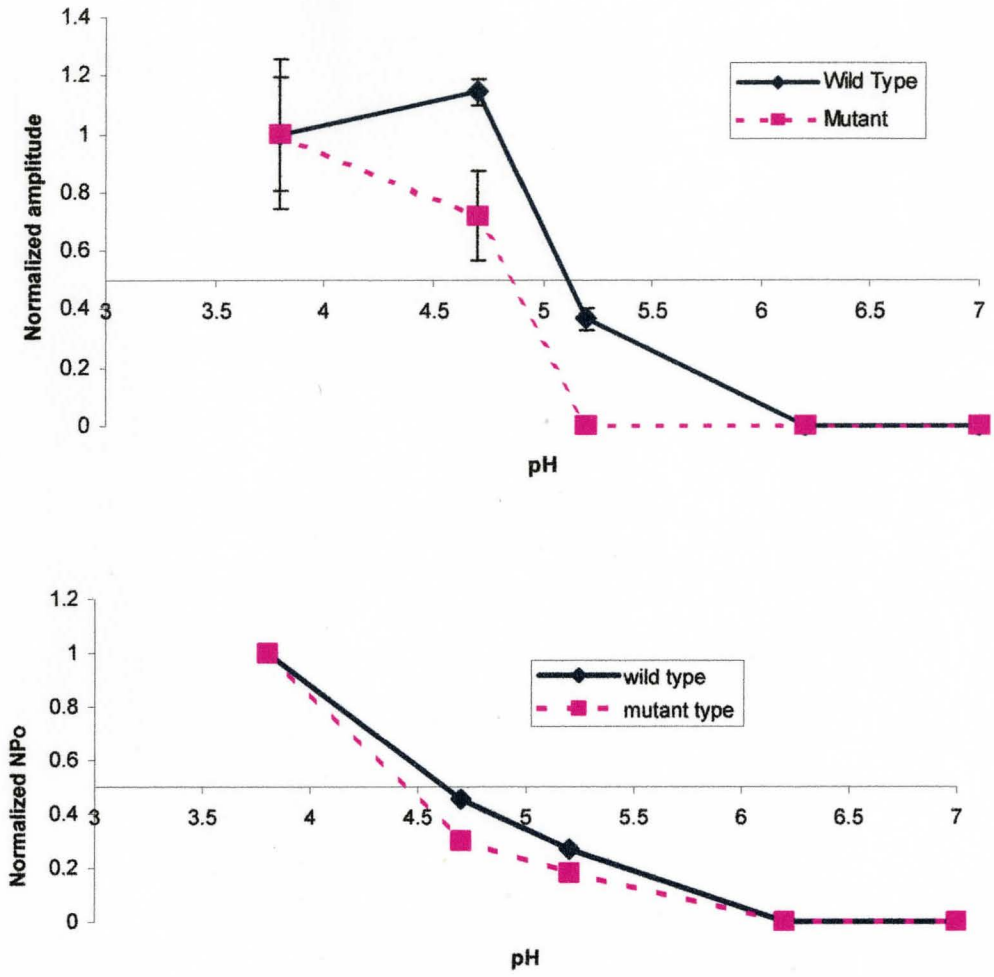


Figure 28: Effect of wild type KcsA and mutant H124A on the pH dependence.
Data were normalized at pH 3.8 based on Table 1, Table 2. X-axis intercepts at a value of 0.5 to facilitate to determine the pH value for the transition. a. pH vs normalized mean amplitude. Data shown wild type KcsA, H124A transition pH value were ~5.1, ~4.7 respectively. Error bars represent the SD and correspond to the Gaussian standard deviation. b. pH vs normalized NPo. Transition pH with wild type and H124A mutant were ~4.7, ~4.4, respectively.

might be involved in the opening event. In the C-terminal domain, there are also seven glutamates whose side chain has a theoretical pKa of 4.25 (David L. Nelson et al., 2000) . When the pH of the cytoplasmic compartment is lower than 5, the glutamates start to be protonated and the C-terminal domain becomes more positively charged leading to channel opening. When the H124 was mutated to alanine, there was one less positive charge on the mutant than wild type at pH 5. Thus, the repulsive force in the C-terminal domain of the mutant was not sufficient to open the channel. To compensate for the loss of positive charge due to H124A mutation, more glutamates have to be protonated. As a result, the opening/closing transition pH of the H124A mutant was lower than wild type.

3.4. Conclusion

In the beginning of our study, we proposed that the C-terminal domain is the pH sensor of KcsA and that histidines in this region are the key residues responsible for the pH-dependent channel opening. Complete protonation of these histidine residues was proposed to be sufficient to cause channel opening. From our results, the opening/ closing transition pH of wild type KcsA was 5 instead of 6.5 reported by Costes et al. in 2001. Our finding is consistent with values reported by other single channel measurements in membrane bilayer studies (Stacey et al., 2002). Since the transition pH is lower than the theoretical pKa of histidine, other residues such as glutamates may also be involved in the channel opening process. It is very likely that full protonation of the histidine residues in the C-terminal domain is not sufficient to induce channel opening. The negative charges on some of the glutamates have to be neutralized by protonation leading to a more positively charged C-terminal domain in order to open the channel. This hypothesis was further justified in the H124A mutant. When the positive charge was removed from the C-terminal domain, more glutamates had to be protonated to cause channel opening and we observed a lower opening/ closing transition pH in the mutant. To sum up, pH-dependent opening of KcsA is based on the charge balance in the C-terminal domain. Protonation of both histidine and glutamate residues in this region is responsible for such event.

Since the data used in our analysis was collected by using the "cell-attached" patching method, and we only had one complete set of data due to time constrain, accuracy in determining the open probability of KcsA was very limited. Other techniques

such as "inside-out" and "outside-out" patch clamp may give us more accuracy in the patched channel's open probability and they should be utilized in future studies.

In spite of these limitations, however, our proposed model offers a first glimpse of the pH-dependent opening mechanism of KcsA. We expect that future studies will further improve our understanding of channel gating activity.

Appendix

Calculation the number of channels in liposome

50 μl 0.2 mg/ml Protein-detergent micelle solution reconstitution into 200 μl 10 mg/ml lipid micelle solution.

Suppose phospholipids/vesicles is $4.19*10^5$ ((Philip l.Yeagle, 1993).

Phosoholipid liposome molecular weight (POPE 718.01g/mol. POPG 770.99g/mol):

$4.19*10^5$/liposome*(1/3*718.01g/mol+2/3*770.99g/mol)=$3.15*10^8$g/mol

[liposome] origin=$10*10^{-3}/10^{-3}/3.15*10^8$ =$3.15*10^{-8}$M

[protein]origin=$0.2*10^{-3}/10^{-3}/64000$=$3.125*10^{-6}$ M

[liposome] mix=$3.15*10^{-8}*200/250$ =$2.52*10^{-8}$M

[protein]mix=$3.125*10^{-6}*50/250$=$6.25*10^{-7}$ M

So the protein/liposome ratio is $6.25*10^{-7}/2.52*10^{-8}$=24.80

If there are only 90% protein reconstituted into liposome, then the ratio is

24.80*90%=22.32

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