Expression and purification of the K2P family of ion channels

Drew Barber

*University at Albany, State University of New York, PALaFrance@aol.com*

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EXPRESSION AND PURIFICATION OF
THE K₂P FAMILY OF ION CHANNELS

by

Drew Barber

A Thesis Submitted to the
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Abstract

The KCNK (two-pore-domain K+ channel) family of potassium channels are the most widely expressed potassium channels in mammals. They are expressed in numerous organs including the brain, heart, adrenal glands, and kidneys. There are fifteen members of this family which can be further divided into six sub groups. These proteins can act as leak channels, setting the rest membrane potential of electrically excitable cells. Due to their function as modulators of excitability these proteins are under control from numerous different chemical and physical signals which include oxygen tension, pH, lipids, mechanical stretch, and neurotransmitters. These channels are not as well characterized as other potassium channels. High resolution, three dimensional structures would greatly enhance the study of the K2P proteins. Currently there are only two published crystal structures; Kcnk1 and Kcnk4. It was the goal of this project to generate soluble protein that could be used for crystallization. Initially eight channels were cloned into Escherichia coli using the Gateway® Cloning system developed by Invitrogen. However, E.coli was unable to express the protein. Pichia Pastoris was then used for expression. Five channels, Kcnk1, Kcnk3, Kcnk5, Kcnk13 and Kcnk18. Kcnk 3 was expressed and successfully purified from P.pastoris.
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Chapter 1. Introduction

Prior to 1995 only single pore forming domain potassium channels were known to exist. It wasn’t until TOK1 was found in Saccharomyces cerevisiae that a two P-domain potassium channel was discovered\(^1\). Following the discovery of TOK1, Kcnk0 was cloned from the neuromuscular tissue of Drosophila melanogaster and soon after the first mammalian gene, KCNK1 which encodes the K\(_2\)P1 (also known as Kcnk1) potassium channel subunit, was also cloned\(^2,3\). Currently there are fifteen known members of the K\(_2\)P\(^3\). These channels differ from other potassium channels, which have one pore spanning loop and form tetramers, in that they have two pore spanning loop domains and exist as dimers\(^4\). K\(_2\)P channels have also been shown to primarily act as leak channels, however, at least one member, Kcnk2, has been shown to function as both a leak channel and in a voltage dependent manner\(^5\). K\(_2\)P channels, because of their primary function as leak channels, act as modulators of excitability and are widely expressed in numerous organs including the brain, heart, adrenal glands, and kidneys. Due to their function as modulators of excitability, these proteins are under control from numerous different chemical and physical signals which include oxygen tension, pH, lipids, mechanical stretch, and neurotransmitters\(^3\).

1.2 K\(_2\)P Subfamilies

The fifteen members of the K2P family can be separated into six groups based on amino acid sequence similarity and functional properties\(^4,3\). Figure 1.1 is an illustration of a Kcnk proteins transmembrane domains and pore spanning loops. Subfamilies and their members are also shown in Figure 1.1. These groups are TASK (tandem of P domains in
a weak inwardly rectifying K+ channel [TWIK]-related acid-sensitive K+ channel),
TREK (TWIK-related K+--channel gene)⁴, TWIK (tandem of P domains in a weak
inwardly-rectifying K channel), THIK (tandem pore domain halothane-inhibited K
channel), TALK (tandem of P domains in a weak inwardly-rectifying K channel related
alkaline pH activated K channel), and TRESK (tandem of P domains in a weak inwardly-
rectifying K channel related spinal cord K channel)⁶. The TASK family consists of Kcnk
3, Kcnk 5, Kcnk 9, and Kcnk 15. The TREK family consists of Kcnk 2, Kcnk 4, and
Kcnk 10. TWIK family members include Kcnk 1, Kcnk 6, and Kcnk 7. The THIK
subfamily is composed of Kcnk 12 and Kcnk 13. TALK family members include Kcnk
16 and Kcnk 17. TRESK is composed of a single member, Kcnk 18³. Members of the
same family are known to form heterodimers with one another, but there is no evidence
that members of different families will dimerize with one another⁷.

TASK channels are especially sensitive to changes in extracellular pH. Acidosis
has been shown to decrease conductance while alkalosis increases current⁴⁴. TASK
channels have been shown to be almost completely inhibited at physiological pH levels
and to reach maximum activity at a pH of 10⁵⁰. These channels are expressed in a wide
range of tissues including the pancreas, brain, lung, prostate, colon, skin, intestine,
skeletal muscle, stomach, and the heart atrium⁴⁵. Due to their wide expression, they are
involved in the regulation of numerous physiological processes including the modulation
of action potential duration in atrial cardiomyocytes⁴⁶, the release of aldosterone from the
adrenal glands⁴⁷, T-cell immunity⁴⁸, and the sleep-wake cycle via modulation of the
thalamus⁴⁹.
TWIK channels are so named because initial studies expressing these channels in *Xenopus laevis* oocytes resulted in weak current generation\textsuperscript{50}. Numerous tissues, including the brain, kidney, lungs, and cardiac tissue, have been found to express TWIK channels\textsuperscript{50}. Recently the structure of Kcnk 1 was solved\textsuperscript{51}. Interestingly, Kcnk1 has been shown to become permeable to sodium ions when extracellular levels of potassium drop below normal levels\textsuperscript{52}. It is currently unclear if Kcnk7 is a functional channel as attempts to express it in *Xenopus* oocytes and COS-7 cells have failed. Additionally, there is an unconventional sequence in the second potassium pore where a glycine residue, which is conserved in all other K\textsubscript{2}P proteins, is changed to glutamic acid residue. The conserved glycine residues are believed to be necessary in forming the selectivity filter of the channel\textsuperscript{50}. TWIK channels are involved in numerous physiological processes including hearing, cardiac function, and proper functioning of the kidneys.

TREK channel activity has been shown to be influenced by several different factors including stretch, lipids including arachidonic acid, linolenic acid, and polyunsaturated acid, temperature, pH, and regulation via G-coupled protein receptor activation and subsequent phosphorylation\textsuperscript{50}. TREK channels are expressed primarily in smooth muscle including the stomach and intestines, neurons and cardiomyocytes\textsuperscript{50}. These proteins play an important role in many physiological processes including nociception, neuroprotection, particularly during an ischemic event, proper heart function, proper functioning of the gastrointestinal tract, regulation of aldosterone, and cortisol release\textsuperscript{50}. Recently the structure of Kcnk4 has been solved\textsuperscript{55}.

TALK channels are highly sensitive to pH changes in the alkaline range reaching 50% activity at a pH of 8.6 or 7.8 when measured at a membrane potential of -50mv or
These proteins have been found to be primarily expressed in the kidney, liver, and in the pancreas with very low expression in the nervous system. TALK channels have been shown to play an important role in bicarbonate reabsorption in the kidney and exocrine function of the pancreas.

THIK channels are expressed in the kidneys, heart, skeletal muscle, pancreas, and the brain. Unlike TALK or TASK channels, THIK proteins are not influenced by changes in pH but are activated by arachidonic acid. THIK proteins have also been found to be inhibited by halothane as well as hypoxia. Regulation of the Purkinje cells of the cerebellum and modulation of the carotid bodies response to hypoxic conditions.

The TRESK family is composed of a single protein which exhibits unique properties. Unlike all other K$_2$P channels, TRESK activity is affected by calcium. Studies have shown that the activation of muscarinic and angiotensin II receptors result in activation of TRESK channels suggesting that calcium influx or release alters TRESK channel activity. Additional studies have shown that the presence acetylcholine, histamine, and glutamate, all of which have receptors that act as calcium permeable ion channels, activate TRESK channels. TRESK channels are not activated by the presence of calcium itself, but through the activation of calcium/calmodulin sensitive phosphatase calcineurin. Calcineurin is believed to dephosphorylate TRESK which allows the channel to become active. Unlike other ion channels, TRESK is able to directly interact with calcineurin through its NFAT-like docking motif. This interaction is facilitated by the presence of calcium as the active form of calcineurin more readily interacts with TRESK. Interestingly enough, TRESK has the highest affinity for calcineurin of any known protein. TRESK expression has been found to be primarily located in the spinal.
cord and the brain. Currently very little is known about the physiological significance of TRESK but it is believed to play a role in regulating trigeminal ganglion and may be involved in migraine headaches\textsuperscript{50}.

Figure 1.1  Kcnk Family Tree
1.3 Kcnk Channel Influence During Development

Central Nervous system development is heavily influenced by the expression of Kcnk channels. The level of expression of Kcnk channels varies throughout development\(^9,11\) and contribute to development in several different ways. One way in which central nervous system development is influenced is through the regulation of calcium entrance into cells by controlling membrane voltage. Calcium entrance is known to influence neuronal growth cone guidance\(^14\) as well as alter gene expression\(^15\). The ability of Kcnk channels to regulate membrane potential helps to determine whether GABA is excitatory or inhibitory during development\(^16\). Kcnk channels have been shown to be involved in the regulation of apoptosis during neuronal development. There are several ways in which apoptosis can be modulated by these channels. The removal of potassium neurons during development through Kcnk3 and Kcnk 5 has been found to be pro-apoptotic\(^17\). The loss of potassium ions results in a decrease in neuron volume which has been implicated in apoptosis signaling\(^18\), while the presence of potassium ions in neurons suppresses the activity of caspases and nucleases\(^19\). Kcnk channels also influence neuronal development by influencing neuron proliferation. Specifically, blocking of potassium channels has been shown to prevent cell proliferation\(^20\). This may be due to the role that potassium channels play in regulating the entrance of calcium into neurons\(^6\). A final way in which Kcnk channels may influence neuronal development is through integration of Kcnk subunits into the cytoskeleton of developing neurons. Integration into the cytoskeleton is not dependent upon potassium conductance by Kcnk channels\(^22\).
1.4 Role of Kcnk Channels in Disease

Due to their wide expression and the role that Kcnk channels play in modulating membrane potential, they have been implicated in various diseases and health problems. These include ischemic stroke, pain disorders, cancer, psychological disorders, heart attack, and migraine headaches.

Various studies have shown that Kcnk 9 plays a role in various types of cancer. Over expression of Kcnk 9 facilitates survival of malignant cells while inhibiting the expression of Kcnk 9 results in a change in cell morphology, a decrease in mitochondrial metabolic activity, and reduced DNA content in melanoma cells\textsuperscript{39}. Kcnk 9 has been implicated in colorectal cancer as well. Studies have shown that over expression of Kcnk 9 in colorectal cancer occurs\textsuperscript{35,36}. In 10\% of breast cancers, Kcnk 9 has been shown to be over expressed by 3 to 10 fold, while 44\% of breast cancers show an over expression of 5 to 100 fold\textsuperscript{40}. Currently, it is believed that the role of Kcnk 9 in cancerous tissue is to promote cell growth and survival by conferring resistance to nutrient deprivation and hypoxia\textsuperscript{36}. This is supported by studies which have mutated the selectivity filter of the channel. This resulted in a decrease in tumor growth, an increase in the occurrence of apoptosis, and a decrease in cell growth in low nutrient conditions\textsuperscript{41}. This would suggest that Kcnk 9 could be a potential target for anti-cancer drugs.

Kcnk 1 has also been shown to play a role in cancer. TAp73, a member of the p53 tumor suppressor family, has been shown to transactivate the gene encoding Kcnk1\textsuperscript{53}. TAp73 inhibits the anchorage-independent tumor growth, and it is believed that Kcnk 1 may be responsible for this effect. Studies have shown that silencing of Kcnk 1
resulted in increased tumor growth that was similar to the growth scene following the silencing of TAp7353.

Kcnk 3 has been shown to play an important role in the modulation of cardiomyocytes. Studies have shown that knocking out Kcnk 3 leads to a prolonged QT interval and a disruption in the depolarization of the left and right ventricles. Individuals with a prolonged QT interval have been shown to have to be at an increased risk for heart attack54.

Kcnk 9, Kcnk 3, and Kcnk 2 have all been implicated in ischemic stroke. One function of potassium channels is to hyper-polarize membranes in order to counter balance the influx of calcium ions by causing a reversal of sodium/calcium antiporters25. Calcium influx may also decrease due to the hyperpolarizing current of potassium by enhancing the magnesium block of NMDA receptors25. Preventing high intracellular levels of calcium is important, since it is toxic. During ischemic stroke, excitotoxicity due to over activation of NMDA receptors by glutamate is believed to be one of the major causes of neuronal loss24. Several studies have found that by knocking out Kcnk 9, Kcnk 3, or Kcnk 2, there is a significant increase in infarct volume25.

Studies have shown that TREK family member Kcnk 2 and Kcnk 10 may play a role in depression. Kcnk 2 is highly expressed in the dorsal raphe nuclei, a major source of serotonin. Knocking out Kcnk 2 in the dorsal raphe has been shown to result in depression resistant phenotypes4. Studies looking at patients who suffer from major depressive disorder have found that these individuals had a high occurrence of polymorphisms in the gene encoding Kcnk2. Additionally, the type of polymorphism also had an impact on treatment efficacy42. Fluoxetine, norfluoxetine, paroxetine are all
commonly used SSRIs have been shown to inhibit Kcnk 2 and Kcnk 10\textsuperscript{50}. Knocking out Kcnk 2 in mice, results in a phenotype that is resistant to depression.

Kcnk 18 is believed to be involved in migraine headaches. It is highly expressed in the trigeminal nerve and inactivating mutations of the gene have been linked to migraine headaches\textsuperscript{50}. Furthermore, a side effect of calcineurin inhibitors, are migraine headaches\textsuperscript{50}. This is likely due to calcineurin activating Kcnk 18 via dephosylation.

1.5 Hypothesis

The K\textsubscript{2}P family is not well studied. These channels are widespread and involved in numerous important physiological processes and are presently linked to several diseases including cancer and stroke. Currently, only two structures have been solved. By solving the remaining structures, the knowledge of these channels will be greatly enhanced and would help in the development of potential medical therapies. The first step in solving these structures is the ability to generate soluble protein. This can be problematic when the protein of interest is a membrane protein like the K2P channels. It is the goal of this project to generate soluble protein. In order to do this, both \textit{E.coli} and the yeast, \textit{P. pastoris} were used for protein expression.

1.6 \textit{Pichia Pastoris} as an Expression System

\textit{P. pastoris} is a widely used system for protein production. This system has highly inducible promoters, AOX1 and AOX2, which encode alcohol oxidases. These enzymes allow \textit{P. pastoris} to use methanol as a carbon source\textsuperscript{57}. In this study the protease deficient \textit{P. pastoris} strain, smd1168, was purchased from Invitrogen (Grand Island, NY) and used to express the proteins of interest.
Chapter 2. Materials and Methods

2.1 Subcloning Human Genes for K$_2$P Proteins into a E.coli Expression vector

The fifteen members of the K$_2$P family (reference sequence numbers in table 2.3) were subcloned into pET-21a expression vectors using the Gateway Cloning System (Invitrogen) (an overview of these method can be found in figure 2.1 and 2.2). This was accomplished by first amplifying the DNA using PCR which was then purified using the GenScript Quick Clean II Gel Extraction Kit. The purified PCR product was then used as the template in a second round of PCR that introduced flanking attB1 sites (all primers used are found in table 2.1). The PCR product was then purified using the GenScript Quick Clean II Gel Extraction Kit. The purified PCR product, along with pDONR221 were used to perform a BP reaction. The reaction was subsequently introduced into E.coli DH5α cells via heat shock. Cells were plated on LB plates containing kanamycin (100ug/ml). Individual clones were selected and inoculated into 5ml cultures which were grown overnight. GenScript QuickClean II Miniprep Kit was used to purify the pDonor221 entry clone. Some of the plasmid was then digested using EcoRI-HF and BamHI-HF (New England Bio Labs) in order to check if the insert was of the correct length. Plasmids containing inserts of the proper length were then sent for sequencing (Genetics Core, Center for Medical Science, Albany, NY). Once verified, the entry clone along with Pet-21a was used to perform an LR reaction which was then introduced into E.coli DH5α cells via heat shock and ampicillin resistant transformants were selected. Individual clones were selected and grown overnight. GenScript QuickClean II Miniprep Kit was used to purify the pET-21a expression clones. Some of the plasmid was then digested using EcoRI-HF and BamHI-HF (New England Bio Labs) in order to check if
the insert was of the correct length. Verified expression plasmid was then used to transform *E.coli* Lemno21CDE3 and Rosetta cells via heat shock. Transformed cells were then plated on LB plates containing ampicillin (100ug/ml) for Rosetta cells or LB plates containing ampicillin (100ug/ml) and chloramphenicol (100ug/ml).
Figure 2.1. Generation of an entry clone via BP reaction. attb1 sites were introduced to gene using PCR. BP reaction was done. Kanamycin resistant transformants were then selected and verified.

Figure 2.2. Generation of expression clone via LR reaction. Entry clone along with pET-21a vector were used to generate expression clone. Ampicillin resistant transformants were then selected and verified.
2.2 Protein Expression Tests in E.coli

Four 5ml cultures of LB with ampicillin (100ug/ml) were inoculated with single colonies and grown at 37°C in shaker (200 rpm) to an O.D.\textsubscript{600} of approximately 1. Three of the cultures were then induced with IPTG (final concentration 1mM), and all four were grown overnight (16 hours). Expression of proteins was then check via SDS PAGE followed by coomassie blue staining.

2.3 One Liter Shake Flask Growth of E.coli

One liter of LB with ampicillin (100ug/ml) was inoculated with a single colony and grown at 37°C in shaker (200 rpm) to an O.D.\textsubscript{600} of approximately 1. Induction was done using IPTG (final concentration 1mM) and culture was grown overnight (16 hours).

2.4a Protein Purification from E. Coli

Cell pellets were placed in VWR 50ml Falcon tubes and re-suspended in 25ml Resuspension Buffer, containing 0.5mM TCEP, DNAse I and PMSF (1mM) (buffer recipes can be found in the proceeding section). Solution was vortexed vigorously in order to dislodge the cell pellet. Five rounds of sonication were used to lyse cells. 10ml of Resuspension Buffer was added containing 1.5% DDM, 0.5mM TCEP and PMSF (1mM) to each 50ml falcon tube. Falcon were mixed tubes end over end at 4°C for one hour (or until lysate was clear). Insoluble material was removed by centrifugation at 5000xg for 20 minutes at 4°C. Then, to separate, fresh, 50ml falcon tubes 200ul 50/50 nickel slurry (equal parts resuspension buffer and Nickel Sepharose Fast Flow) was added. Centrifuged and cleared lysates were poured into fresh falcon tubes containing the slurry.
Tubes were then mixed end over end for one hour at 4°C to allow proteins to bind to the nickel resin. Falcon tubes were then centrifuged at 500xg for 5 minutes at 4°C. Supernatant was discarded and the pelleted resin was pipetted into an unsealed 96 well Thompson filter plate and allowed to drip dry. The filter plate was then sealed. 1ml ATP wash buffer, containing 5mM ATP, 0.1mM TCEP, 0.05%DDM was then added to the well. The plate was then shaken at 600 rpm on a plate shaker for 30 minutes at 4°C. The plate was then unsealed and was allowed to drip dry via gravity. 1ml of Wash Buffer B, containing 0.1mM TCEP and 0.05% DDM was added to the filter plate and allowed to drip dry via gravity. The plate was then resealed and 1ml Wash Buffer B was added to each well. The plate was then shaken at 600 rpm overnight at 4°C. The following day the filter plate was unsealed and allowed to drip dry via gravity. The resin was washed with with 1ml Wash Buffer B and drained by gravity. This was repeated with another 1ml of Wash Buffer B. A 96 well round bottom plate was placed beneath the unsealed filter plate and centrifuge at 500xg for two minutes to remove all remaining wash buffer.

The filter plate was then resealed and 100ul Elution Buffer, containing 0.1mM TCEP and 0.05%DDM was added. The plate was then shaken at 600 rpm for 30 minutes at 4°C. A fresh 96 well round bottom plate was placed beneath the filter plate and centrifuge at 500xg for two minutes. Another 100ul of Elution Buffer was added to the filter plate. A second, fresh 96 well round bottom plate was placed underneath the filter plate and shaken at 600rpm at 4°C. The plate was then centrifuge at 500xg for two minutes and the eluates were combined. Eluates were then checked via SDS-PAGE followed by coomassie staining.
2.4b *E. coli* Purification Buffers

Resuspension Buffer:
50mM Hepes pH 7.8
300 mM NaCl
20mM Imidazole pH 7.8
5% glycerol
1mM MgCl₂.

ATP Wash Buffer:
50mM Hepes pH 7.8
300 mM NaCl
40mM Imidazole pH 7.8
5% glycerol
5mM MgCl₂.

Wash Buffer B:
25mM Hepes pH 7.8
500mM NaCl
75mM Imidazole pH 7.8
5% glycerol

Elution Buffer:
25mM Hepes pH 7.8
200mM NaCl
500mM Imidazole pH 7.8
5% glycerol

2.5 Subcloning of Kcnk 1, 3, 5, 13, and 18 into *P. pastoris*

Genes for the selected proteins were subcloned into the pPICZ C vector (Invitrogen). This was done by using PCR to mutate predicted glycosylation (List of Primers can be found in Table 2.2). NetOGlyc and NetNGlyc programs were used to predict potential glycosylation sites. In addition to eliminating glycosylation sites, PCR was used to generate GFP-KCNK fusion DNA sequences that were flanked by *EcoRI*-HF and *XhoI* sites. Between the GFP and KCNK segments was an *NdeI* site. PCR products were purified using GenScript Quick Clean II Gel Extraction Kit. Following purification, PCR products were digested with *EcoRI*-HF and *XhoI* (New England Bio...
Labs), along with the pPICZ C vector. Once the digest of the vector and PCR product was complete, ligation was done using T4 DNA ligase (New England Bio Labs). The ligation reaction was introduced into *E.coli* DH5α cells by heat shock. Transformed cells were then plated onto LB plates with Zeocin (100 ug/ml). Individual clones were selected and used to inoculate 5ml cultures of LB with Zeocin (100 ug/ml). Plasmids were purified from 5ml cultures using GenScript QuickClean II Miniprep Kit. Some Plasmid was digested using *Eco*RI-HF and *Xho*I. Plasmids with the correct insert were sent for sequencing (Genetics Core, Center for Medical Science, Albany, NY).

In the case of Kcnk 3, the GFP-Kcnk13 construct was digested with *Nde*I and *Xho*I, the digested vector was then purified from a 1% agarose gel by GenScript Quick Clean II Gel Extraction Kit. The Kcnk 3 PCR product was digested in the same manner. Ligation of the digested GFP containing pPICZ C vector and Kcnk 3 PCR product was accomplished using T4 DNA ligase. The ligation reaction was introduced into *E.coli* DH5α cells by heat shock. Transformed cells were then plated onto LB plates with Zeocin (100 ug/ml). Individual clones were selected and used to inoculate 5ml cultures of LB with Zeocin (100 ug/ml). Plasmids were purified from 5ml cultures using GenScript QuickClean II Miniprep Kit. Some Plasmid was digested using *Eco*RI-HF and *Xho*I. Plasmids with the correct insert were sent for sequencing (Genetics Core, Center for Medical Science, Albany, NY).

*P. pastoris* was prepared for transformation by first inoculating an individual colony in 5ml of YPD in a 50ml conical tube and incubating at 30°C overnight. Fresh 500ml YPD was inoculated with 0.5ml of overnight culture and grown to an OD600 of between 1.3-1.5. Cells were then centrifuged at 1500xg for 5mins at 4°C. Pellet was then
resuspended in 500ml of sterile, cold water (0-4°C). Cells were pelleted again and then resuspended in 250ml of cold water. Again the cells were pelleted and resuspended in 20ml of cold 1M sorbitol (0-4°C). Finally, the cells were pelleted and resuspended in 1ml of cold 1M sorbitol. 10ug of plasmid was then linerized using SacI (New England Bio Labs) and mixed with 80ul. The mixture was then transferred to a cold electroporation cuvette (0-4°). Cuvette was then incubated on ice for 5mins. Electroporation was done using a BioRad Gene Pulser Xcell according to the devices parameters for *P. pastoris*. 1ml of ice cold 1M sorbitol was immediately added to cuvette and then transferred to the cell/sorbitol mix to a 15ml Falcon tube. The tube was then incubated at 30°C for 1 hour without shaking. 200ul of the transformed cells were then plated on YPDS (YPD + sorbitol) plates containing Zeocin. (100ug/ml). Plates were incubated at at 30°C for 2-3 days (until colonies appeared).

### 2.6 *P. pastoris Expression Test*

Individual clones as well as the transformation cell mixture were grown at 30°C at 200rpms in MGY (minimal glycerol) pre-induction media to an OD$_{600}$ of 1 and then re-suspended in MMH (minimal methanol and histidine) induction media so that the OD$_{600}$ of 1. Cells were then induced with 0.5% methanol, 5% methanol, or 10% methanol (final concentration) which was added every 24hrs. Cultures were grown at 30°C at 200rpms. 1ml samples were taken from each culture at intervals of 24hrs over a period of 4 days. 200ul of each sample were then placed in 96 well plates and the GFP signal was measured using a BioTek FLx 800 Microplate reader.
2.7 **Cell Sorting**

Fluorescence assisted cell sorting (FACS) was accomplished by transforming *P. pastoris* and growing the transformed cells in 10ml of YPDS with Zeocin (100ug/ml) for two days. After two days, cells were pelleted and re-suspended in MGY pre-induction media and grown at 30°C at 200rpm to an OD$_{600}$ of 1. Cells were then pelleted and re-suspended in MMH induction media so that the OD$_{600}$ of 1. Cells were then induced with 0.5% methanol (final concentration) which was added every 24hrs. After 48hrs cells were sent for FACS by the Flow Cytometry Core (David Axelrod Institute, Albany, NY). Following sorting, cells were plated on YPD plates.

2.8 **P. pastoris Culture Growth and Purification**

Following cell sorting, an individual clone was grown in 1L of MGY media at 30°C at 200rpm to an OD$_{600}$ of 4. Cells were then pelleted and re-suspended in 4 1L cultures of MMH media to an OD$_{600}$ of 1. Cultures were then induced with 0.5% methanol (final concentration) and grown for 48hrs before being pelleted, placed in 50ml Falcon tubes and stored at -80°C.

Cell lysates were prepared using a French Press. Cell pellets were re-suspended in Lysis buffer (Buffer recipes appear in the following section) at a ratio of 1:1 (cell wet weight to buffer). 5ul of 1M MgCl$_2$ per ml and 1ul of DNase per ml of cell suspension was then added in order to reduce viscosity. The sample was then applied to French Press pressure cell and was brought to 10,000psi of pressure. This was repeated 5 times for the sample. The sample was then centrifuged at 20,000rpm for 20mins. Lysate was then collected and placed in a fresh 50ml Falcon tube. 1ml of Nickel-NTA resin was added to sample and mixed, end over end at 4°C for 3 hrs. The sample was then added to a pre-
equilibrated column (pre-equilibrated with 5x column volume). Flow through was collected in fresh 50ml Falcon tube and stored at 4°C. The column was then washed with 20mls (20x column volume) Wash Buffer. 1ml Elution Buffer was added to column and elute was collected in fresh 1.5ml Eppendorf tubes. Elution was repeated a total of 4 times. Flow through, wash, elution samples, and 20ul of resin was checked using by SDS-PAGE. Samples were then concentrated using a pressure concentrator.

2.8b *P. pastoris* Purification Buffers

**Lysis Buffer:**
50mM Tris-HCl pH 7.5  
500mM NaCl  
10mM Imidazole pH 8  
1mM PMSF  
70ul Protease Inhibitor Cocktail (Sigma Aldrich)  
1.5% (W/V) DDM

**Preequilibration Buffer:**
50mM Tris-HCl pH 7.5  
500mM NaCl  
10mM Imidazole pH 8  
5mM β-mercaptoethanol  
0.5% (W/V) DDM

**Elution Buffer:**
50mM Tris-HCl pH 7.5  
500mM NaCl  
500mM Imidazole pH 8  
5mM β-mercaptoethanol  
0.5% (W/V) DDM

2.9 Primers and Ascension Numbers

<p>| KCNK1-INS-TH-F | CAAAAAAGCAGGCTCCACCCTGGTGTTCCGCTGGATCCCATATGCTGCAGTCCCCTGGCC |
| KCNK1-INS-TH-R | GGCCAGGGACTGCAGCATATGGGATCCACGGGAACCAGGTGAGGCTGCTTTTTTG |
| KNCK2-N-3C | CATCACCCTGGAAAGGTTCTGTTCCAGGCGCCCTGGATCCCATATGCGGACCTGACTTG |</p>
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<th>Sequence</th>
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<tr>
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<tr>
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<td></td>
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<tr>
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<td></td>
<td>GGGGACCACCTTTGTACAAGAAAGCTGGTGCAATCTCAT AAACGGACTCCGGCGTCC</td>
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**Note:** The sequences are alignments of primers and their targets, indicating regions of high conservation and potentially important for PCR analysis.
| KCNK10-N-F     | CATCACCCACATCACCTGGTTCCGCCTGGATCCCATATGGAGATGGATTATAGGGGAG    |
| KCNK10-C-R     | GGGGACCCTTTTGTACAAGAAAGCTGGGTTGGCGCCGCCTTTAGTTTTCTGTTCTCTAAAG   |
| KNCK12-N-3C-F  | CATCACCTGGGAAGTTCTGGAAGGATCCCATATGGCTCTTCCCAGCGACGCTTCTCTCC   |
| KNCK12-C-R     | GGGGACCCTTTTGTACAAGAAAGCTGGGTTGGGAATTCGAGCTGAGGCCCTTCTCC       |
| KCNK13-INS-TH-F | CAAAAAGCAGGCTCCACCTGGTTCCGCCTGGATCCCATATGGCTCTTGCCCAGCGACGCTTCTC |
| KCNK13-INS-TH-R | GAAACCCCGGCCAGCCTGATTGGATCCCATATGGCTCTTGCCCAGCGACGCTTCTCTCCC   |
| KNCK15-N-3C    | CATCACCTGGGAAGTTCTGGAAGGATCCCATATGGCTCTTCCCAGCGACGCTTCTCTCCC   |
| KNCK15-C-R     | GGGGACCCTTTTGTACAAGAAAGCTGGGTTGGGAATTCGAGCTGAGGCCCTTCTCC       |
| KCNK16-BAM-F   | AGCCAGCAGATCCCAGGCTTCTCTCCAGAAAGTGAGCTGCTC                   |
| KCNK16-BAM-R   | GAGCTCACTTTTCTGGGAAGGAGCTGGGATCTGCTGCTGCT                  |
| KCNK17-INS-TH-F | CAAAAAGCAGGCTCCACCTGGTTCCGTTGGATCCCATATGGCTCTTGCCCAGCGACGCTTCTC |
| KCNK17-INS-TH-R | GGCTCGCGGTCGGTACATATGGGATCCACGCGAAACCAGGGTGAGGCTGGCCTTGCTTTTTTG |
| KCNK18-INS-TH-F | CAAAAAGCAGGCTCCACCTGGTTCCGTTGGATCCCATATGGGAGGTCTCGGGCAGCC |
| KCNK18-INS-TH-R | GTGCCCCGAGACCTCCATATGGGATCCACGCGAAACCAGGGTGAGGCTCTTGCTTTTTTG |

**TABLE 2.2**

| HIS_PICZC_F | GAGGAATTTCGACATGGCACACCACCACTACACCATACCCACCATACCCACCATATGGGAGCAGAAG |
| **EMGFP_PICZ_F** | CATCACCACATGTTGAGCAAGGGCGAG |
| **EMGFP_PICZ_R** | GGGCCCTGGGAACAGAACTTCCAGACCACCAGGAGCCACCCTTTGTACAGCTCGTCCATG |
| **PICZ_LINKER_F** | GTTCTGTTCACAGGGCCCTGCCGAGGCTTCCAATTCCGGCCACCATGTTCCGCTTCT |
| **KCNK1_PICZ_F** | CCACCATGTTCGGCTCTGTGGCT |
| **KCNK1_N95Q_F** | GCGTGTCGGGTCTGAGCCAGCCTCGGGCAACTG |
| **KCNK1_N95Q_R** | GTTCAATGTTGGCCAGGCTGCTGAGACAGAC |
| **KCNK1_289TER_R** | CCGCTCGAGCTAGTGACCTCCTTGC |
| **KCNK3_N_PICZ_F** | GTTCTGTCCAGGGCCCTTGGGATCCAATGAGCCAGGAGACG |
| **KCNK3_A270TER_R** | CTCGCTCGAGCTACGCTCGACCCGTTG |
| **KCNK3_N53Q_F** | CTGCGGGCCGCTACCTGGAGGCCAGACGGCGG |
| **KCNK3_N53Q_R** | GCCGCCCTGGCTGAGCTGTAAGCGACCCGCG |
| **KCNK5_N_PICZ_F** | GTTCTGTCCAGGGCCCTTGGAATCCATAGGACCCCCGAGCC |
| **KCNK5_G317TER_R** | CTCGCTCGAGCTACCCTCGCTTTCATGG |
| **KCNK5_PICZ_C_R** | CTCGCTAGCTGTGAGCCCTGCCTGGGGTTAGCCTTGTGTA |
| **KCNK5_N77Q_F** | GTGTGGCCATCACAGGGCAGACCCCTTAACAACG |

22
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<td>Kenk 18</td>
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Chapter 3. Results

3.1 Gateway Cloning Results

Using the Gateway cloning system 8 genes encoding Kcnk proteins, KCNK 1, KCNK 3, KCNK 6, KCNK 7, KCNK 9, KCNK 13, KCNK 16, and KCNK 17, were successfully cloned into pET-21a expression vectors. Figure 3.1 and Figure 3.2 are representative gels of successful BP and LR reactions checked via enzymatic digest.
Figure 3.1: Successful BP reaction of Kcnk 9. DNA standards are located in the far left lane. 4 clones were verified by enzymatic digest. The upper band is characteristic of digested pDONR221, approximately 2600bp. The lower band is the correct size for Kcnk 9, 1124bp.

Figure 3.2: Successful LR reaction of Kcnk 9. DNA standards are located in the far right 4 clones were verified via enzymatic digest. The upper band is characteristic of digested pET-21a, approximately 5000bp. The lower band is the correct size for Kcnk 9, 1124bp.
3.2 *E.coli* Expression Test Results

5ml cultures were grown in order to test protein expression in both Lemno21cde3 cells and Rosetta cells. The expression for all 8 clones was checked via SDS PAGE and coomassie blue staining. In order to determine how well the protein was being expressed, non-induced cultures were compared to IPTG induced cultures. No difference in protein levels for any of the 8 proteins was found between non-induced or induced Lemno21cde3 cells or Rosetta cells. Figure 3.3 is a representative figure of the expression test done for the 8 channels.
Figure 3.3: Expression test for 5ml culture of Kcnk 15 (36.9 kDa) and Kcnk 17 (39.2 kDa) expressed in Lemno21cde3 cells.
3.3 Purification of Kcnk proteins from 1L Growth of Lemno21cde3 Cells

1L cultures of all Kcnk proteins expressed in lemno21cde3 cells were grown and purified. The purified samples were then checked on an SDS-PAGE gel. The gel revealed proteins where present but they were not the correct size. Thus it was concluded that the proteins were not being expressed in the *E.coli*. Figure 3.4 is a representative gel following purification of Kcnk 9 and Kcnk 13.
Figure 3.4: Purification of Kcnk 9 and Kcnk 13. From left to right, lane 1 Kcnk 9 first elution, lane 2 Kcnk 9 second elution, Lane 3 standards, lane 4 Kcnk 13 first elution, and lane 5 Kcnk 13 second elution.
3.4 Mutagenesis and Ligation of KCNK genes

The GFP-KCNK13 DNA sequence was successfully ligated into the pPICZ C vector. However, mutagenesis was unsuccessful so the KCNK13 DNA was wild type. Figure 3.5 illustrates the successful digest of the GFP-KCNK13 vector. This construct was then digested and wild type KCNK 3 was successful ligated into the pPICZ C-GFP vector. This is represented in Figure 3.6. Following ligation into the vector mutagenesis of KCNK3 was successful. Mutagenesis of KCNK 1 was successful but ligation was not. Mutagenesis of KCNK5 and KCNK18 was not successful nor was ligation into the pPICZ-C vector.
Figure 3.5: Enzymatic digest of the ligation of KCNK13 (1600bp) into pPICZ C vector. The correct ligation is found in lane 3.

Figure 3.6: Enzymatic digest of the ligation of Kcnk 3 (759bp) into the pPICZ C-GFP vector.
3.7 *P. pastoris* Expression Test

In order to determine the optimal conditions for expression in *P. pastoris* an expression test using yeast expressing Kcnk 13 was conducted. Expression was determined by the GFP signal. The expression test was repeated twice. Based on the results of the first test, which are shown in figure 3.7, cells induced with 5% methanol and grown for 24hrs had the highest expression but it dropped significantly after 48hrs. The second highest expression occurred in cells induced with .5% methanol and grown for 48hrs. The results of the second test, shown in figure 3.8, revealed that cells induced with .5% methanol and grown for 48hrs had the highest expression. The results of the second test show expression levels much lower than the first. This is most likely due to a higher KCNK13 copy number found in the clone used in the first test. Based on these results induction with .5% methanol and a growing time of 48hrs was determined to be the best. Additionally, the copy number seemed to play a significant role in the level of protein expression.
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<th>Pre-Induction</th>
<th>Day 1</th>
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</tr>
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<td>.5% Single Colony</td>
<td>414</td>
<td>590</td>
<td>1108</td>
</tr>
<tr>
<td>5% Single Colony</td>
<td>409</td>
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<td>1184</td>
</tr>
<tr>
<td>10% Single Colony</td>
<td>434</td>
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<td>422</td>
</tr>
</tbody>
</table>

**Figure 3.7:** Expression test of Kcnk 13. Blank was just induction media and was measured to be 408, the single colony conditions were grown using the same clone. Measurements are taken in RFUs.

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<th>% Methanol</th>
<th>Pre-Induction</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
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<td>562</td>
<td>518</td>
<td>454</td>
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**Figure 3.8:** Expression test of Kcnk 13. Blank was just induction media and was measured to be 366, the conditions were grown using the same clone. Measurements are taken in RFUs.
3.8 Cell Sorting

Following successful mutagenesis of KCNK3, *P. pastoris* was transformed with the mutated KCNK3 gene and FACS was done. 13.1% of the cells were found to be highly expressing GFP. The top one percent of cells that were expressing GFP were selected. The purity of the collected cells was determined to be 95.2%. The sorted cells were then re-cultured. The results of FACS are found in figure 3.9 and 3.10.
Figure 3.9: KCNK3 Cell sorting results. Events detected in Q4, left figure, indicate cells that are GFP positive. The right figure is a quantification of the events shown in the figure on the right. 13.1% of cells were found to be GFP positive. The top 1% of these cells were collected.

Figure 3.10: Purity of the top 1% of collected cells highly expressing KCNK3. Events detected in Q4, left figure, indicate cells that are GFP positive. The right figure is a quantification of the events shown in the figure on the right. Purity was determined to be 95.2%.
3.9 Purification of Kcnk 3

Following cell sorting, 4 1L cultures of Kcnk3 were grown and purified. The purified sample was checked via SDS PAGE and coomassie blue staining. The gel revealed, figure 3.11, that Kcnk 3 was successfully expressed in and purified from Pichia Pastoris. However, the yield was low. Following pressure concentration of the four elutes, a 500ul sample with a concentration of .812mg/ml was acquired. The GFP signal of the concentrated sample was then determined to be approximately 25 times that of the elution buffer alone (Figure 3.12). These results suggest the presence of soluble protein.
Figure 3.11: SDS PAGE of Kcnk 3 elutes. The bands found in lanes 4-7 are the correct size (59.7 kDa).

<table>
<thead>
<tr>
<th>Elution Buffer</th>
<th>Kcnk 3 Concentrated Elution</th>
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<td>3032</td>
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Figure 3.12: GFP signal of the eluted protein compared to elution buffer alone. The signal from the elute protein is approximately 25 times greater.
3.10 Kcnk 3 Western Blot

In order to further show the presence of Kcnk 3, a western blot was done using an anti-His primary anti-body (Figure 3.12). The western blot revealed the presence of an approximately 58 kDa protein which suggests the presence of Kcnk 3. The blot also revealed a protein (approximately 46 kDa) that was not visible on the gel. Because the antibody for His is not very specific, it also bound to a protein in the standard, the protein is probably contaminate.
Figure 3.13: Western Blot using anti-his antibody. The band at approximately 58 kDa is most likely Kcnk 3 (59.7 kDa).
3.12 Discussion

The goal of this project was to express and purify soluble Kcnk protein. We attempted to do this by expressing Kcnk proteins in both *E.coli* and *P. pastoris*. Our experiments show that bacteria did not express the ion channels while *P. pastoris* was capable of producing protein at detectable levels. Poor expression of mammalian membrane proteins in bacteria may be due to the inability of bacteria to properly fold and insert the proteins into membranes\(^5\). Additionally, the accumulation of foreign proteins in the membranes of prokaryotes has also been shown to be toxic and may also account for the lack of expression in bacteria\(^5\).

*P. pastoris* was able to successfully express Kcnk 3, suggesting it would be a viable expression system for the remaining Kcnk proteins. We had attempted to clone five channels but were only successful with Kcnk 3 and Kcnk 13. While the mutagenesis of Kcnk 3 was successful, Kcnk13 was not. We believe this may be due to the primer design. When some of the mutagenesis reaction was run on a gel no product was detected. This suggests that the primers were not setting down properly. Varying the annealing temperature or the duration of the melting step did not increase the product yield. Addition of DMSO did not help either. This suggests the primers may have some secondary structure problems and should be redesigned. Kcnk 1, Kcnk 5, and Kcnk 18 were not successfully ligated into the pPICZ C vector. This was due to improper digestion of the PCR products. This is most likely due to problems with the digest sites.

Verification of the presence Kcnk 3 was done using coomassie blue staining. This revealed the presence of a band corresponding to the size Kcnk 3. No other proteins were detected by coomassie blue staining in the eluted samples. Following concentration of the elution samples a GFP signal was detected, suggesting the presence of our fusion
protein. The presence of Kcnk 3 was further confirmed by western blot. We used an anti-his antibody as an antibody to Kcnk3 was not readily available. The blot also revealed the presence of another protein that was not detected on the gel. This protein is most likely a contaminate that has a long stretch of histidine residues.
3.13 Conclusions and Future Studies

We have demonstrated that Kcnk proteins can be expressed and purified from *P. pastoris*. Expression and purification of soluble membrane protein is difficult as relatively few structures of membrane proteins have been solved when compared to the solved structures of water soluble proteins.

Due to their wide expression Kcnk proteins most likely play an important role in many physiological processes. However very little is known about these proteins. If the structures of these proteins were solved it would greatly enhance what is known about these channels and their study. In order to determine the structure of these proteins future studies could focus on large scale growth via fermentation, protein crystallization, and X-ray diffraction.


47. Czirjak, G, Fischer T, Spat A, Lesage F, Enyedi P. TASK (TWIK-Related Acid-Sensitive K+ channel) is expressed in glomerulosa cells of rat adrenal cortex and inhibited by angiotensin II. Molecular Endocrinology, 14(6), 863 (2000).


49. Meuth SG, Budde T, Kanyshkova T, Broicher T, Munsch T, Pape HC. Contribution of TWIK-related acid-sensitive K+ channel 1 (TASK1) and TASK3 channels to the control of activity modes in thalamocortical neurons. Journal of Neuroscience, 23(16), 6460-6469 (2003).


