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An endocytic signal for the potassium channel KCNK3

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An endocytic signal for the potassium channel KCNK3

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UNIVERSITY OF MASSACHUSETTS MEDICAL SCHOOL

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

by

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Date:

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Prof. Elizabeth Ryder, Advisor

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Abstract

Membrane trafficking is a major cellular mechanism for the regulation of surface expression of plasma membrane proteins. Recently, a carboxyl terminus motif, FREKLAYAIA, has been shown to control the internalization of the dopamine transporter (DAT), a critical protein in synaptic transmission. Sequence comparison across BLAST identified a similar carboxyl motif, SREKLQYSIP, in the leak potassium channel KCNK3. This raised the possibility that the KCNK3 protein may also undergo a regulated internalization, and that this sequence may be important in modulating its surface expression. The current study tested whether this motif was necessary and sufficient for the internalization of the channel. Our results suggest that the carboxyl terminus of KCNK3 is sufficient for the internalization of an endocytic deficient reporter molecule, Tac. Further studies will examine whether or not KCNK3 internalizes and whether the SREKLQYSIP is necessary for its internalization.

Acknowledgements

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INTRODUCTION

Project Context

Many important neuronal functions are controlled by protein trafficking mechanisms. It is important for proteins involved in neuronal excitability to be present at the plasma membrane at the correct concentration. When smaller concentrations of these proteins are required on the surface of the plasma membrane, short amino acid motifs can signal their endocytosis. One such protein is the dopamine transporter (DAT). The dopamine transporter is responsible for removing the neurotransmitter dopamine from the synaptic cleft of neurons and thus terminating the signal transmission. Internalization of DAT would hence result in prolonged postsynaptic responses. Therefore, the importance of understanding the factors that cause DAT internalization becomes evident. Previous experiments performed on DAT in the Melikian laboratory, showed that in order to internalize, DAT requires an internalization sequence, FREKLYAIA, which was found to be on its carboxyl terminus (Holton et al., 2005). This sequence was compared against the human genome using the Basic Local Alignment Search Tool (BLAST) and a type of potassium channel, called the KCNK3 channel, was found to have a FREKLYAIA-type sequence on its carboxyl terminus; more specifically the sequence SREKLQYSIP. The goal of our project was to determine whether this channel internalizes and if so, whether the SREKLQYSIP sequence is responsible for its internalization.

Membrane Proteins

Membrane proteins are divided into two classes based on their interaction with the membrane. Integral membrane proteins or transmembrane proteins are embedded directly within the lipid bilayer. Peripheral membrane proteins are not inserted into the

lipid bilayer, but associate with the membrane indirectly by interactions with the integral membrane proteins. Many ions and polar molecules cannot diffuse through the membrane freely, but can only pass through the membrane with the help of transport proteins. Transport proteins have multiple membrane-spanning regions that form a passage through the lipid bilayer, facilitating the transport of charged molecules across the hydrophobic core of the membrane. One such class of membrane transport proteins is the channel proteins that form open pores through the membrane. Ion channel proteins regulate the movement of ions such as Na^+ , K^+ , Cl^- and Ca^{2+} , and are highly selective to their specific ions (Cooper and Hausman, 2004).

Trafficking

Since membrane proteins are inserted into a fluid lipid bilayer, they are sometimes able to diffuse laterally through the membrane and enter the cell through a process called endocytosis. One type of endocytic membrane trafficking, called receptor mediated endocytosis, provides means for the selective uptake of specific molecules. As the name suggests, this type of trafficking is involved with receptor recycling and is used to maintain the correct composition of proteins in the plasma membrane and to deliver molecules to the appropriate compartments in the cell.

The first step in receptor mediated endocytosis is the binding of ligands to specific cell surface receptors that are concentrated in clathrin-coated pits in the plasma membrane (Fig. 1). These pits then bud off from the membrane to form small clathrin-coated vesicles containing the receptors and the bound ligand. The sorting endosome, which sends molecules to their correct destination, is usually the first compartment with which these vesicles merge. From there, some molecules will directly return to the

plasma membrane, whereas others will remain in the endosome until it matures to become a late endosome or a lysosome. Another possible route from the sorting endosomes is the endocytic recycling compartment, from which almost all of the molecules return to the plasma membrane (Maxfield and McGraw, 2004).

In neurons, the arrival of an action potential at their terminals signals the fusion of the synaptic vesicles with the plasma membrane to release the neurotransmitters that carry the signal to the synaptic cleft. The empty synaptic vesicles are recovered from the plasma membrane in clathrin-coated vesicles, which fuse with early endosomes. The synaptic vesicles are then regenerated directly by budding from endosomes and then they accumulate a new amount of neurotransmitters from the cytosol for the next synaptic transmission (Cooper and Hausman, 2004).

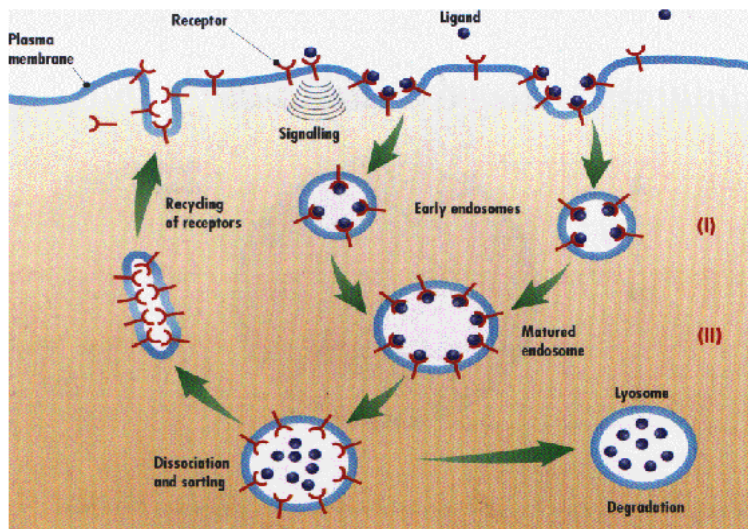


Figure 1: *Receptor mediated endocytosis.* Upon binding of the ligand, the receptor sends a signal that begins its internalization into early endosomes through the use of clathrin-coated pits. From there the receptors can either be transported back to the plasma membrane or remain in the endosome to be degraded. (Taken from Time Resolved Microfluorimetry, n.d.)

Sorting of transmembrane proteins to endosomes and lysosomes is mediated by signals present within the cytosolic domains of the proteins. Most signals consist of short, linear sequences of amino acid residues (Bonifacino and Traub, 2003). These internalization sequences bind to adaptor proteins, which then bind clathrin on the cytoplasmic tails of receptors (Maxfield and McGraw, 2004). Currently, two main groups of internalization signals are known, the tyrosine-based signals and the dileucine-based signals. These signals are usually found in the cytosolic domain of transmembrane proteins, and their most important residues are bulky and hydrophobic. The signals are often recognized by adaptor proteins, such as the ones coating clathrin and forming clathrin-coated pits.

There are two types of tyrosine-based signals (NPXY and YXXØ) and two types of dileucine-based signals ([DE]XXXL[LI] and DXXL). The NPXY motif can be found within cytosolic domains of about 40 to 200 amino acids and is responsible for the rapid internalization of some integral membrane proteins. It is important to note that this motif is usually not found at the carboxyl terminus of proteins. The other tyrosine motif, YXXØ, is found in large cytosolic domains and is particularly important for the rapid internalization of endocytic receptors, intracellular sorting receptors and lysosomal membrane proteins, among others. The tyrosine residue on both of these motifs is necessary to maintain function of the signal. The function of both the dileucine-based sorting signals is to target proteins to endosomal and lysosomal compartments. The [DE]XXXL[LI] motif is usually located very close to the transmembrane domain, and even though the first leucine is invariant for the maintenance of activity, the second leucine can be replaced by isoleucine without abrogating activity. However, mutating the

leucine residue in the DXXL motif to isoleucine causes a loss of activity. The main function of DXXL is to mediate the incorporation of proteins into clathrin-coated pits (Bonifacino and Traub, 2003).

The trafficking of DAT follows a receptor-mediated endocytosis. However, its internalization follows a clathrin-independent mechanism that does not conform to either tyrosine-based or dileucine-based motifs (Holton et al., 2005). Instead, the Melikian laboratory has shown that there is a sequence, namely FREKLAYAIA (DAT 587-596), in the carboxyl terminus of DAT that is both necessary and sufficient to drive DAT internalization. The Isoleucine at position 595 has been shown to be particularly important for the constitutive internalization of DAT (Holton et al., 2005). Notably, protein kinase C (PKC) downregulates DAT to prolong synaptic transmission, through the use of a clathrin-dependent mechanism. However, this mechanism is dependent on the FREKL part of the sequence (DAT 587-591). PKC not only increases the rate of DAT endocytosis but also slows down the process of redelivering DAT to the cell surface.

Potassium Channels

K⁺ channels are found in all cells, and they play an important role in maintaining membrane potential. They allow the flux of K⁺ ions in and out of the cell and thus are essential for the generation of electric current across excitable membranes. The closure of K⁺ channel leads to membrane depolarization, whereas the activation of K⁺ channel leads to hyperpolarization of the cell membrane. KcsA, which has similar amino acid sequences to other K⁺ channels, will serve here as a general model for the structure of eukaryotic K⁺ channels (Doyle et al., 1998).

The KcsA channel is a tetramer with four-fold symmetry about a central pore (Fig. 2A). Each subunit has two transmembrane segments and one pore. The four inner C-terminal helices form the pore and they are packed against each other near the cytoplasmic side of the membrane whereas the four outer helices, which face the lipid bilayer, support the inner helices. The channel has two layers of aromatic amino acids positioned to extend into the lipid bilayer (Fig. 2B).

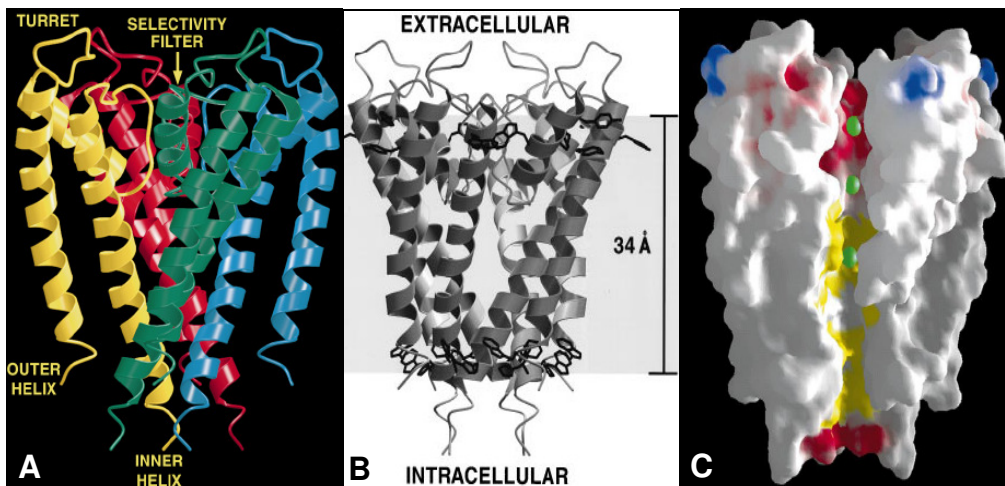


Figure 2: Three representations of KcsA channel tetramer. The cytoplasm is below and the extracellular region above. (A) Ribbon diagram. Each of the four identical subunits is colored differently. Each subunit has two transmembrane α -helices connected by the roughly 30 amino acid pore region, which consists of the turret (that projects out into the extracellular space), the pore helix, and the selectivity filter. (B) Ribbon diagram of the KcsA channel tetramer as an integral membrane protein. The length of the channel is 34 Å. Aromatic amino acids on the membrane-facing surface are shown in black. (C) Solvent accessibility of the K⁺ channel pore. The surface of the K⁺ channel is colored according to its physical properties with negatively charged areas red, uncharged areas white, positively charged areas blue and hydrophobic areas of the central pore yellow. K⁺ ions are represented as green spheres (Modified from Doyle et al., 1998).

The central pore of the K⁺ channel has variable width. It starts at its cytoplasmic side as a 6-Å-diameter and 18- Å-long tunnel, which is called the internal pore, and is

lined with four anionic side chains (red area at the bottom of Fig. 2C). The internal pore then widens to form the ~10-Å-diameter central cavity that is wide enough to let a K⁺ ion pass through in its hydrated state. The upper part of the pore, called the selectivity filter, narrows to 3 Å, forcing a transiting K⁺ ion to shed its waters of hydration. The walls of the internal pore and the central cavity are lined with hydrophobic groups that interact minimally with the diffusing ions. The selectivity filter (red area near the top of Fig. 2C) is lined with closely spaced main chain carbonyl oxygens of residues that are highly conserved in all K⁺ channels- the TVGYG signature sequence. Mutation of these amino acids disrupts the channel's ability to discriminate between K⁺ and Na⁺ ions (Pratt et al., 2006).

There are three main structural classes of potassium channels: the voltage gated potassium channels (Kv), the inwardly rectifying K⁺ channels (Kir) and the KCNK channels. As suggested by their name, Kv channels (Fig. 3A) open in response to voltage changes in their surroundings. The Kir channels (Fig. 3B), may be open at all membrane potentials and favor the influx of K⁺ ions rather than their efflux.

The KCNK channels (Fig. 3C), a new family of potassium channels, are active at resting membrane potential and show little voltage dependence. They are responsible for 'leak' K⁺ currents that contribute to the resting membrane potential of cells and exert control over excitability by shaping the duration, frequency and amplitude of action potentials (Patel and Honorè, 2001). Increased K⁺ leak currents stabilize cells at hyperpolarized voltages below the firing threshold of nerves and muscles, whereas leak suppression allows depolarization and excitation (Goldstein et al., 1996). Each KCNK channel subunit possesses two pore domains and has four transmembrane segments,

which means that only two subunits are required to form a functional channel. The TWIK-related acid-sensitive K⁺ channels (TASK) are a subgroup of the KCNK channel family, with KCNK3 and KCNK9 being very similar and co-expressed in many areas of the brain.

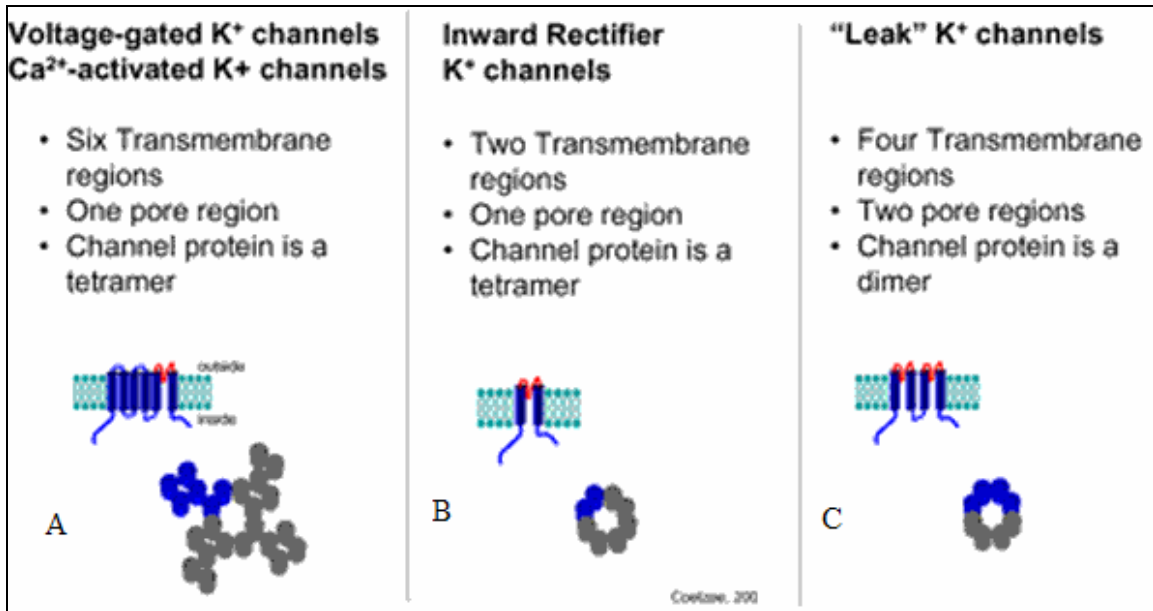


Figure 3: Three structural classes of potassium channels. (A) The Kv subunits have one pore (P) domain and six transmembrane fragments. The fourth transmembrane fragment, which is responsible for the channel's voltage sensitivity, has positively charged residues at every third or fourth position. These amino acids move with changes in membrane potential to open the channels. Four subunits have to come together to form a functional Kv channel. (B) The Kir channels, which also require four subunits to become functional, have one P domain and only two transmembrane segments per subunit. (C) Each KCNK channel subunit possesses two pore domains and has four transmembrane segments, which means that only two subunits are required to form a functional channel. (Adapted from Potassium Channels, 2003).

KCNK3

KCNK3 is an open rectifier leak channel, a channel that is continuously open and is used to maintain the correct balance of K⁺ ions in and out of the cell. In physiological conditions, where there is a higher K⁺ concentration inside the cell, the channel produces

an outward current. However, when K^+ concentration is the same inside and outside the cell membrane, the channel produces currents proportional to the membrane voltage (Goldstein et al., 2001).

Each KCNK3 channel subunit has four transmembrane segments, two pore domains (P1 and P2) and its N and C termini are intracellular (Fig. 6). Since a functional K^+ selectivity filter requires four pore domains, KCNK3 works as a dimer. The significance of this is that both the P1 and P2 domains are active in the selectivity pore (Czirják and Enyedi, 2002).

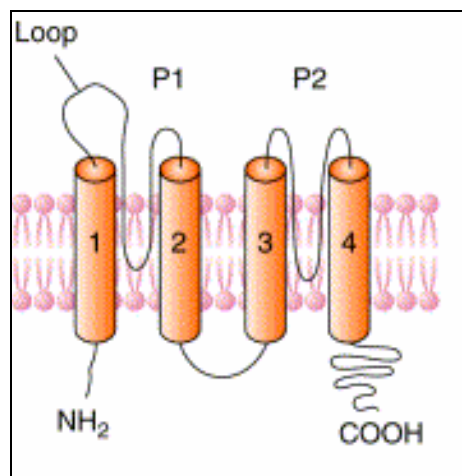


Figure 4: *Topology and Structure of a KCNK3 subunit.* The subunit has four transmembrane segments and two pore domains (2P/4TMS). The extracellular loop is called the M1P1 loop because it is located between the first transmembrane segment and the first pore domain. Both its termini are intracellular. (Taken from Patel and Honorè, 2001).

Until recently, it was believed that all K^+ channels were formed by homodimers. However, recent research has shown that functional heterodimers can form between KCNK3 and KCNK9, at least in the oocytes of *Xenopus laevis* (Czirják and Enyedi, 2002) and in HEK 293 cells (Berg et al., 2004). The characteristics of these heterodimers, for

the most part, lie somewhere between the characteristics of the two homodimers depending on the percentage expression of KCNK3 and KCNK9. Recent papers have also shown that the extracellular M1P1 loop in the subunit is an α -helix that contains hydrophobic and charged residues arranged in a regular sequence. A cysteine residue on this loop is responsible for the formation of disulphide bridges between the two subunits, holding the dimer together (Lesage, 2003).

In humans, KCNK3 channels mostly appear in the brain and the spinal cord, and can be found synaptically and postsynaptically. The highest levels of KCNK3 expression are found in somatic motoneurons in the central nervous system. In rats and mice, KCNK3 channels are present in cardiac tissue as well as neuronal tissue. Several factors have been shown to affect these channels. Neurotransmitters acting through muscarinic receptors can inhibit KCNK3 in rat hypoglossal motoneurons and cerebellar granule cells. As a result, excitability of such cells is increased (Goldstein et al., 2001). Other inhibitors of KCNK3 are G-protein-coupled receptors (Czirják and Enyedi, 2002) and the endocannabinoid anandamide, which specifically and directly blocks KCNK3 in only submicromolar concentrations (Lesage, 2003). On the other hand, sensitivity to anesthetics, which can be attributed to the C-terminus (Patel and Honorè, 2001), allows anesthetics such as halothane to enhance KCNK3 leak channel activity, prolonging hyperpolarization and thus decreasing cell excitability. KCNK3 is also quite sensitive to extracellular pH, illustrated by the fact that at extracellular pH 7.3 fifty percent of all KCNK3 channels are closed. A histidine residue located at position 98 near P1 is responsible for this sensitivity (Patel and Honorè, 2001). In contrast, extracellular alkalization activates KCNK3 (Berg et al., 2004).

Project Goals

The –COOH terminus of KCNK3 contains the sequence SREKLQYSIP in its 322 to 331 amino acid residues. This sequence is similar to the FREKLYAIA consensus sequence found in the 587 to 596 residues of the dopamine transporter that is responsible for the internalization of the transporter (Holton et al., 2005). To date no other studies have taken place to show whether similar sequences are responsible for the internalization of proteins other than the ones belonging to the SLC6 family.

The goals of this project were to determine whether the SREKLQYSIP sequence is sufficient to induce KCNK3 internalization and if so, whether the entire sequence is necessary or if certain residues within it are more important than others. We also wanted to examine whether KCNK3 internalizes physiologically, and if so to perform experiments to determine whether the sequence is solely responsible for the internalization or if it works with other parts of the channel to co-induce it.

This project's results could prove important in future research involving KCNK3 channels and their medical applications. The identification of the KCNK3 residues responsible for its internalization will allow for the development of mechanisms to either induce or block it, decreasing or increasing the effects of anesthetics on these channels. Changing the amount of KCNK3 channels in the cell membrane will also change the excitability of a neuron since these leak channels are responsible for neuron repolarization.

MATERIALS AND METHODS

Cell Culture

PC12 cells were maintained at 37°C, 10% CO₂ in PC12 media (DMEM supplemented with 5% BCSS, 5% HS, 2mM glutamine, 100 U/ml penn/strep). CHO cells were maintained at 37°C, 5% CO₂ in CHO media (HAMS F-12 nutrient mixture supplemented with 10% FBS, 2mM glutamine, and 100U/ml penn/strep).

Tac Internalization Assay

24-well plate transfection

Coverslips were placed in 24-well plates and covered with 1mg/ml poly-D-lysine. The excess poly-D-lysine was removed and the plates were left to incubate for fifteen minutes at room temperature. At the end of the incubation, the wells were washed three times with 1-2 ml of PBS (Appendix A). PC12 cells were plated at a density of 210,000 cells/well in 0.5 ml of penn/strep free PC12 media. After a 24 hour incubation, the cells were transfected with Lipofectamine 2000™ (Invitrogen) using 0.8 µg of Tac, Tac-DAT, Tac-KCNK9 or Tac-KCNK3 DNA/well at a Lipofectamine: DNA ratio of 2.5:1 (volume/weight) with 50 µl of OPTIMEM/well.

Assay

Forty-eight to seventy-two hours after the PC12 transfection the 24-well plates were moved to the cold room and the cells were washed three times with ice-cold PBS²⁺ (Appendix A). The cells were incubated for 1hour at 4°C with 200µl /well of mouse anti-Tac antibody (Upstate; conc. 0.4×10^{-3} mg/ml) in the Primary Antibody Solution (Appendix A). All the plates, except the one containing the controls at 4°C, were then

quickly transferred to room temperature and washed three times with antibody solution brought to 37°C, and placed in the 37°C incubator for either twenty, thirty or forty minutes (Fig. 5). All plates were then washed three times with 1X PBS, followed by the addition of 500 µl of Fixing Solution (Appendix A) and a 10 minute incubation at room temperature with light shaking. The cells were washed two times with 0.5 ml of 1X PBS, and 200 µl of Blocking Solution for Tac Internalization Assay (Appendix A) were added to each well. Blocking continued for 30 minutes at room temperature with light shaking, followed by the addition of 200 µl of the Secondary Antibody Solution (Appendix A). The plates were covered in aluminum foil and incubated for 45 minutes at room temperature with light shaking.

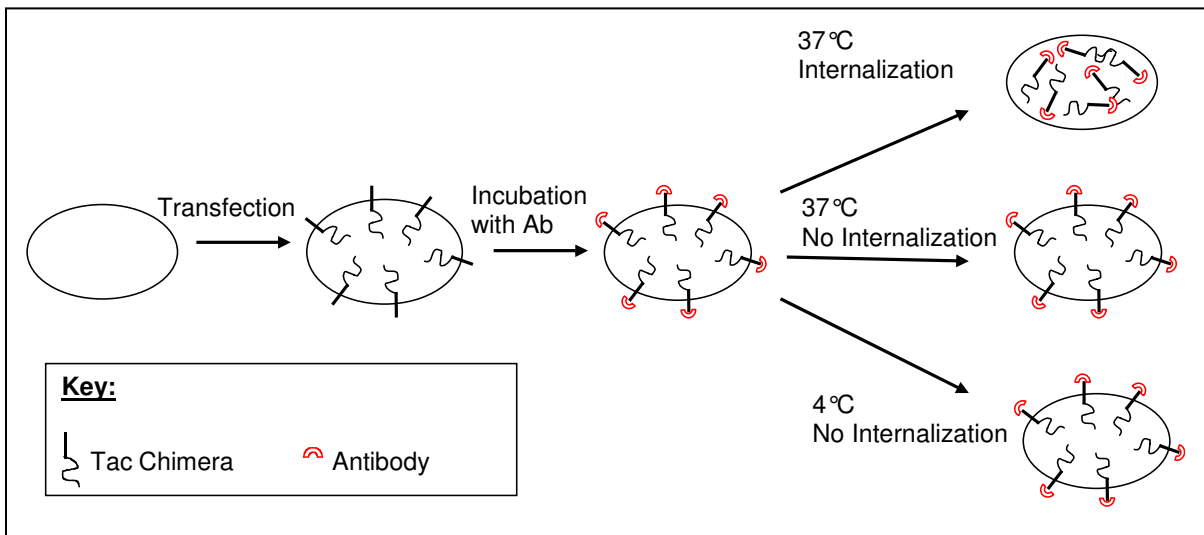


Figure 5: *Tac internalization assay schematic.* A schematic representation of the Tac internalization assay showing the possible outcomes. Antibody to Tac (anti-Tac) was bound at 4°C to cells transfected with the indicated Tac chimeras, and cells were warmed to 37°C for 20, 30 or 40 minutes. Possible outcomes are shown.

The cells were washed two times with 1X PBS with incubations for 5 minutes at room temperature with shaking, and a final wash with 1X PBS for 45 minutes covered in foil and lightly shaking. Finally, the plates were allowed to dry for 20 minutes in the 37°C incubator and the dry coverslips were mounted on slides using Invitrogen ProLong Gold.

The slides were left to dry overnight at room temperature covered with aluminum foil. The results of the assay were visualized using the Zeiss Axiovert 200M inverted microscope using Texas Red reflective with a 63X magnification. Micrographs were taken at 0.4 μm intervals across the cell and deconvolved using a constrained iterative algorithm, using the software Slidebook 4.0.

Immunoblots

6-well plate transfection

PC12 cells were plated at 10^6 cells /well in 2ml of penn/strep free PC12 media. After a 24 hour incubation, the cells were transfected with Lipofectamine 2000TM (Invitrogen) using 4 μg of DAT or KCNK3 DNA/well at a Lipofectamine: DNA ratio of 2:1 (volume/weight) with 250 μl of OPTIMEM (Invitrogen) per well. CHO cells were plated at 360,000 cells/well in a 6 well plate in 2 ml of penn/strep free CHO media. After 24 hours, the cells were transfected with LipofectamineTM Reagent (Invitrogen) using 1 μg of DAT or KCNK3 DNA/well at a Lipofectamine: DNA ratio of 3:1 (volume/weight) with 250 μl of OPTIMEM/well.

Preparation of protein lysate

Cells were gently washed 3 times with 2 ml 1X PBS. Then 300 μl of the RIPA/PI (Appendix A) were added to the wells. The plates were shaken in the cold room for 20 minutes. The lysates were transferred to microfuge tubes and spun at 9,520 x g for 10 minutes in the cold room. The supernatant was then transferred to clean microfuge tubes to perform a protein assay.

Bicinchoninic Acid (BCA) Protein assay (Pierce)

BSA (bovine serum albumin) standards for the protein assay were made from a 5 mg/ml stock of BSA in RIPA/PI. The protein assay was performed following kit instructions on a 96 microwell plate at 562 nm. All standards and samples were assayed in triplicate.

SDS-PAGE gel

The protein samples were run on two 8% SDS-PAGE gels. 25 µg of protein sample was loaded onto each lane along with 6X Sample Buffer (Appendix A). The gel was run at 100V through the stacking gel and 200V through the running gel.

Immunoblotting

After the protein samples were electrophoretically separated in the 8% SDS-PAGE gels, they were transferred to nitrocellulose membranes for 1 hour at 100V or 30V overnight in the cold room. Chilled 1X Transfer Buffer (Appendix A) was used as the buffer. The nitrocellulose membranes were stained with Ponceau Stain and shaken for 5 minutes on the shaker. The stain was then rinsed off with dI water. The membranes were then blocked for 45 minutes using Blocking Solution for Western Blot (Appendix A). The nitrocellulose membranes were probed for 45 minutes at room temperature with rabbit anti-KCNK3 antibody (Bayliss Lab, University of Virginia) at 1:200 dilution in Blocking Solution for Western Blot or rat anti-DAT 1^o Ab (Chemicon) at 1:500 dilution in Blocking Solution for Western Blot. Three quick washes were made with 10 ml Blot Wash (Appendix A) and then three 5 minutes washes were made with 20 ml Blot Wash. The KCNK3 membrane was probed with a horseradish peroxidase-conjugated goat anti-rabbit 2^o Ab (Chemicon) at 1:1000 dilution, and the DAT membrane was incubated with a horseradish peroxidase-conjugated goat anti-rat 2^o Ab (Chemicon) at 1:1000 dilution. The membranes were washed again as before and were developed using Pierce SuperSignal

West Dura (Pierce) at 1:2 dilution for 5 minutes. CCD camera gel documentation system was used to capture immunoreactive bands, which were later quantified using Quantity One software (Bio-Rad).

Construction of the HA-tag

Insertion of KCNK3 into pcDNA 3.1(+)

The KCNK3 cDNA (cloned into pcDNA3.1 Topo(+) vector) was donated to us by the Bayliss lab (University of Virginia). The sequence of pcDNA3.1 Topo(+) was not available and hence in order to locate the restriction sites, the construct was sequenced using primers KCNK3-649R primer (Invitrogen) (5'-CTCATAGTAGGAGAAGGCAG-3') and BGHrev (5'-TAGAAGGCACAGTCGAGG-3') from the Nucleic Acid Facility at the University of Massachusetts Medical School. The restriction enzymes BamHI (NEB) and XbaI (NEB) were used to excise the KCNK3 coding region sites from pcDNA3.1 Topo(+) so that it could be ligated into pcDNA 3.1(+)
(Invitrogen). In order to do this a double digest was performed at 37°C. The reactions were carried out in a final volume of 20 µl with 5µg each of KCNK3 pcDNA3.1 Topo(+) DNA or the pcDNA 3.1(+)
vector, along with 0.5 µl of each of the restriction enzymes and 2 µl of 10X Buffer 2 (NEB). The digests were then run on a 0.9% agarose gel. The separated KCNK3 band and the digested pcDNA 3.1(+)
band were cut from the gel and purified (Qiagen Gel Extraction Kit) and ligated using 50 ng of the digested pcDNA 3.1(+)
vector at 5:1 insert (KCNK3) to vector molecular ratio. T4 DNA Ligase (NEB) was used for this reaction which was carried out overnight at 16°C.

Insertion of HA (YPYDVPDYA) tag to N terminus of KCNK3

A PCR reaction was carried out to add the HA tag on the N terminus of KCNK3. The forward primer BamHI-HA-KCNK3 104 (5'-ACCGAGCTCGGATCCATGTACCCCTACGACGTGCCCGACTACGCGAAGCGGCAGAATGTGCGCACG-3') contains a BamHI site, followed by the HA tag sequence and part of the KCNK3 sequence until nucleotide 104. 1 μ M of the forward primer, 1 μ M of 649 Reverse primer, 200 μ M of dNTPs, 50ng of template KCNK3 DNA, 0.5 μ L Pfu enzyme and 5 μ L Pfu buffer were added in a final reaction volume of 50 μ L. The PCR was carried out in a thermocycler for 30 cycles of the following settings: 94°C for 30 seconds, 45°C annealing 30 sec and 72°C extension for 8 minutes. The resulting PCR product (Fig. 6) containing the BamHI site, the HA tag sequence and part of the KCNK3 sequence was digested with BamHI and XcmI. The pcDNA3.1(+) was digested with these same enzymes, so that they could be ligated by means of sticky ends.

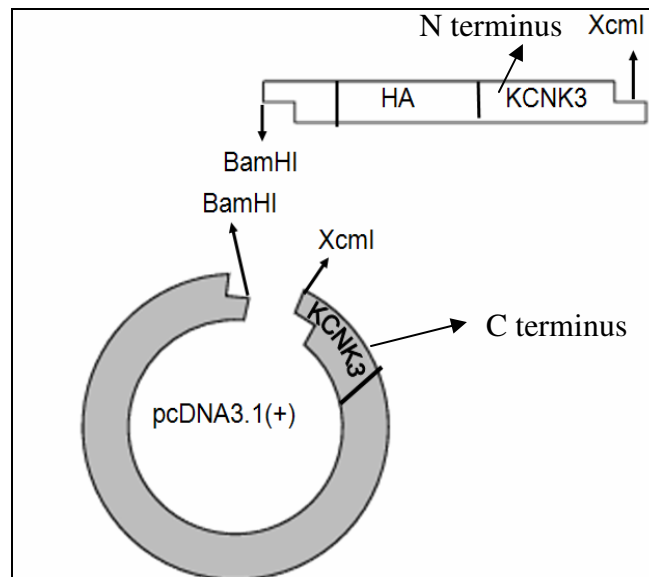


Figure 6: *Ligation Schematic to get the HA tagged KCNK3.* The PCR product should be ligated into pcDNA3.1(+) after restriction digestion with BamHI and XcmI. (Modified from http://www.ornl.gov/sci/techresources/Human_Genome/publicat/primer/fig11a.html)

Mutagenesis of Tac-KCNK3 322-331

Two sets of primers were designed, one set to mutate the first five amino acids in the SREKLQYSIP sequence to alanines and the second set to mutate the last five amino acids to alanines. The first set of primers were named Tac-KCNK3 322-326 (5A)F (5'-CGTGCCTCTGTAAAGGCCGCCGCGGCGGCAGTACTCCATCCCCATG-3') and Tac-KCNK3 322-326 (5A)R (5'-CATGGGGATGGAGTACTGCGCCGCCGCGGC GGCCTTGTACCAGAGGCACG-3') (T_m= 78.7°C). The second set of primers were named Tac-KCNK3 322-331 (10A)F (5'-GGCCGCCGCGGCGGCGGCGGCCGCCGCCATGATCATCCCGCGGG-3') and Tac-KCNK3 322-331 (10A)R (5'-CCCGCGGGATGATCATGGCGGCGGCGGCCGCCGCCGCGGCGGCC -3') (T_m= 85.8°C). For the Tac-KCNK3 322-326 (5A) mutation, 25ng of Tac-KCNK3 DNA in pcDNA 3.1+ were used, as well as 125ng of each of the 5A primers in a solution of buffer and dNTP mix, using the Pfu Turbo polymerase (Stratagene QuikChange Mutagenesis Kit). The reaction mixture was prepared according to the kit instructions and placed in a thermocycler for 18 cycles of the following settings: 95°C for 30 seconds, 55°C annealing for 1 minute and 65°C extension for 6 minutes and 24 seconds. The resulting PCR product was treated with DpnI, which digested the methylated template DNA, and transfected in XL1-Blue competent cells. The restriction endonuclease and the competent bacteria are part of the kit. The Pfu Polymerase creates mutated plasmids that have staggered nicks on each end. The XL1-Blue bacteria have the ability to repair the nicks in the mutated plasmids. The resulting transformants were mini-prepped and sequenced. To create the 10A mutant, the reaction was repeated using 25ng of the newly formed Tac-KCNK3 322-326 (5A) DNA as a template and 125ng of each of the 10A

primers. The thermocycler settings were set at 18 cycles: 95°C for 30 seconds, 50°C annealing for 1 minute and 65°C extension for 7 minutes.

Ligation of Tac-KCNK3 mutants into parental plasmid

The restriction endonucleases Bsu36I (NEB) and ApaI (NEB) were used to excise the mutated part of the gene and ligate it back into the parental plasmid. Bsu36I cuts once in about the middle of the Tac-KCNK3 gene, whereas ApaI cuts once in the vector, about five bases after the end of the gene, resulting in an excised piece of approximately 610 base pairs (Fig. 7). For the digest, two 30µl reactions were set up at 37°C, one containing the parental plasmid and one containing the plasmid with the mutated DNA. Each of the reactions required 0.5µl of Bsu36I and 1.5µl of ApaI. Since ApaI has a half life on one hour, 0.5µl were added to each of the reactions every one hour. At the end of the digest the parental plasmid was treated with 1µl of CIP (NEB). The resulting reactions were run on a 0.7% agarose gel. After the parental vector and the mutated insert were cut from the gel and purified, a 5:1 insert to vector molecular ratio ligation reaction was set up using 50ng of parental vector and 24.8ng of insert for the Tac-KCNK3 (5A) mutant, and 75ng of parental vector with 37.1ng of insert for the Tac-KCNK (10A) mutant. T4 DNA ligase (NEB) was used for this reaction.

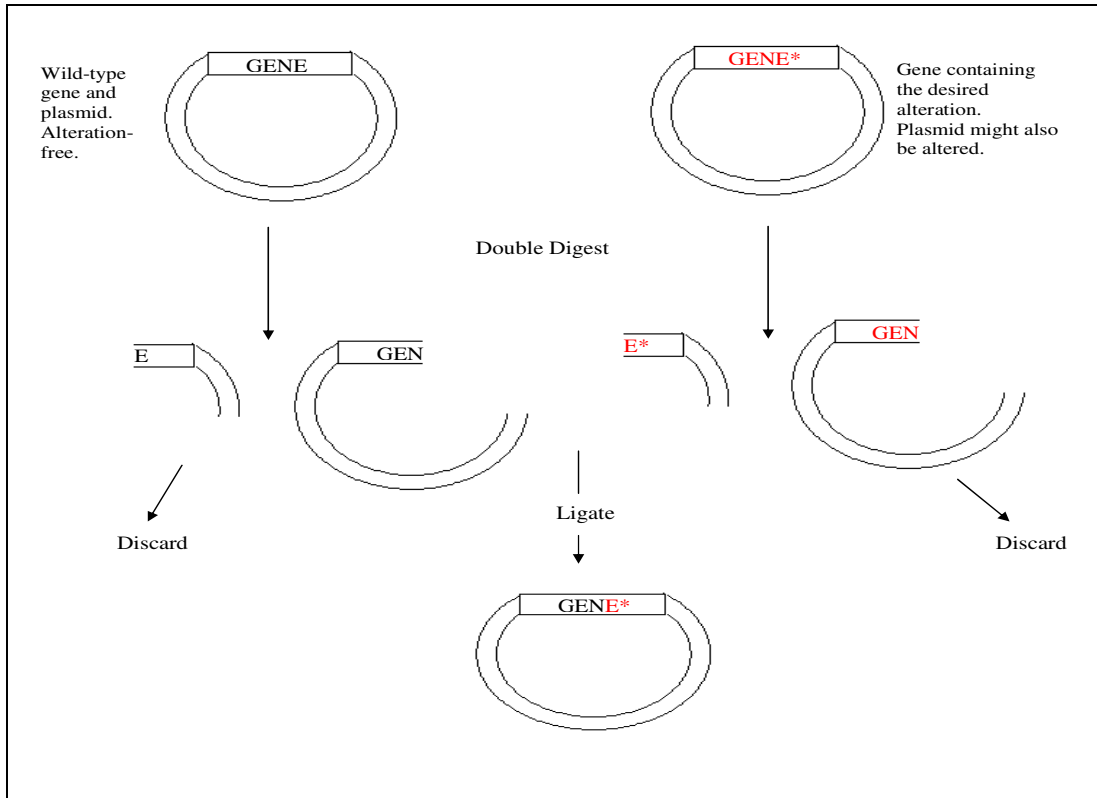


Figure 7: Ligation schematic. After both the parental and the altered plasmid were digested using single cutting restriction endonucleases, the parental vector and the altered gene were recovered from a gel and ligated together to give a new plasmid where only the gene is altered.

RESULTS

The carboxyl terminus of KCNK3 is sufficient to cause Tac internalization

To determine whether the carboxyl terminus of KCNK3 would induce Tac (IL-2 α receptor) internalization, PC12 cells were transfected with four different DNAs, followed by an internalization assay. Tac is a cell membrane receptor that does not internalize unless another molecule attached to it directs it to do so. The carboxyl terminus of DAT causes Tac to internalize; therefore, PC12 cells transfected with the Tac-DAT chimera were used in this experiment as positive controls (Holton et al., 2005). Conversely, PC12 cells transfected with only Tac DNA were used as a negative control. As mentioned earlier, KCNK9, which is a KCNK3 homolog, does not contain the suspected internalization motif. Thus, cells transfected with the Tac-KCNK9 chimera were used as a specificity control. To test the hypothesis that the carboxyl terminus of KCNK3 will drive Tac internalization, PC12 cells were transfected with the Tac-KCNK3 chimera. The Tac internalization pattern was viewed using a Tac antibody (Holton et al., 2005) (Fig. 8).

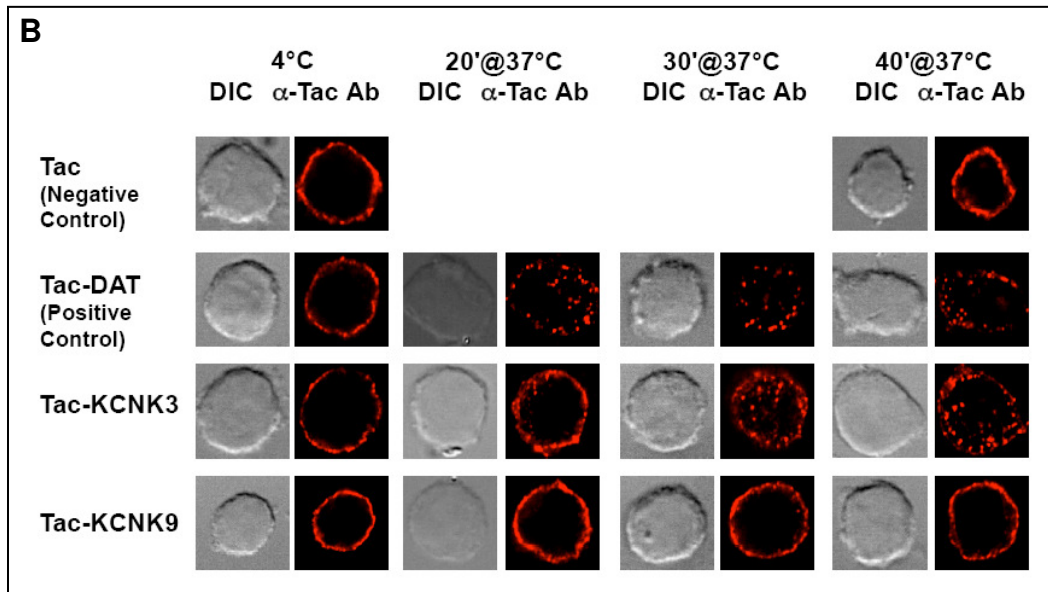
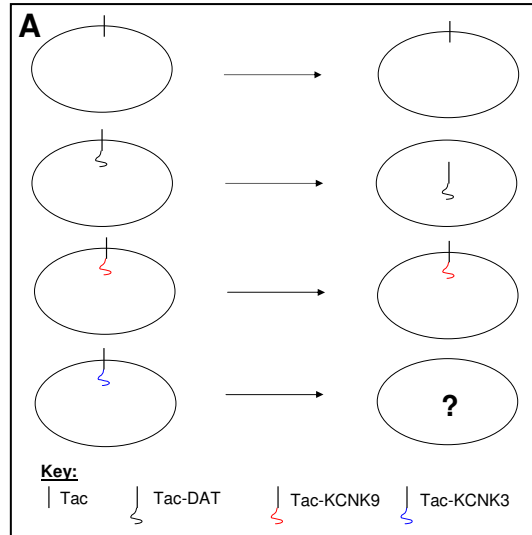


Figure 8: *The carboxyl terminus of KCNK3 is sufficient to cause internalization of Tac.* (A) A schematic representation of the expected results when cells are transfected with each of the Tac chimeras. (B) Results of Tac internalization assay. Antibody to Tac (anti-Tac) was bound at 4°C to cells transfected with the indicated Tac chimeras, and cells were warmed to 37°C for 20, 30 or 40 minutes. Internalized anti-Tac was detected with fluorescently labeled secondary antibodies using wide-field microscopy and image deconvolution. Internalization was scored in cases where Tac surface staining was lost, with a parallel appearance of intracellular puncta. All pictures shown are of the middle of the cell. Representative cells are shown.

Tac construct	Condition	Total number of cells imaged	Percentage of cells showing internalization
Tac	4 °C	12	0.0 ± 0.0
	20'@37 °C	1	0.0 ± 0.0
	40'@37 °C	11	28.3 ± 11.7
Tac-DAT	4 °C	11	11.1 ± 11.1
	20'@37 °C *	21	81.1 ± 7.4
	30'@37 °C *	11	100.0 ± 0.0
	40'@37 °C *	6	83.3 ± 16.7
Tac-KCNK3	4 °C	15	0.0 ± 0.0
	20'@37 °C	17	20.8 ± 20.8
	30'@37 °C	11	61.7 ± 21.7
	40'@37 °C *	12	61.1 ± 27.8
Tac-KCNK9	4 °C	17	0.0 ± 0.0
	20'@37 °C	13	0.0 ± 0.0
	30'@37 °C	13	0.0 ± 0.0
	40'@37 °C **	12	0.0 ± 0.0

Figure 9: Summary of the results from the Tac internalization assay. The conditions signify the amount of time and the temperature where incubation took place. Tac-DAT 20' @37°C, Tac-DAT 30' @37°C, Tac-DAT 40' @37°C and Tac-KCNK3 40' @37°C were significantly different from Tac 4°C. Tac-KCNK9 40' @37°C was significantly different from Tac-KCNK3 40' @37°C (ANOVA, Bonferroni posthoc).

As expected, internalization was observed for the Tac-DAT chimera at all time intervals of the 37°C incubations, as was evident from the disappearance of surface staining and the simultaneous appearance of puncta inside the cells. Interestingly, the Tac-KCNK3 chimera also internalized but it seemed to do so more slowly, as can be seen by the increasing percentages of cells showing internalization as the incubation time increased (Fig. 9). Curiously, we observed about 27% of the cells transfected with Tac and incubated at 37°C for 40 minutes to show some internalization; however, this was not nearly as robust as the internalization demonstrated by the Tac-DAT and Tac-KCNK3 chimeras, and may be attributed to membrane fluidity. It is important to note that the

cells transfected with the Tac-KCNK9 chimera only exhibited surface staining, consistent with the hypothesis that the SREKLQYSIP motif in the carboxyl terminus of KCNK3 might be important for internalization.

Tac-KCNK3 mutants were constructed

To determine whether the SREKLQYSIP sequence is responsible for the internalization of the Tac-KCNK3 chimera, this sequence was mutated to encode either 10 alanines or 5 alanines (see Methods), and the mutated sequence was ligated back into the parental plasmid (Fig. 10). These mutants will be used in Tac internalization assays in the future.

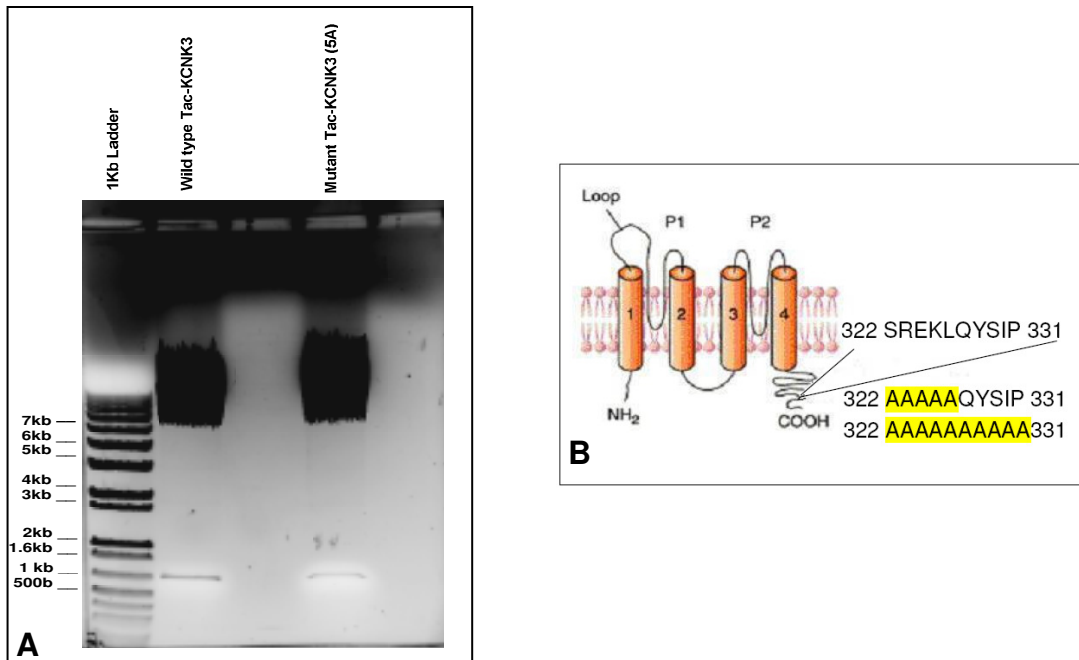


Figure 10: *Construction of the Tac-KCNK3 mutants.* (A) Gel used for the ligation of the Tac-KCNK3 (5A) mutant insert into the Tac-KCNK3 parental plasmid. After the wild-type Tac-KCNK3 and the Mutant Tac-KCNK3 were cut by restriction endonucleases, a 0.7% agarose gel was run to separate the pieces. The large vector band (~7kb) from the wild-type lane and the small (~600b) band from the mutant lane were extracted, purified and ligated. The same procedure was followed for the Tac-KCNK3 (10A) mutant. (B) Schematic representation of the mutated amino acids in the SREKLQYSIP motif. The (5A) mutant is shown directly below the wild type sequence and the (10A) mutant is shown directly below that. The mutated amino acids are highlighted.

Available rabbit anti-KCNK3 antibody does not specifically recognize KCNK3 by immunoblot.

In order to perform biochemical studies we needed a specific antibody that recognized KCNK3 to measure its trafficking. The antibody that was tested in this experiment was rabbit anti -KCNK3 that was generously donated by the Bayliss lab at University of Virginia. To characterize the KCNK3 Ab, two immunoblots were performed. The first (Fig. 11A) was the KCNK3 immunoblot, to determine if the KCNK3 Ab binds specifically to the KCNK3 protein. The other immunoblot was the DAT blot (Fig. 11B) which served as the control for transfection and the blotting techniques. DAT primary Ab (Chemicon) was already successfully used previously (Melikian et al, 1999). Both immunoblots were done at the same time.

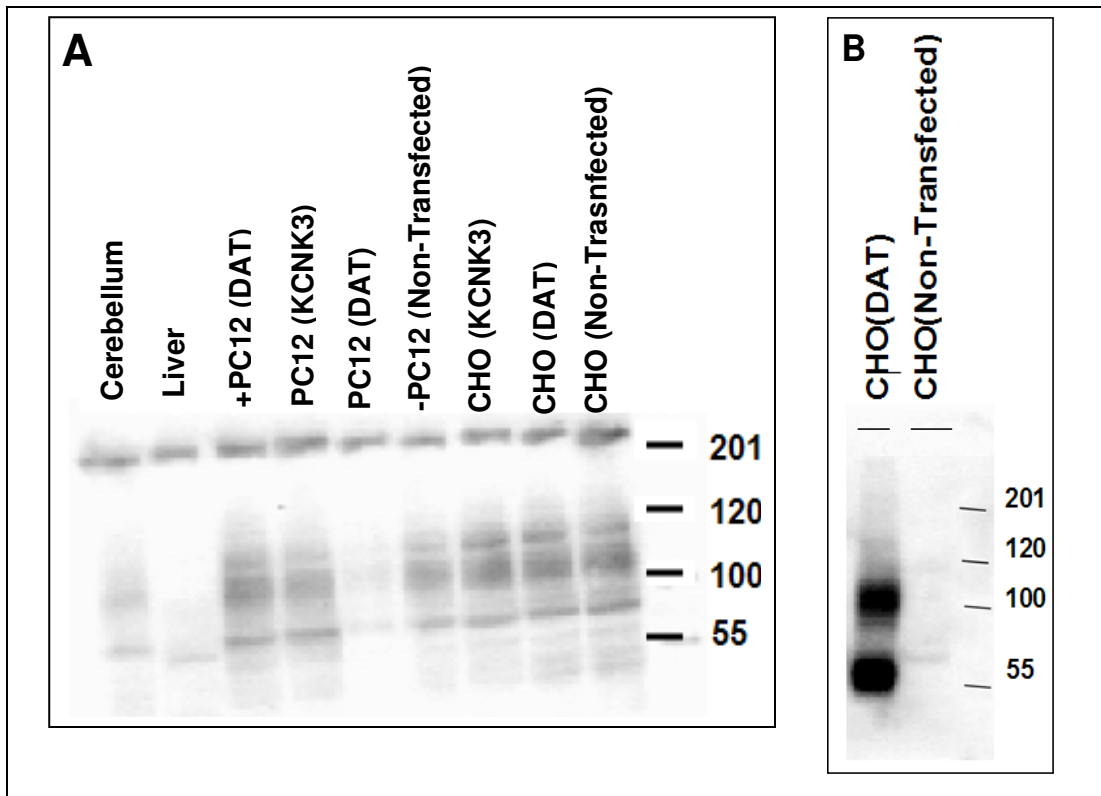


Figure 11: *Characterization of the KCNK3 Ab by Immunoblotting.* (A) Available Ab rabbit anti -KCNK3 does not specifically recognize KCNK3. Lanes were loaded with 25 μ g of protein. Lanes 1 and 2- protein lysate from Rat cerebellum and liver. Lane 3, lysate from PC12 cells that were stably transfected with DAT DNA. Lane 4 and 5, lysate of PC-12 cells transfected with KCNK3 or DAT DNA. Lane 6, non-transfected PC12 cells. Lane 7 and 8, CHO cells transfected with KCNK3 or DAT DNA. Lane 9 non-transfected CHO cells. Lane 10, the molecular weight ladder in kDa. (B) DAT immunoblot as transfection control- Lane1, CHO cells transfected with DAT DNA and lane 2, non-transfected cells. Both lanes were loaded with 25 μ g of protein.

Figure 11A depicts the KCNK3 immunoblot. According to the Bayliss lab at University of Virginia, KCNK3 immunoreactive bands were seen around 45-55 kDa, and a non-specific band was also present in non-transfected cells (Berg et al., 2004). In figure 11A, rat cerebellum was used as the positive control (Berg et al., 2001) while the liver lysate was used as the negative control. PC12 cells and CHO cells transfected with KCNK3 DNA also served as positive controls. As demonstrated in Figure 11A, the

rabbit anti-KCNK3 Ab was not specific in our conditions. All the lanes had non-specific bands on the immunoblot from 130 kD to 45 kD.

As seen from the results obtained in the DAT immunoblot (Fig. 11B), lane 1, transfection of CHO cells followed by immunoblotting revealed two bands. The rat anti-DAT Ab specifically recognizes DAT at a broad band at 90 -100 kDa band and a lower band at 56 kDa (Fig. 11B) that is a biosynthetic intermediate as determined by enzymatic deglycosylation (Melikian et al., 1999). Thus this transfection control was successful, suggesting that technical problems in the experiment were not responsible for the rabbit anti-KCNK3 non-specificity to KCNK3 in our conditions.

Because the immunoblots were not successful, an HA tag will be constructed on the N-terminus of KCNK3 so that KCNK3 can be visualized by immunoblotting.

PCR to add the HA tag

Since the available Ab, rabbit anti-KCNK3 was not specific to KCNK3, an HA tag will be added to the N terminus of KCNK3. In order to do this, a PCR reaction was carried out with the forward primer containing the HA tag sequence (See methods). The PCR product was expected to be ~ 675 bp. The PCR reaction was successful twice (Fig. 12), but the gel purification gave lower yields of the PCR product when the PCR product was digested with BamHI and XcmI. Thus when the digested products were run on a gel again, no PCR product band was visible.

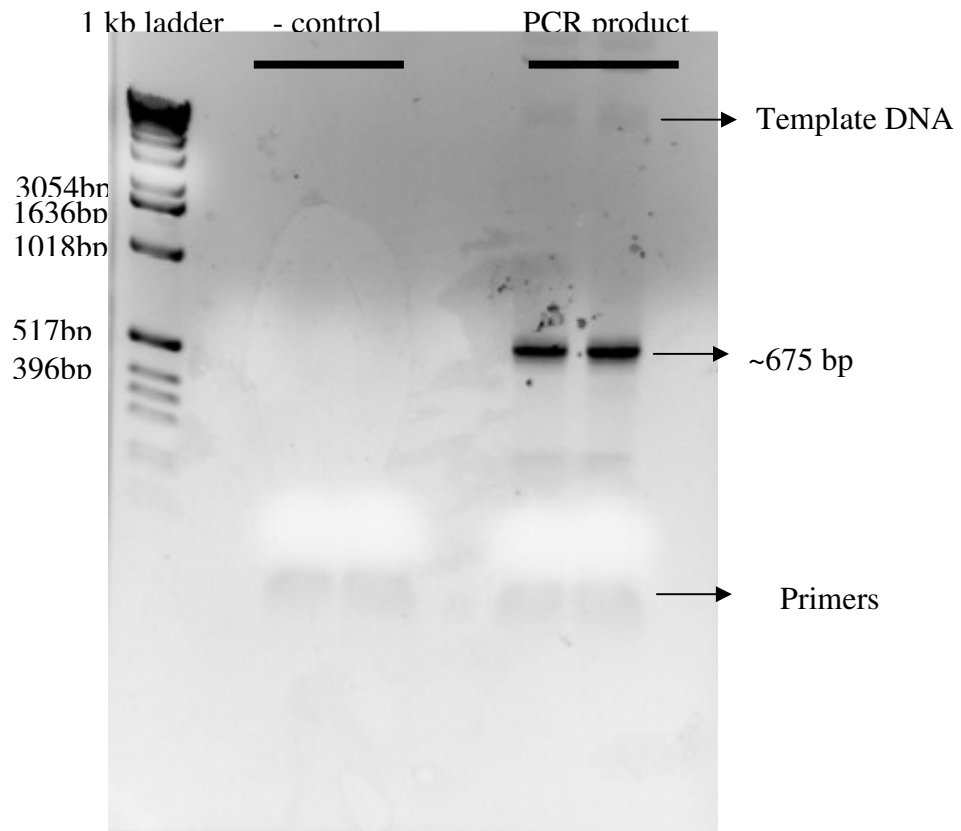


Figure 12: *Visualization of the PCR product.* The PCR product (~675bp) was separated from the template DNA and the primers on a 0.9% agarose gel.

DISCUSSION

In this project we sought to define whether the carboxyl terminus of the leak potassium channel KCNK3 would be sufficient to induce the internalization of an endocytic deficient reporter molecule and whether the entire KCNK3 channel is able to constitutively internalize. In both cases we wanted to determine whether the SREKLQYSIP motif on the carboxyl terminus of KCNK3 would be important for such internalization. Our results show that indeed the carboxyl terminus of KCNK3 is sufficient to cause the internalization of the interleukin 2 α receptor, Tac.

Since the carboxyl terminus of DAT, which contains the newly identified internalization motif FREKLAYAIA, has been shown to be sufficient for the rescue of Tac internalization, the first step in our investigation was to determine whether the carboxyl terminus of KCNK3 would be able to do the same, through the Tac internalization assays. In fact, we have shown that fusing the carboxyl terminus of KCNK3 to the Tac caused the whole chimera to internalize. However, we saw that longer incubations than those required for the Tac-DAT chimera were necessary to make this internalization evident. For Tac-DAT twenty minutes at 37°C were enough to induce internalization in approximately 80% of cells imaged; however, it took a forty minute incubation at 37°C for internalization to be evident in 75% of the cells transfected with Tac-KCNK3. This result may suggest that the mechanism governing the internalization of Tac-KCNK3 is different than the one for Tac-DAT, or that the signal for internalization is not as strong.

The results of the internalization assay also suggest that much like the DAT FREKLAYAIA motif, the SREKLQYSIP motif in KCNK3 may be responsible for the internalization of the reporter protein. This suggestion is supported by the fact that none

of the cells transfected with Tac fused to the carboxyl terminus of the homologous KCNK9 showed internalization. It is important to note that KCNK3 and KCNK9 have very similar carboxyl termini; however, KCNK9 contains a completely different sequence in the place of the SREKLQYSIP motif. To further test the hypothesis that the SREKLQYSIP motif is responsible for this internalization, we constructed one Tac-KCNK3 mutant, where amino acids 322-331, which correspond to the SREKLQYSIP motif were mutated to alanines, and another Tac-KCNK3 mutant where amino acids 322-326, corresponding to the SREKL part of the motif, were mutated to alanines. Due to time constraints we were not able to test what effects these mutations would have on the internalization of Tac. However, we suggest that both of these mutants be used in the internalization assay to see whether the internalization pattern is affected.

In order to show that the whole KCNK3 channel internalizes, we needed a specific Ab that can bind KCNK3, so that internalization studies could be performed on KCNK3. The rabbit anti-KCNK3 from the Bayliss lab was shown to be non-specific to KCNK3 in our experimental conditions (Fig. 11A). This might be because of the different methods by which the experiments were carried out, or the inability of the Ab to bind KCNK3 in the cell lysates that were used. In order to perform further studies on the channel, an HA tag will be added to the N-terminus of KCNK3, so that the channel internalization can be studied by cell surface biotinylation assays. Initial efforts to add the HA tag on the N terminus of KCNK3 haven't been completed and should be continued in the future.

KCNK channels are modulated by a variety of physical and chemical stimuli such as temperature, intracellular/extracellular pH, oxygen tension, osmolarity and breathing.

Deletional analysis has shown that the carboxyl termini of leak potassium channels are important for all of the above functions. KCNK3 sensitivity to anesthetics, which can be attributed to the carboxyl terminus, allows anesthetics such as halothane to increase KCNK3 leak channel activity, inhibiting cell excitability (Patel and Honorè, 2001). Thus, the SREKLQYSIP sequence that is present on the carboxyl terminus might be a critical sequence in affecting anesthetic mechanisms. Would mutating the sequence affect neuronal excitability? The opening of 2P pore regions of KCNK3 by inhalation anesthetics might also indicate that they are involved in the mechanism of general anesthesia (Patel and Honorè, 2001). Our results provide only an initial step in understanding the mechanisms that govern KCNK3 endocytosis.

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APPENDIX A

Solution Ingredients

1. Blocking Solution for Tac Internalization Assay

1X PBS pH 7.4, 5% Normal Goat Serum, 1% BSA (IgG and Protease free), 0.2% Triton X-100.

2. Blocking Solution for Western Blot

5% non-fat dried milk (w/v) in Blot Wash (1X PBS with 0.1% Tween-20).

3. 5X Electrode Running Buffer

0.125M Tris Base, 0.960M Glycine, 0.5% SDS (premade in lab).

4. Fixing Solution

4% Paraformaldehyde in 1X PBS.

5. PBS

Phosphate Buffered Saline

6. PBS²⁺

1.0 mM CaCl₂, 0.1mM MgCl₂ in 1X PBS.

7. Primary Antibody Solution -PBS²⁺/g/BSA

0.18% glucose, 0.2% BSA (IgG and Protease free) in PBS²⁺.

8. RIPA/PI (RIPA buffer with Protease inhibitors)

10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.1 % SDS, 1% Triton X-100, 1% Na deoxycholate, 150 mM NaCl, 1.0mM PMSF with 1.0 µg/ml each of leupeptin, pepstatin and aprotinin.

9. Running gel (8%)

30% acrylamide, 1.5M Tris-HCl (pH8.8), 10% SDS(w/v), 10% of Ammonium persulfate solution(w/v), TEMED, H₂O.

10. 6X Sample Buffer

1.0M Tris-HCl (pH6.8), 12% SDS powder, 45% glycerol, 0.012% bromophenol blue ,
9.3% DTT.

11. Secondary Antibody Solution

Alexa 594 diluted 1:5000 in Blocking Solution for Tac Internalization Assay.

12. Stacking gel

30% acrylamide, 1.0M Tris-HCl (pH6.8), 10% SDS (w/v), 10% Ammonium persulfate
solution (w/v), TEMED, H₂O.

13. 5X Transfer Buffer

0.25M Tris Base, 1.25M glycine