2-13-2009

Dual Roles for Rhoa/Rho-Kinase in the Regulated Trafficking of a Voltage-Sensitive Potassium Channel

Lee Stirling
University of Vermont

DUAL ROLES FOR RHOA/RHO-KINASE IN THE REGULATED TRAFFICKING OF A VOLTAGE-SENSITIVE POTASSIUM CHANNEL

A Thesis Presented

by

Lee David Stirling

to

The Faculty of the Graduate College

of

The University of Vermont

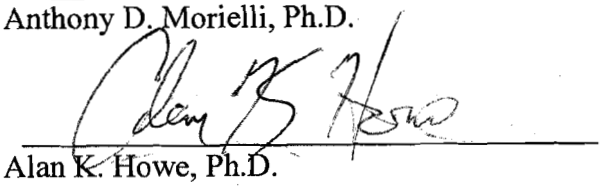
In Partial Fulfillment of the Requirements
for the Degree of Master of Science
Specializing in Cell and Molecular Biology

February, 2009

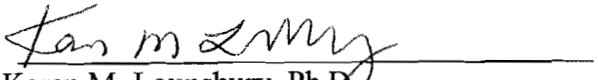
Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Master of Science, specializing in Cell and Molecular Biology.

Thesis Examination Committee:

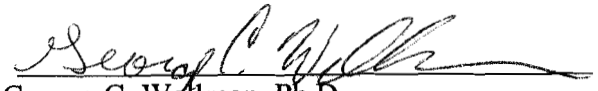
Anthony D. Morielli, Ph.D. **Advisor**



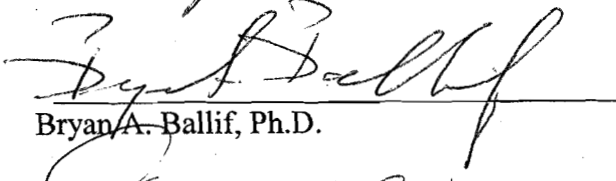
Alan K. Howe, Ph.D.



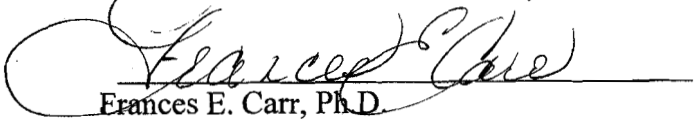
Karen M. Lounsbury, Ph.D.



George C. Wellman, Ph.D.



Bryan A. Ballif, Ph.D. **Chairperson**



Frances E. Carr, Ph.D. **Vice President for Research and Dean of Graduate Studies**

Date: November 19, 2008

ABSTRACT

Kv1.2 is a member of the *Shaker* family of voltage-sensitive potassium channels and contributes to regulation of membrane excitability. The electrophysiological activity of Kv1.2 undergoes tyrosine kinase-dependent suppression in a process involving RhoA. We report that RhoA elicits suppression of Kv1.2 ionic current by modulating channel endocytosis. This occurs through two distinct pathways, one clathrin-dependent and the other cholesterol-dependent. Activation of RhoA downstream effectors Rho-kinase (ROCK) or protein kinase N (PKN) via the lysophosphatidic acid (LPA) receptor elicits clathrin-dependent Kv1.2 endocytosis and consequent attenuation of its ionic current. LPA-induced channel endocytosis is blocked by ROCK inhibition , dominant negative PKN, or by clathrin RNAi. In contrast, steady-state endocytosis of Kv1.2 in un-stimulated cells is cholesterol-dependent. Inhibition of basal ROCK with Y27632 or basal PKN with HA1077 increases steady-state surface Kv1.2. The Y27632-induced increase persists in the presence of clathrin RNAi and, in the presence of the sterol-binding agent filipin, does not elicit an additive effect. Temperature block experiments in conjunction with studies that perturb trafficking of newly synthesized proteins from the Golgi demonstrate that basal ROCK affects cholesterol-dependent trafficking by modulating the recycling of constitutively endocytosed Kv1.2 back to the plasma membrane. Both receptor-stimulated and steady-state Kv1.2 trafficking modulated by RhoA/ROCK require the activation of dynamin as well as the ROCK effector LIM kinase, indicating a key role for actin remodeling in RhoA-dependent Kv1.2 regulation.

ACKNOWLEDGEMENTS

I wish to take this opportunity to thank the Cell and Molecular Biology Program for providing me the opportunity to pursue an interesting and fulfilling research project. Thanks also to the Department of Pharmacology for accepting me as one of their own, graciously hosting me, and providing a comfortable, constructive environment for my growth as a scientist. Special thanks to my advisor, Dr. Anthony Morielli, who has been a consistent source of encouragement, support, and insight. I also wish to thank the other members of the Morielli laboratory; Emilee Connors, Megan Doczi, and Michael Williams for all the help they have provided from the start of this research project.

I am deeply grateful to the members of my thesis studies committee for their contributions and commitment on my behalf; Dr. Bryan Ballif, Dr. Alan Howe, Dr. Karen Lounsbury, and Dr. George Wellman.

Finally, this project would have been impossible without the consistent, unwavering support of my family and friends. Thanks to you all, especially Aline, Greta, and Eve who make each day brighter than the one before.

TABLE OF CONTENTS

LIST OF FIGURES

Page

Chapter 1

Chapter 2

Chapter 3

GLOSSARY

α-CHCm: Mouse monoclonal antibody that recognizes the heavy chain of clathrin
α-EEA1m: Mouse monoclonal antibody that recognizes the early endosome marker EEA1
α-Kv1.2e: Rabbit polyclonal antibody that recognizes the first extracellular loop of Kv1.2
α-pMYPT1p: Rabbit polyclonal antibody that recognizes Thr696 phosphorylated myosin phosphatase target subunit 1
α-RhoAm: Rat monoclonal antibody that recognizes total RhoA
BFA: Brefeldin A
CCh: Carbachol
DMSO: Dimethyl sulfoxide
EEA1: Early endosome antigen 1
GAP: GTPase activating protein
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GDI: Guanine nucleotide dissociation inhibitor
GDP: Guanosine di-phosphate
GEF: Guanine nucleotide exchange factor
GM130: Golgi matrix protein of 130 kD
GTP: Guanosine tri-phosphate
HA1077: Protein kinase N inhibitor
HEK-K: Human embryonic kidney 293 cells stably expressing the m1 acetylcholine receptor, Kv1.2α, and Kvβ2.
LIMK: LIM kinase
LPA: Lysophosphatidic acid
MeOH: Methanol
NT: Non-targeting
pMYPT1: Myosin phosphatase target subunit 1
PKN: Protein kinase N
RNAi: Ribonucleic acid interference
ROCK: Rho-kinase
SRE: Serum response element
Y27632: Rho-kinase inhibitor

CHAPTER 1: LITERATURE REVIEW

Potassium Channels

Regulation of cellular excitability and resting membrane potential is important in many different cell types and critical for processes such as neurotransmission, cardiovascular regulation, and learning and memory. This regulation is accomplished by the combined contribution of many different ion channels. Among these, potassium channels are an important participant.

Excitable cells are set up with a high concentration of potassium in the cytoplasm, such that when potassium channels open, positively charged ions flow out of the cell, down their concentration gradient (Figure 1) (Kandel *et al.*, 2000). The end result is a reduction of positive charge inside the cell, making the membrane potential more negative, thus the cell becomes less excitable, or hyperpolarized (Kandel *et al.*, 2000).

All potassium channels are comprised of multiple alpha subunits that make up the pore-forming portion of the channel. These alpha subunits can contain varying numbers of trans-membrane domains, depending on the channel type, along with cytoplasmic N and C termini. Intracelluar beta subunits interact with the hydrophilic alpha subunit N-terminus (Rhodes *et al.*, 1995; Jan and Jan, 1997) and can modulate channel kinetics and trafficking (Shi *et al.*, 1996; Shi and Trimmer, 1999; Wissmann *et al.*, 1999; Long *et al.*, 2005). When functioning properly, potassium channels have a flow rate that approaches $1x10^{8}$ ions sec^{-1} (Grottesi *et al.*, 2005).

All potassium channels have a highly conserved selectivity filter that allows only potassium ions to flow through the pore (MacKinnon, 1995). This filter is comprised of extracellular loops from the alpha subunits, P-loops, dipping into the plasma membrane (MacKinnon, 2003). In order for potassium ions to flow through the channel, they must first be dehydrated, creating an energetic barrier for ion flow. Channels overcome this barrier by lining the interior of the filter with carbonyl oxygens that interact with potassium ions, mimicking hydration (Figure 2) (Doyle *et al.*, 1998; Choe, 2002). Other positively charged cations, like sodium, have different atomic radii than potassium and cannot effectively interact with the carbonyl oxygens lining the filter (MacKinnon, 2003).

There are four interaction sites for potassium ions within the filter (S1-S4) and two exterior sites, one on each side of the pore (Choe, 2002). Only two internal sites are occupied at any one time, S1 and S3 or S2 and S4. As such there is concerted ion flow through the filter with repulsive forces between positively charged ions aiding the flow of potassium (Berneche and Roux, 2001; Morais-Cabral *et al.*, 2001; MacKinnon, 2003). In addition, it has been reported that the selectivity filter transitions between two conformation states depending upon interaction with potassium. When potassium is present, channels adopt a conductive conformation with sites S1 thru S4 available for interaction. When potassium is not present, channels adopt a non-conductive conformation with sites S2 and S3 pinched shut to prevent ion flow (Valiyaveetil *et al.*, 2006). Potassium channels therefore have two levels of ion selectivity.

Kv1.2 Overview

Several types of potassium channel exist. Of particular importance to the regulation of membrane potential and cellular excitability are the Shaker family voltage-sensitive potassium channels, of which Kv1.2 is a member. Other Shaker family channels include Kv1.1, 1.3, 1.4, 1.5, and 1.6 (Chandy, 1991; Roberds and Tamkun, 1991). These channels were originally isolated from *Drosophila* during studies of the Shaker gene (Kamb *et al.*, 1987; Solc *et al.*, 1987). Kv1.2 can be found in hippocampal and cerebellar neurons (Tsaur *et al.*, 1992; Sheng *et al.*, 1994; Veh *et al.*, 1995; Grosse *et al.*, 2000), vascular smooth muscle (Adda *et al.*, 1996; Wang *et al.*, 1997; Wang *et al.*, 2005), and cardiac muscle (Cox *et al.*, 2001; Hong *et al.*, 2004) where it functions as a delayed rectifier. Kv1.2 regulates diverse physiological functions, including the maintenance of action potential proliferation along myelinated axons (Rasband *et al.*, 2001; Rasband and Trimmer, 2001; Rasband, 2004), transduction of pain in peripheral sensory neurons (Ishikawa *et al.*, 1999; Kim *et al.*, 2002; Yang *et al.*, 2004) and modulation of tone in vascular smooth muscle cells (Adda *et al.*, 1996; Wang *et al.*, 1997; Wang *et al.*, 2005). Dysregulation of Kv1.2 has been implicated in disease states such as hypertension (Cox *et al.*, 2001; Hong *et al.*, 2004), neuropathic pain (Ishikawa *et al.*, 1999; Kim *et al.*, 2002; Yang *et al.*, 2004) and seizure activity in the central nervous system (Lambe and Aghajanian, 2001; Brew *et al.*, 2007). These are all loss-of-function phenotypes.

Kv channels are comprised of 4 alpha and 4 beta subunits. Each alpha subunit has six trans-membrane domains (S1-S6 helices). The tetramer forms a single pore (Papazian

et al., 1991; Papazian *et al.*, 1995; Seoh *et al.*, 1996). The P-loop occurs between helices S5 and S6. The N and C-terminus of the alpha subunit are cytoplasmic, with the N-terminus containing a tetramerization (T1) domain (Choe, 2002) which interacts with the beta subunit (Rhodes *et al.*, 1995; Jan and Jan, 1997). Kv1.2 is most commonly associated with the Kv beta 2 subunit (Shi *et al.*, 1996). The S4 helix acts as the voltage sensor. It contains four positively charged arginines at positions 294, 297, 300, and 303 that allow the helix to move in relation to the rest of the channel when voltage across the plasma membrane changes. Movement of the S4 helix allows the pore region of the channel to open or close (Long *et al.*, 2005) (Figure 3).

Kv1.2 Regulation

Kv1.2 is regulated by a diverse array of signals. It was the first potassium channel for which tyrosine phosphorylation was demonstrated to control activity (Pearson and Kemp, 1991; Huang *et al.*, 1993). Phosphorylation of tyrosine 132 results in suppression of Kv1.2 ionic current. This occurs in response to stimulation of the M1 muscarinic acetylcholine receptor (M1 mAChR) with carbachol (CCh) in both *Xenopus* oocytes and human embryonic kidney 293 (HEK-K) over-expression systems (Huang *et al.*, 1993). Kv1.3 and Kv1.5 can be tyrosine phosphorylated, also resulting in ionic current suppression (Lev *et al.*, 1995; Holmes *et al.*, 1996; Fadool *et al.*, 1997; Mason *et al.*, 2002). Alternatively, threonine phosphorylation of Kv1.2 by protein kinase A (PKA) at position 46 also occurs, resulting in enhanced ionic current (Huang *et al.*, 1994).

Phosphorylation is catalyzed by enzymes called kinases that specifically transfer the γ terminal phosphate from ATP to the hydroxyl group of a particular serine, threonine, or tyrosine of the target protein (Alberts *et al.*, 2002). The addition of a phosphate group, which contains four oxygen atoms, allows for complex hydrogen bonding to occur between different, sometimes distant, amino acids in the protein. This, coupled with the fact that the phosphate group has two negative charges at physiological pH, allows for significant conformational changes of the protein structure to occur (Johnson and O'Reilly, 1996).

One common structural change that occurs upon protein phosphorylation is hydrogen bonding between the phosphate group oxygens with nitrogens of the peptide backbone at the start of protein alpha-helical regions. There tends to be more glycine residues at these locations, whose lack of a bulky side chain allows for tight hydrogen bonds to form (Hol, 1985; Chakrabarti, 1993; Copley and Barton, 1994). Another common structural change occurs when the negatively charged phosphate group interacts with the positively charged side chain of an arginine residue, a tight electrostatic interaction (Chakrabarti, 1993; Copley and Barton, 1994). Both types of phosphate group binding result in significant structural changes to the phosphorylated proteins compared to their non-phosphorylated counterparts, which in turn can affect the activation or inhibition of protein function or the protein-protein interactions that occur (Johnson and O'Reilly, 1996). It is not clear whether any conformational changes to the channel occur following Kv1.2 phosphorylation, but phosphorylation of Kv1.2 regulatory proteins can be crucial for channel function.

Kv1.2 trafficking has emerged as a key mechanism for regulating channel activity. Following CCh stimulation, Kv1.2 is internalized to early endosomes. This process is dynamin-dependent and requires an intact actin cytoskeleton. Alterations to the channel that prevent tyrosine phosphorylation-dependent ionic current suppression also prevent Kv1.2 endocytosis (Nesti *et al.*, 2004). Further research has demonstrated that the cyclic-AMP (cAMP) pathway regulates the level of Kv1.2 on the cell surface in both PKA-dependent and independent manners. Basal levels of cAMP modulate steady-state homeostasis of the channel through PKA signaling. Increasing cAMP levels by application of the adenylate cyclase activator forskolin, increases Kv1.2 at the cell surface by negatively regulating homeostatic turnover of the channel in a PKA-independent manner. Essential for the cAMP-mediated effects is dynamin and the actin-regulating protein cortactin (Connors *et al.*, 2008). Cortactin is an actin-binding protein known to activate the Arp2/3 complex, leading to actin filament branching and dynamic cytoskeletal structure formation (Wu and Parsons, 1993; Huang *et al.*, 1997). Other studies have demonstrated a direct interaction between Kv1.2 and cortactin that is reduced upon channel tyrosine phosphorylation. The interaction is prevalent at the edge of cells, while their interaction is significantly reduced under conditions that favor Kv1.2 endocytosis (Hattan *et al.*, 2002; Williams *et al.*, 2007). Cortactin binds the Kv1.2 C-terminus between positions 445 and 463. An intact cortactin C-terminus is also essential because it contains specific tyrosines that are important for proper channel trafficking. These tyrosines in the C-terminus of cortactin are also potential targets of Src kinase and may serve a scaffolding function to recruit other cytoskeletal proteins or components of

the endocytosis machinery. Mutations that abolish the ability of cortactin to bind F-actin or activate the Arp2/3 complex reduce steady-state levels of surface Kv1.2 or the ability of CCh to suppress Kv1.2 respectively (Williams *et al.*, 2007).

This is not the first evidence implicating the actin cytoskeleton as an important player in the regulation of Kv1.2. Previously, it was also demonstrated that RhoA, another actin-modulating protein, could affect Kv1.2 function (Cachero *et al.*, 1998). As evidenced by two-hybrid screen analysis, RhoA interacts with Kv1.2 requiring an intact channel N-terminus. Over-expression of RhoA suppresses Kv1.2 ionic current while pharmacological inhibition of RhoA enhances Kv1.2 ionic current. These effects are specific to RhoA, as over-expression of other Rho GTPases do not alter Kv1.2 ionic current (Cachero *et al.*, 1998). Evidence suggests that RhoA functions as part of the tyrosine phosphorylation-dependent suppression pathway. Mutation of tyrosine 132, required for tyrosine phosphorylation-dependent Kv1.2 suppression, significantly diminishes the ability of RhoA to reduce channel currents. Furthermore, inhibition of RhoA with C3 exoenzyme can prevent CCh-induced Kv1.2 current suppression (Cachero *et al.*, 1998). Little is known, however, about the mechanism of Kv1.2 suppression downstream of RhoA.

Rho GTPases

RhoA is member of the Rho GTPase family of actin-regulating proteins which are members of the larger Ras superfamily of monomeric, 20-30 kilo dalton GTP-binding proteins. In mammals the Ras superfamily is comprised of five major groups, those

being Ras, Rho, Rab, Arf, and Ran proteins (Etienne-Manneville and Hall, 2002). There are twenty genes that encode the Rho GTPases which can be further broken down into smaller classes, those being Rho, Rac, Cdc42, Rnd, RhoD, and TTF (Hall, 1998; Aspenstrom, 1999). These proteins undergo dynamic cycling between GDP and GTP-bound states. When GTP-bound, Rho GTPases are active and can regulate various cytoskeletal structures within the cell or certain signaling pathways. Most Rho GTPases also have a geranyl-geranyl post-translational modification on their C-terminus which allows them to associate with cellular membranes (Adamson *et al.*, 1992; Cox and Der, 1992; Seabra, 1998). These proteins have demonstrated, critical roles for processes such as cytokinesis, phagocytosis, pinocytosis, cell migration, cell morphogenesis, and axon guidance. Rho GTPases have also been shown to regulate Serum Response Factor (SRF), JNK/p-38 MAP Kinase, NADPH oxidase, and cell cycle progression signaling pathways (Ridley and Hall, 1992; Cox *et al.*, 1997; Drechsel *et al.*, 1997; Luo *et al.*, 1997; Nobes and Hall, 1999; Settleman, 1999; Prokopenko *et al.*, 2000). Clearly, Rho GTPases are important regulators of cellular function.

Because of their involvement in so many critical processes, tight regulation of Rho GTPase activity is necessary. Guanine nucleotide cycling and cellular localization of the GTPases is regulated by the combined activity of three sets of proteins known as GEFs, GAPs, and GDIs (Kjoller and Hall, 1999) (Figure 4). GEFs, or guanine nucleotide exchange factors, catalyze the exchange of bound GDP for GTP. All GEFs contain a catalytic Dbl-homology (DH) domain responsible for nucleotide exchange as well as an adjacent pleckstrin-homology (PH) domain which can modulate the

interaction of the GEF with membranes (Hart *et al.*, 1994; Zheng *et al.*, 1996; Rameh *et al.*, 1997; Cherfils and Chardin, 1999). GAPs, or GTPase activating proteins, enhance the intrinsic GTPase activity of the Rho protein. GAPs function by insertion of an arginine finger motif into the active site of the GTPase which stabilizes a molecular transition state during the hydrolysis of GTP to GDP, thus speeding up the reaction (Lamarche and Hall, 1994; Rittinger *et al.*, 1997a; Rittinger *et al.*, 1997b). GDIs, or guanine nucleotide dissociation inhibitors, prevent the exchange of GDP for GTP and can sequester Rho GTPases in the cytoplasm of the cell (Olofsson, 1999). GDIs have an immunoglobulin-like, hydrophobic pocket on their C-terminus which can bind and mask the geranyl-geranylation of Rho GTPases, making them soluble, while their N-terminus interacts with the GTPase, preventing nucleotide exhange (Gosser *et al.*, 1997; Keep *et al.*, 1997; Longenecker *et al.*, 1999; Olofsson, 1999; Hoffman *et al.*, 2000). There are currently over sixty known GEFs and 70 known GAPs for Rho GTPases (Etienne-Mannville 2002). Clearly, Rho GTPases are subject to tight regulation.

RhoA Overview

RhoA is involved in a large array of important cellular processes. By virtue of its ability to signal through many different downstream effectors, RhoA signaling is an important initiating upstream event (Figure 5). To activate RhoA it must bind GTP, which results in a conformational change of the protein. This conformational change occurs primarily in regions of the GTPase known as switch I and switch II (amino acids 28-44 and 62-69 in human RhoA respectively) (Ihara *et al.*, 1998). When bound to GTP,

hydrogen bonds within the switch regions occur to stabilize the conformational change. (Wei *et al.*, 1997; Rudolph *et al.*, 1999). Switch I and II regions are also areas of contact between RhoA and downstream effectors such as Rho-kinase (ROCK) and protein kinase N (PKN). In general, these effectors interact directly with RhoA via anti-parallel coiled coil (ACC) finger folds at these sites (Flynn *et al.*, 1998; Maesaki *et al.*, 1999; Zong *et al.*, 1999; Bishop and Hall, 2000). Interaction with RhoA leads to effector activation, typically by relieving intra-molecular auto-inhibition (Bishop and Hall, 2000).

In order for RhoA to interact with downstream effectors though, RhoA itself must first become activated and it is widely accepted that activation of RhoA occurs through signaling of G protein-coupled receptors. Interestingly, it has been shown that stimulation of the M1 muscarinic acetylcholine receptor (M1 mAChR) with CCh leads to RhoA activation via $G_{\alpha q}$ through an EGF receptor-dependent pathway (Kjoller and Hall, 1999; Hilal-Dandan *et al.*, 2004; Nishida *et al.*, 2005; Riobo and Manning, 2005). This pathway has previously been implicated in the tyrosine kinase-dependent suppression of Kv1.2 ionic current (Huang *et al.*, 1993). Another critical means of RhoA activation is through G protein-coupled receptors that signal through $G_{12/13}$. Specific pharmacological agents known to stimulate $G_{12/13}$ include lysophosphatidic acid (LPA), thrombin, bombesin, sphingosine-1-phosphate (S-1-P) and endothelin, which results in recruitment of p115 RhoGEF and subsequent GTP loading of RhoA (Hart *et al.*, 1998; Kozasa *et al.*, 1998; Kjoller and Hall, 1999). Activation of RhoA results in more of the protein becoming membrane-bound by virtue of C-terminal geranyl-geranylation unmasking as interactions with Rho-GDIs are reduced (Fleming *et al.*, 1996; Donovan *et al.*, 1997;

Gong *et al.*, 1997; Aoki *et al.*, 1998; Seasholtz *et al.*, 1999). In fact, CCh stimulation of M1 mAChR has been shown to reduce cytosolic and increase membrane-bound RhoA in HEK293 cells in the presence of GTPγS (Keller *et al.*, 1997).

RhoA activity has been shown to affect many different cellular processes, some examples of which will be given below. This speaks to the significant contribution of RhoA signaling to the normal functioning of our cells. Initial studies of RhoA in Swiss 3T3 fibroblasts primarily demonstrated its ability to modulate the cytoskeleton, such as induction of stress fiber formation, focal adhesion complex formation, cellular contraction, and actin-myosin interaction through ROCK (Paterson *et al.*, 1990; Ridley and Hall, 1992; Noda *et al.*, 1995; Amano *et al.*, 1996a; Kimura *et al.*, 1996). Subsequent studies have shown activity of RhoA and its effectors do much more. For example, LPA-induced RhoA activity is necessary for proper NF-κB transcription factor activity in 3T3 and COS-7 cells. (Pan *et al.*, 1996; Perona *et al.*, 1997; Pan *et al.*, 1998; Shahrestanifar *et al.*, 1999). Additionally, RhoA downstream effectors ROCK and PKN have been shown to activate serum response element (SRE)-mediated gene transcription (Quilliam *et al.*, 1996; Chihara *et al.*, 1997; Sahai *et al.*, 1998). RhoA activity also plays a key role in processes such as normal cellular migration and for invasion of tumor cells as evidenced by unsuccessful scrape-wound closure of NRK49F fibroblasts in the presence of dominant negative ROCK, the prevention of chemotactic neutrophil migration in the presence of the ROCK inhibitor Y27632, and the ability of LPA stimulation to elicit cell monolayer penetration by rat hepatoma cells (Imamura *et al.*,

1993; Fukata *et al.*, 1999; Niggli, 1999). Clearly, appropriate signaling of RhoA and its effectors is very important and, when perturbed, can have profound cellular effects.

Another important example of how RhoA signaling can have profound effects is one in which regulation and function of Kv1.2 is also paramount. It has been shown that RhoA and ROCK expression and activity are enhanced in cardiac myocytes and vascular smooth muscle cells in disease states such as hypertension. (Kobayashi *et al.*, 2002; Satoh *et al.*, 2003; Torsoni *et al.*, 2003; Hattori *et al.*, 2004). ROCK phosphorylates and inhibits the action of myosin light chain phosphatase, resulting in higher levels of myosin light chain phosphorylation, increased myosin filament contraction, and hypertension. (Noda *et al.*, 1995; Kimura *et al.*, 1996). In fact, treatment with the ROCK inhibitor Y27632 has been shown to lower blood pressure (Uehata *et al.*, 1997; Seasholtz *et al.*, 2001; Seko *et al.*, 2003; Moriki *et al.*, 2004). It is also known that in a rat model of hypertension, vascular smooth muscle cells have increased levels of calcium influx and higher intracellular calcium concentrations which have been shown to suppress the function of the voltage-sensitive potassium channel Kv1.2 (Lozinskaya and Cox, 1997). As stated previously, it is known that RhoA signaling itself can modulate the function of Kv1.2 (Cachero *et al.*, 1998). It appears, therefore, that the potential exists for RhoA signaling to perform dual roles in the context of the cardiovascular system by controlling smooth muscle constriction and ion channel function simultaneously.

Rationale

The mechanism by which RhoA modulates Kv1.2 function is not yet known, but trafficking of the channel has emerged as an important method for its regulation. This study examines the contribution of RhoA and its effectors to the well-established endocytosis-based regulation of Kv1.2. RhoA can exert its effects through direct protein-protein interaction or through downstream effectors. Rho-kinase (ROCK) is perhaps the most well understood downstream effector of RhoA and therefore received the most attention, however we do investigate the role of protein kinase N (PKN) as well. ROCK is a serine/threonine kinase whose activity is enhanced by interacting with active RhoA (Ishizaki *et al.*, 1996; Matsui *et al.*, 1996). ROCK is responsible for modulating many actin-based processes in cells via phosphorylation of proteins such as myosin phosphatase (MYPT) and LIM kinase (LIMK) (Kaibuchi *et al.*, 1999; Riento and Ridley, 2003). Information on the role of ROCK in endocytosis is limited but examples include its necessity for sodium, potassium-ATPase endocytosis upon RhoA activation in alveolar epithelial cells (Dada *et al.*, 2007) and for rearward clathrin structure polarization during the migration of T-lymphocytes (Samaniego *et al.*, 2007). ROCK activation also inhibits epidermal growth factor (EGF) receptor endocytosis in PC12 cells (Kaneko *et al.*, 2005).

RhoA has multiple, differential roles in the trafficking of a variety of other membrane proteins. For example, in alveolar epithelial cells clathrin-dependent sodium, potassium-ATPase endocytosis in response to hypoxia is dependent upon RhoA

activation (Dada *et al.*, 2007). In HeLa cells, activation of RhoA inhibits clathrin-dependent endocytosis of EGF and transferrin (Tf) receptors (Lamaze *et al.*, 1996). In lymphocytes RhoA is required for clathrin-independent, cholesterol-dependent internalization of IL-2 receptors (Symons and Rusk, 2003). Thus, RhoA signaling plays both positive and negative roles in a variety of endocytosis pathways.

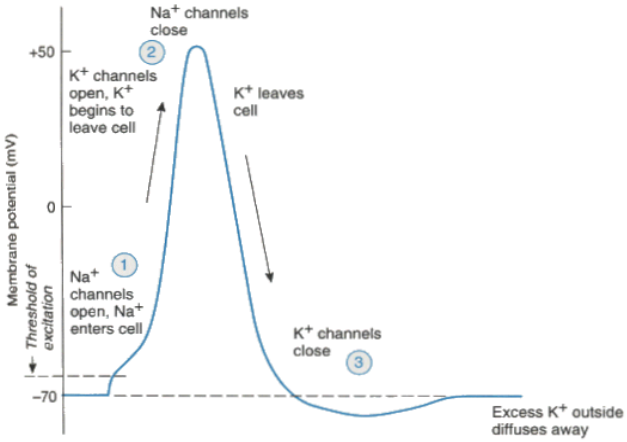
Since Kv1.2 regulation was previously shown to be trafficking-dependent (Nesti *et al.*, 2004; Williams *et al.*, 2007; Connors *et al.*, 2008), the central hypothesis of this thesis is that RhoA regulates Kv1.2 function via modulation of channel trafficking. We report that RhoA does control the trafficking of Kv1.2 and it does this by acting through its downstream effectors, primarily ROCK, but also PKN. Interestingly, we have discovered that RhoA controls multiple forms of Kv1.2 trafficking, both under steady-state conditions and in response to G protein-coupled receptor signaling. We also show that endocytosis of Kv1.2 occurs through different mechanisms, one being cholesterol-dependent, the other clathrin-dependent and we implicate RhoA as critical for channel recycling. In total, this research has established RhoA and its downstream effectors as key regulators of Kv1.2 function, with the ultimate implication being that RhoA signaling can control cellular excitability. RhoA, therefore, can potentially serve as a therapeutic target for a variety of neuronal and cardiovascular ailments.

A

	Inside	Outside
K^+	400mM	20mM
Na^+	50mM	440mM

Ion concentrations across the membrane of the squid giant axon Kandel & Schwartz. Principles of Neural Science. 2002.

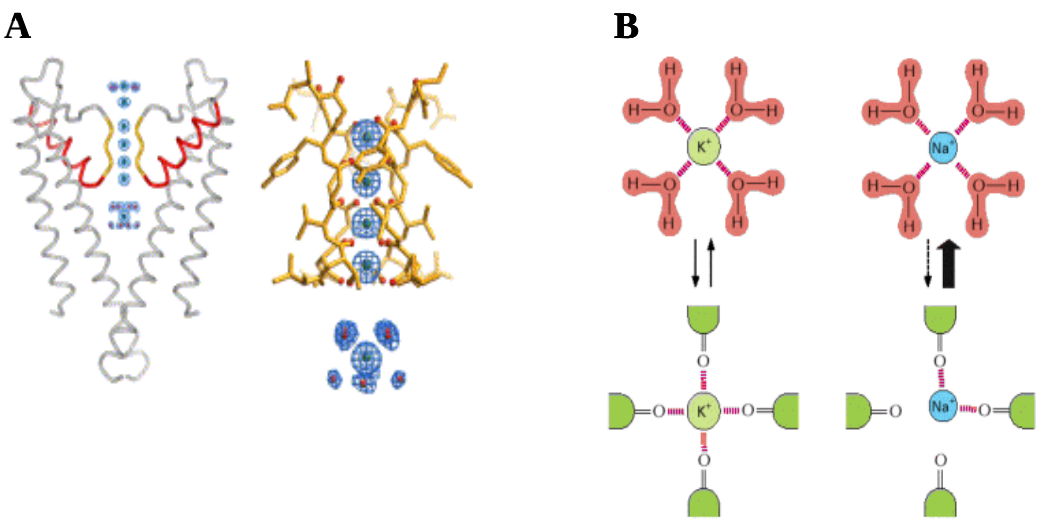
B



http://www.lhsc.on.ca/critcare/ucicu/educate/orient/neuroap.htm

Figure 1. Steps of an Action Potential

(A) Sodium and potassium ions across the plasma membrane of excitable cells exist along opposing concentration gradients. (B) When an excitatory stimulus depolarizes such a cell beyond its excitation threshold, fast-gated sodium channels open, allowing positively charged sodium ions to flood into the cell, resulting in further depolarization. Slow-gated potassium channels that respond to the voltage change across the plasma membrane open as the sodium channels are inactivated, allowing positively charged potassium ions to rush out of the cell. This effectively moves the membrane potential back towards the resting level. Another action potential cannot occur until the cell has returned to its original resting membrane potential.

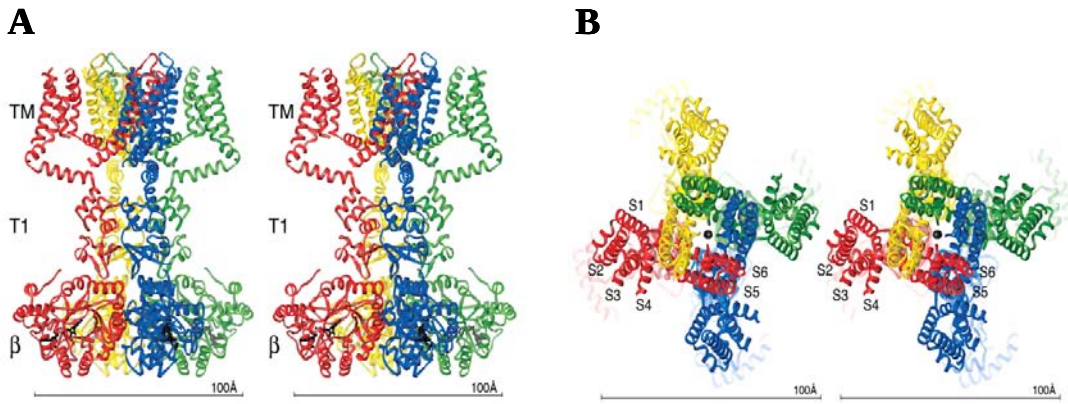


MacKinnon, FEBS Letters 555, 62-65 (2003).

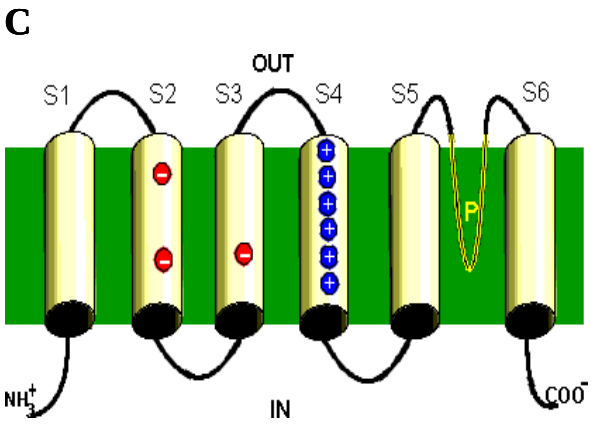
Alberts et al. Molecular Biology of the Cell. 2002.

Figure 2. Design and Function of the K^+ Selectivity Filter

(A) The pore region of potassium channels contains a highly conserved selectivity filter (gold). Within the selectivity filter are four binding sites for potassium ions. (B) Before potassium ions enter the selectivity filter, they must be dehydrated, costing energy. The radius of the potassium ion is such that once in the filter, it interacts with carbonyl oxygen atoms at the binding sites, mimicking hydration, offsetting the cost of dehydration. Other positively charged cations, like sodium are not the proper size to interact with these carbonyl oxygens, thus conferring potassium specificity.



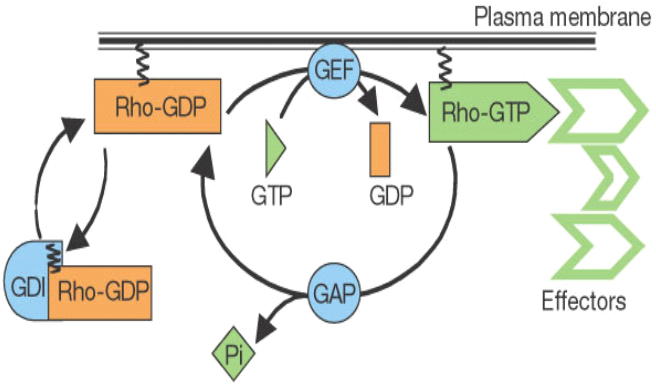
Long et al., Science 309, 897-903 (2005).



http://nerve.bsd.uchicago.edu/KHTML/sub_struct.htm

Figure 3. Architecture of Kv1.2

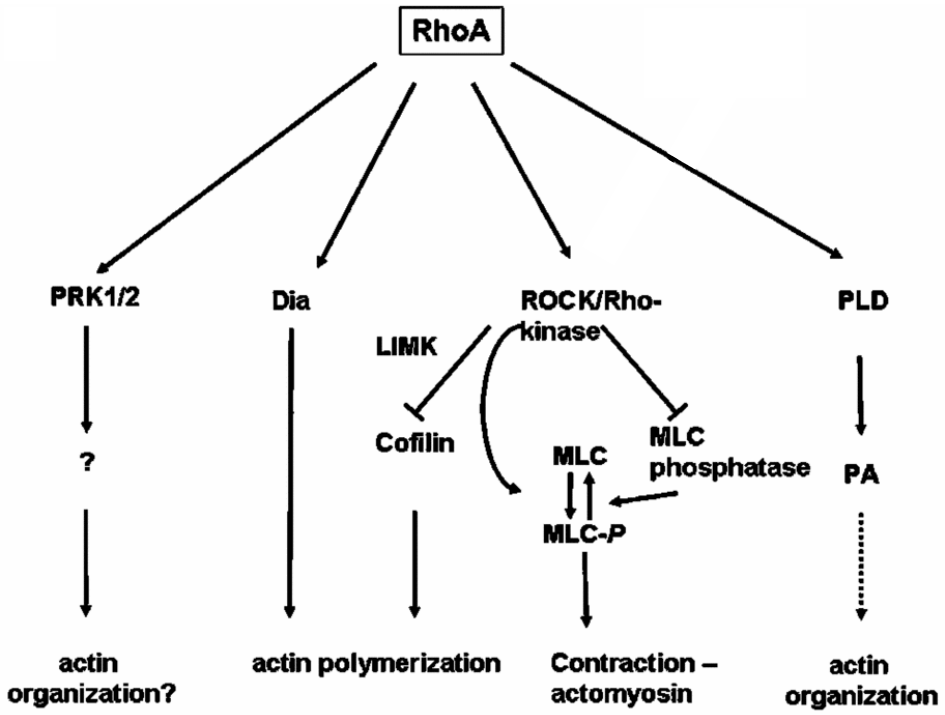
(A) Side-view of the Kv1.2 holochannel illustrating the trans-membrane, pore-forming region (TM) of the channel, the intracellular tetramerization domain (T1), and the associated beta subunits (β). (B) Top-view of the trans-membrane portion of Kv1.2, illustrating the tetramerization of alpha subunits. (C) Shematic cartoon of an individual alpha subunit, clearly showing six alpha-helical membrane-spanning domains (S1-S6), the positively charged voltage sensor helix (S4), and the pore-forming loop between helices S5 and S6 that dips into the membrane (P)



Etienne-Mannville et al. *Nature* 420, 629-635 (2002)

Figure 4. The Rho GTPase Cycle

Rho GTPases cycle between an active (GTP-bound) and in inactive (GDP-bound) conformation. When bound to GTP, they interact with one of many downstream proteins, called effectors, and signal through various pathways. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, causing activation. GTPase-activating proteins (GAPs) increase the rate of GTP hydrolysis, leading to deactivation. Guanine nucleotide dissociation inhibitors (GDIs) prevent exchange of GDP for GTP and can mask the Rho GTPase C-terminal lipid modification, thus holding the protein in an inactive conformation while sequestering it in the cytoplasm. Thus, there are many levels of Rho GTPase regulation.



Riento et al. *Biochem. Soc. Trans.* 33, 649-651 (2005)

Reproduced with Permission

Figure 5. Downstream Effectors of RhoA

RhoA has the ability to interact with many downstream effectors. These downstream effectors signal through various pathways but a key theme is their ability to regulate the cytoskeleton. The extent to which RhoA signals through a particular effector depends on the activity level of RhoA, the spatio-temporal dynamics of RhoA activation, and the availability of a particular effector at sites of RhoA activity.

CHAPTER 2: DUAL ROLES FOR RHOA/RHO-KINASE IN THE REGULATED TRAFFICKING OF A VOLTAGE-SENSITIVE POTASSIUM CHANNEL

Introduction

The voltage-gated potassium channel Kv1.2 regulates diverse physiological functions, including the maintenance of action potential proliferation along myelinated axons (Rasband *et al.*, 2001; Rasband and Trimmer, 2001; Rasband, 2004), transduction of pain in peripheral sensory neurons (Ishikawa *et al.*, 1999; Kim *et al.*, 2002; Yang *et al.*, 2004) and modulation of tone in vascular smooth muscle (Adda *et al.*, 1996; Wang *et al.*, 1997; Wang *et al.*, 2005). Dysregulation of Kv1.2 has been implicated in disease states such as hypertension (Cox *et al.*, 2001; Hong *et al.*, 2004), neuropathic pain (Ishikawa *et al.*, 1999; Kim *et al.*, 2002; Yang *et al.*, 2004) and seizure activity in the central nervous system (Lambe and Aghajanian, 2001; Brew *et al.*, 2007). Kv1.2 is important for maintaining neuronal resting membrane potential (Dodson *et al.*, 2003) and is heavily expressed in neurons of the hippocampus and cerebellum (Tsaur *et al.*, 1992; Sheng *et al.*, 1994; Veh *et al.*, 1995; Grosse *et al.*, 2000) where it influences neuron firing (Laube *et al.*, 1996; Southan and Robertson, 1998; Haghdoust *et al.*, 2007). Commensurate with its role as a regulator of cellular physiology, Kv1.2 is itself highly regulated. Understanding the pathways involved in Kv1.2 regulation is therefore fundamental to understanding a wide array of physiological processes.

Negative regulation of Kv1.2 involves tyrosine phosphorylation-dependent suppression of its ionic current (Huang *et al.*, 1993). This can occur in response to a

variety of stimuli, including the activation of G-protein coupled receptors (Huang *et al.*, 1993). Channel suppression by this pathway requires the activity of the small GTPase RhoA (Cachero *et al.*, 1998); inhibition of RhoA GTPase activity with C3 exoenzyme or over-expression of a dominant negative form of RhoA prevents the suppression of Kv1.2 ionic current in response to channel tyrosine phosphorylation (Cachero *et al.*, 1998). The finding that RhoA modulates Kv1.2 was significant because it provided a new link between signaling pathways governing actin dynamics and pathways governing membrane excitability. For example, in vascular smooth muscle, both RhoA and Kv1.2 regulate contractility. In this tissue, it is well accepted that RhoA acts through direct effects on the contractile machinery (Uehata *et al.*, 1997; Nakamura *et al.*, 2003) and that Kv1.2 affects contractility indirectly through its effects on membrane potential (Yuan, 1995; Yuan *et al.*, 1998). The finding that RhoA affects Kv1.2 function raised the intriguing possibility that the effects of Kv1.2 and RhoA on smooth muscle contractility may not be distinct, but instead may be tightly connected. Subsequent studies have begun to support this idea (Luykenaar *et al.*, 2004). Similar links may exist between RhoA-regulated actin dynamics and membrane potential in other tissues expressing Kv1.2, including neuronal synapses (Dodson *et al.*, 2003). Despite the evident importance of this signaling nexus, the mechanism by which RhoA regulates Kv1.2 is not known.

Tyrosine phosphorylation-dependent suppression of Kv1.2 ionic current involves endocytosis of the channel protein from the cell surface (Nesti *et al.*, 2004). Kv1.2 endocytosis has at least two distinct components: steady-state channel endocytosis and

receptor-induced endocytosis (Williams *et al.*, 2007; Connors *et al.*, 2008). Both forms of Kv1.2 endocytosis require direct association of Kv1.2 with the actin-regulating protein cortactin (Hattan *et al.*, 2002; Williams *et al.*, 2007). Strikingly, each pathway is affected by distinct actin-regulatory domains within cortactin (Williams *et al.*, 2007). Disruption of the F-actin binding region within cortactin affects steady-state and receptor induced channel endocytosis. In contrast, disruption of cortactin's ability to stimulate dendritic actin polymerization via Arp2/3 selectively blocks receptor-induced channel endocytosis but has no effect on steady-state channel trafficking. Despite being clearly separable based on distinct actin-regulatory functions of cortactin, the mechanisms involved in steady-state vs. receptor-induced Kv1.2 endocytosis have remained unclear.

Since Kv1.2 regulation involves actin-dependent endocytosis, and since RhoA is a key modulator of both actin dynamics and endocytosis, I hypothesized that RhoA affects Kv1.2 ionic current by modulating channel endocytosis. This idea is consistent with involvement of RhoA in the trafficking of a variety of other membrane proteins. For example, in alveolar epithelial cells clathrin-dependent Na,K-ATPase endocytosis in response to hypoxia is dependent upon RhoA activation (Dada *et al.*, 2007), in HeLa cells activation of RhoA inhibits clathrin-dependent endocytosis of epidermal growth factor (EGF) and transferrin (Tf) receptors (Lamaze *et al.*, 1996), and in lymphocytes RhoA is required for clathrin-independent internalization of IL-2 receptors (Symons and Rusk, 2003). In some instances, RhoA modulation of endocytosis involves Rho-kinase (ROCK). Perhaps the most well understood RhoA effector, ROCK is a serine/threonine kinase that is activated by RhoA (Ishizaki *et al.*, 1996; Matsui *et al.*, 1996). Examples of

RhoA/ROCK dependent endocytosis include a requirement for ROCK in Na,K-ATPase endocytosis in alveolar epithelial cells (Dada *et al.*, 2007) and for rearward clathrin structure polarization during the migration of T-lymphocytes (Samaniego *et al.*, 2007). ROCK activation inhibits epidermal growth factor (EGF) receptor endocytosis in a process involving LIM kinase/cofilin (Kaneko *et al.*, 2005). In this pathway, RhoA activates ROCK, which in turn phosphorylates and activates LIM kinase. LIM kinase then phopshorylates its major substrate, the F-actin severing protein cofilin, thereby inactivating it (Bernard, 2007). This leads to alteration of actin dynamics and modulation of endocytosis. This finding is crucial because it provides a direct link between regulated actin dynamics and RhoA-mediated endocytosis. Despite these intriguing findings, evidence linking RhoA, actin dynamics, and endocytosis remains sparse.

Here we report that RhoA is a determinant of Kv1.2 trafficking. We show that RhoA activity is inversely correlated to the level of channel at the cell surface and that this reduction in surface Kv1.2 occurs via channel endocytosis. These findings reveal that steady-state homeostasis of Kv1.2 is achieved through a balance of constitutive, cholesterol-dependent endocytosis and channel recycling which is distinct from clathrin-dependent channel endocytosis elicited by G protein-coupled LPA receptor activation. RhoA/ROCK signaling contributes to both forms of trafficking, but does so through distinct mechanisms in each case. In addition, we demonstrate that dynamin is necessary for both forms of Kv1.2 trafficking. Finally, we show that RhoA/ROCK modulates Kv1.2 trafficking through LIM kinase/cofilin, thus establishing a direct link between RhoA-regulated actin dynamics and the functional regulation of Kv1.2.

Materials and Methods

Materials

Rabbit polyclonal antibody directed against the first extracellular loop of Kv1.2 (α-Kv1.2e) was developed in conjunction with Biosource International (Camarillo, CA). EEA1 monoclonal (α-EEA1m) and clathrin heavy chain monoclonal (α-CHCm) antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). RhoA monoclonal antibody (α-RhoAm) was a generous gift of Dr. S. Yonemura (Center for Developmental Biology, RIKEN, Kobe, Japan). Phospho-MYPT1 (α-pMYPT1p) polyclonal and GAPDH (α-GAPDHm) monoclonal antibodies were purchased from Millipore (Billerica, MA). Alexa Fluor-conjugated transferrin, secondary antibodies, and phalloidin were purchased from Invitrogen (Carlsbad, CA). Non-targeting RNAi and clathrin heavy chain RNAi were purchased from Dharmacon (Lafayette, CO). Y27632 was purchased from EMD Biosciences (San Diego, CA), C3 exoenzyme was purchased from Cytoskeleton Inc. (Denver, CO), Lysophosphatidic acid (LPA), forskolin, and filipin were purchased from Sigma-Aldrich (St. Louis, MO). RhoA (T19N), ROCK (RB/PH-TT), LIM kinase (D460A), and cofilin (S3A) were generously provided by Dr. K. Hahn (University of North Carolina at Chapel Hill), Dr. K. Kaibuchi (Nagoya University), Dr. K Suzuki (National Institute of Health Sciences, Tokyo, Japan), and Dr. P. Caroni (Friedrich Miescher Institite, Basel, Switzerland) respectively. Dynasore was generously provided by Dr. T. Kirchhausen (Harvard Medical School).

Cell culture and transfection

Human embryonic kidney 293 cells stably expressing Kv1.2-α, Kvβ2 and the M1 muscarinic acetylcholine receptor (HEK-K) were utilized and cultured as previously reported (Nesti *et al.*, 2004). Experimental plasmid constructs were co-transfected into HEK-K cells with pEGFP-N1 using calcium phosphate. Confluent cultures were plated to a density of 3.3 x 10^4 cells per cm^2 onto tissue culture plates or glass cover slips (Corning Glass Works, Corning, NY) coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO) (0.1 mg/ml) or plasma fibronectin (0.015 mg/ml) for biochemistry, flow cytometry, electrophysiology, and immunofluorescence 12-16 h in serum-free media before use.

Electrophysiology

All recordings were made on HEK-K cells using the whole-cell patch-clamp method. Data were collected using an Axopatch 200 patch-clamp amplifier using pClamp 9.2 (Axon Instruments, Burlingame, CA). Currents were collected with step pulses from -70 to +50 mv in increments of 10 mv from a holding potential of -60 mV and were leak subtracted with a P/4 protocol. The pipette solution contained 60 mM K_2SO_4, 1.2 mM KCl, 5 mM $MgSO_4$, 5 mM Na-HEPES, and 35 mM sucrose (pH 7.1). The external (bath) solution contained 118 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl2, 0.8 mM $MgCl_2$, 5 mM Na-HEPES, and 23 mM glucose (pH 7.4). All recordings were made at 37ºC.

Detection of surface Kv1.2

For flow cytometric detection of surface Kv1.2, HEK-K cells were treated with saline, vehicle control solution, or an appropriate stimulus, then 154 mM sodium azide as in (Nesti *et al.*, 2004). After azide, the cells were lifted and placed in a test tube where surface Kv1.2 was labeled with 0.33 μg/ml α-Kv1.2e. Antibody binding was detected with fluorescently conjugated anti-rabbit IgG (0.1 μg/ml). Fluorescence was detected by flow cytometry as described below.

Flow cytometry

A single laser flow cytometer (Easy Cyte PCA-96; Guava Technologies, Hayward, CA) was used. In all HEK-K cells, α-Kv1.2e primary antibody was detected by labeling with a goat α-rabbit secondary antibody conjugated to PE/Cy^{TM}5 with an excitation peak of 488nm and an emission peak of 667nm (Southern Biotechnology, Birmingham, AL). Surface Kv1.2 in transfected cells was taken as the distribution of cells emitting at 667 nm (indicating surface Kv1.2) in cells also emitting at 508 nm (indicating GFP expression) minus background signal. Background staining was quantitated in cells using goat α-rabbit-PE/Cy^{TM}5 without prior application of α-Kv1.2e.

Immunoblot

Western analysis of HEK-K whole-cell lysates was performed as previously reported. (Connors *et al.*, 2008).

Immunofluorescence

Confluent cultures were plated as for flow cytometry and pre-treated as indicated at 37ºC. α-Kv1.2e was applied to live cells at 0.33 μg/ml for 10 min at 37ºC. Cells were fixed

with 4% paraformaldehyde (Polysciences, Warrington, PA) in PHEM 6.1 buffer (60 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA; pH to 6.1 with KOH) at room temperature. Cell were extracted with ice cold acetone. Coverslips were incubated in blocking buffer (PHEM 6.9, 3% goat serum, 0.1% fish skin gelatin) at room temperature. Other primary antibodies were then applied (α-EEA1 = 1.25 μg/ml, α-RhoAm = 35.1 μg/ml, α-CHCm = 2.5 μg/ml) diluted in blocking buffer. Alexa Fluor-conjugated secondary antibodies were applied (4 μg/ml in blocking buffer) (Invitrogen, Carlsbad, CA) at room temperature. F-actin was detected with Alexa Fluor 350 or 488 phalloidin (Invitrogen) (4 U/ml). Coverslips were mounted on glass slides with ProLong Gold mounting medium (Molecular Probes) according to the provided protocol. Images were obtained with the DeltaVision reconstruction microscopy system (Applied Precision).

Transferrin endocytosis assay

Confluent cultures were plated as for flow cytometry. Coverslips were washed with warm serum-free media, chilled, then incubated for 45 min in cold serum-free media containing 25μg/ml Alexa 488-conjugated transferrin. Coverslips were washed then moved to 37ºC for 0 or 15 min. Coverslips were chilled, washed, and processed for immunofluorescence.

Statistical analysis

Descriptive statistics are provided in figures as line or bar graphs indicating the sample mean with error bars indicating the standard error of the mean (SEM). Detection of statistical difference between two independent measurements was by one-way t-test.

Comparison of percent changes between pairs of independent measurements was by two-way ANOVA. Sample populations were considered to be significant at $p \leq 0.05$

Results

RhoA affects Kv1.2 surface expression

Tyrosine kinase-dependent suppression of Kv1.2 by M1 muscarinic acetylcholine receptors requires RhoA (Cachero *et al.*, 1998). In independent studies, this process was shown to occur by channel endocytosis from the plasma membrane (Nesti *et al.*, 2004; Williams *et al.*, 2007; Connors *et al.*, 2008). However it is not known whether these two processes are mechanistically linked. We therefore asked whether Kv1.2 trafficking at the plasma membrane requires RhoA. Using flow cytometry as previously described (Nesti *et al.*, 2004; Connors *et al.*, 2007; Williams *et al.*, 2007; Connors *et al.*, 2008), steady-state levels of Kv1.2 at the cell surface were measured in the presence or absence of the RhoA-specific inhibitor, C3 exoenzyme. Treatment of HEK-K cells with increasing concentrations of C3 exoenzyme produced a concentration-dependent increase of steady-state Kv1.2 at the cell surface (Figure 1A; n=4, *$p<0.05$, **$p<0.01$). In a complementary experiment designed to confirm that the C3 exoenzyme effect is specific to RhoA, increasing amounts of dominant negative RhoA (T19N) (Subauste *et al.*, 2000) were transfected. This too caused a dose-dependent increase of steady-state surface Kv1.2 (Figure 1B; n=18, **$p<0.001$). The effectiveness of C3 exoenzyme and RhoA (T19N) suggest that RhoA is at least partially active in un-stimulated HEK-K cells. Since RhoA translocation to the plasma membrane occurs upon activation (Takaishi *et*

al., 1995), we used immunofluorescence to assess the sub-cellular distribution of RhoA in un-stimulated cells. Strong RhoA immunofluorescence was detected at the cell periphery and areas of cell-cell contact (Figure 2, arrows). RhoA in these areas was coincident with Kv1.2 immunofluorescence, consistent with a role for RhoA in regulating steady-state trafficking of Kv1.2 at the cell surface.

RhoA activation triggers Kv1.2 endocytosis

The lysophosphatidic acid (LPA) receptor couples to $G_{12/13}$, to potently activate RhoA (Moolenaar, 1995; Bian *et al.*, 2006). Stimulation of endogenous LPA receptors in HEK-K cells elicited a significant, concentration-dependent reduction of surface Kv1.2 (Figure 3A; n=5, *p<0.05, **p<0.01). Whole-cell patch clamp studies confirmed that the loss of surface Kv1.2 induced by LPA (10 μM, 15 min) correlates with a reduction of Kv1.2 function. The mean steady-state current amplitude measured between 60-80 ms after depolarization to +20 mV was reduced from 9.4±1.1 nA in saline treated cells to 4.3±1.4 nA in cells treated with LPA (Figure 3B; n≥11, p<0.01). To determine whether the LPA effect on Kv1.2 surface expression results from its ability to activate RhoA, cells were pre-treated with C3 exoenzyme to block RhoA prior to application of LPA. C3 pre-treatment reduced the LPA-mediated loss of surface Kv1.2 by approximately 50% (Figure 4A, Figure 4B; n=31, *p<0.05, **p<0.01), supporting the assertion that LPA activation of RhoA elicits Kv1.2 endocytosis. To confirm this finding, we took an alternate approach whereby RhoA activity was blocked not with C3 exoenzyme, but instead by activation of an endogenous inhibitory pathway. RhoA activity is potently inhibited by phosphorylation of serine 188 by protein kinase A (PKA) (Dong *et al.*, 1998;

Forget *et al.*, 2002). Pre-treatment of HEK-K cells with forskolin, an agent shown in another study to activate PKA under these experimental conditions (Connors *et al.*, 2008), strongly inhibits the LPA-induced loss of surface Kv1.2 (Figure 4C; n=15, **$p<0.001$). Therefore, LPA-induced reduction of functional Kv1.2 channels from the cell surface is at least partially dependent on receptor-induced activation of RhoA.

M1 muscarinic acetylcholine receptor stimulation causes endocytosis of Kv1.2 to EEA1-containing structures (Nesti *et al.*, 2004). To confirm that LPA-induced loss of surface Kv1.2 also occurs by channel endocytosis, immunofluorescence was used to visualize Kv1.2 trafficking in HEK-K cells treated with saline or LPA. Surface Kv1.2 was labeled in live cells with α-Kv1.2e before addition of saline or LPA (10 μM, 15 min), followed by fixation and analysis by immunofluorescence. LPA treatment resulted in translocation of Kv1.2 from the cell periphery to intracellular puncta. Internalized Kv1.2 puncta co-stained with EEA1 or were adjacent to EEA1-containing structures (Figure 5), confirming that LPA treatment elicited channel endocytosis. We also noted that LPA-induced internalization of surface Kv1.2 disrupts its distinct co-localization with RhoA at the cell periphery (Figure 5). These data are consistent with an inverse relationship between RhoA activity and the level of Kv1.2 at the cell surface and support the hypothesis that RhoA exerts its effect on channel function by modulating channel endocytosis.

Rho-kinase (ROCK) is required for LPA-induced Kv1.2 endocytosis

A major downstream effector of RhoA is Rho-kinase (ROCK), a serine/threonine kinase that modulates the actin cytoskeleton and regulates the trafficking

of certain membrane proteins (Narumiya *et al.*, 1997; Kaneko *et al.*, 2005; Dada *et al.*, 2007; Samaniego *et al.*, 2007). To test the hypothesis that RhoA effects on Kv1.2 involve ROCK, we took two distinct approaches to inhibiting ROCK. First, DNA encoding a dominant negative form of ROCK (RB/PH-TT) (Amano *et al.*, 1999) was transfected in into HEK-K cells and the effect on Kv1.2 surface levels and trafficking was determined. Over-expression of dominant negative ROCK resulted in a significant increase in levels of Kv1.2 at the cell surface in un-stimulated cells (Figure 6A; n=69, **p<0.001). Strikingly, LPA-induced Kv1.2 endocytosis was significantly reduced in ROCK (RB/PH-TT)-expressing cells (Figure 6B; n=69, *p<0.001). To confirm that these effects were caused by blockade of ROCK and not by over-expression of ROCK (RB/PH-TT) *per-se*, we took the complementary approach of blocking ROCK with the pharmacological inhibitor Y27632. Inhibiting ROCK in this way significantly increased the steady-state level of surface Kv1.2 (Figure 6C; n=40, *p<0.01, **p<0.001) and completely blocked LPA-induced Kv1.2 endocytosis (Figure 6C; n=40, *p<0.01, **p<0.001). To confirm that LPA activates ROCK and that Y27632 exerts its effect on Kv1.2 by inhibiting that activation, we used Thr696-phosphorylated myosin phosphatase-1 (MYPT1) as an indirect measure of ROCK activity (Feng *et al.*, 1999). LPA treatment (10 μM, 15 min) of HEK-K cells increased detectable pMYPT1 while treatment with the ROCK inhibitor Y27632 (10 μM, 30 min) reduced levels of pMYPT1 relative to saline control (Figure 6D). Immunofluorescence experiments shown in Figure 7 confirm that the effects on surface Kv1.2 observed in flow cytometry experiments (Figure 6C) arise from alterations of Kv1.2 trafficking. LPA elicited a translocation of Kv1.2 from the cell

periphery to intracellular puncta, indicative of channel endocytosis (Nesti *et al.*, 2004), an effect completely blocked by Y27632 (Figure 7). Collectively, these findings indicate that ROCK is an essential downstream effector for receptor-induced, RhoA-mediated endocytosis of Kv1.2. Since both ROCK (RB/PH-TT) over-expression and Y27632 treatment cause an increase of steady-state surface Kv1.2, these findings also indicate that basal ROCK activity is important downstream of RhoA for maintaining steady-state Kv1.2 at the cell surface as well.

LPA-induced Kv1.2 endocytosis is clathrin-dependent

Although Kv1.2 endocytosis can be elicited by a variety of signals (Huang *et al.*, 1993; Nesti *et al.*, 2004; Williams *et al.*, 2007; Connors *et al.*, 2008), the specific endocytosis pathways involved are not known. Many cell-surface proteins and ion channels are internalized in a clathrin-dependent manner, among them the transferrin (Tf) receptor, epidermal growth factor (EGF) receptor, as well as the epithelial sodium channel (ENaC) (Sorkin and Waters, 1993; Shimkets *et al.*, 1997; Rotin *et al.*, 2001). We therefore sought to explore the role of clathrin in RhoA/ROCK-mediated endocytosis of Kv1.2. Knockdown of clathrin protein with RNAi inhibits Tf receptor internalization (Hinrichsen *et al.*, 2003). We chose this approach to explore the role of clathrin in Kv1.2 endocytosis. To validate the efficacy of clathrin RNAi in HEK-K cells, clathrin protein levels were determined in cells transfected with non-targeting (NT) RNAi or with RNAi targeting clathrin. Transfection of clathrin RNAi reduced clathrin protein levels by 50% compared to cells transfected with NT RNAi, as detected by immunoblot (Figure 8A; n=8, **p=0.008). Comparable results were obtained from immunofluorescence

experiments (Figure 8B, Figure 8C), where clathrin signal intensity was reduced by 30% compared to NT control (Figure 8C; n=37, *p=0.035). This level of reduction was sufficient to interfere with clathrin-dependent endocytosis since Tf receptor internalization, as viewed by immunofluorescence (Figure 9A), was reduced approximately 3-fold in cells transfected with clathrin RNAi compared to NT RNAi control (Figure 9B; n=17, *p<0.05, **p<0.001). Thus, knockdown of clathrin protein in HEK-K cells is an effective way to inhibit clathrin-dependent endocytosis. Application of this technique to Kv1.2 revealed that clathrin RNAi reduced Kv1.2 endocytosis in response to LPA by 50% (Figure 10A, Figure 10B; n=33, *p<0.05, **p<0.001). This result is consistent with a 50% knockdown of clathrin protein by RNAi transfection. Interestingly, despite its effect on LPA-induced Kv1.2 endocytosis, inhibiting clathrin-mediated trafficking had no detectable effect on the steady-state level of surface Kv1.2 (Figure 10A). Moreover, clathrin knockdown had no effect on the ability of Y27632 to elevate surface Kv1.2 levels (Figure 10C; n=33, p=0.493). These findings suggest the intriguing hypothesis that LPA-induced endocytosis of Kv1.2 is clathrin-dependent while the trafficking mechanism responsible for maintaining the steady-state level of surface channel is clathrin-independent, despite both processes being regulated by RhoA/ROCK.

Steady-state endocytosis of Kv1.2 is cholesterol-dependent

We next sought to determine the trafficking pathway involved in steady-state regulation of Kv1.2 at the cell surface. To assess the role of cholesterol in steady-state trafficking, we made use of the sterol-binding agent filipin to disrupt cholesterol-rich regions of the plasma membrane (Chang *et al.*, 1992; Rothberg *et al.*, 1992; Schnitzer *et*

al., 1994; Smart *et al.*, 1994). Steady-state surface levels of Kv1.2 were significantly increased in the presence of filipin while subsequent Y27632 application had no additive effect (Figure 11A; n=32, *p<0.01, **p<0.001). In contrast, filipin did not block LPA-induced Kv1.2 endocytosis, rather it was significantly enhanced, suggesting that higher levels of steady-state surface Kv1.2 creates a larger pool of channel available for LPA-induced, clathrin-dependent endocytosis (Figure 11B, Figure 11C; n=5, *p<0.05, **p<0.001). These findings also suggest that basal ROCK activity modulates steady-state surface Kv1.2. Additionally, cholesterol-dependent endocytosis operates constitutively, controlling the steady-state surface level of Kv1.2 independently of the clathrin-dependent mechanism elicited by G protein-coupled LPA receptor stimulation.

In many instances, steady-state levels of proteins at the plasma membrane represent a homeostasis between endocytosis and recycling back to the plasma membrane (Dunn and Hubbard, 1984; Morrison *et al.*, 1996; Leterrier *et al.*, 2004). While filipin is widely used to block cholesterol-dependent endocytosis, it can have other effects on trafficking, including modulation of intracellular vesicular trafficking and recycling pathways (Kwik *et al.*, 2003). We therefore asked whether filipin's effect on Kv1.2 involved one or both of these processes. To do so, we used low temperature (16° C) to block intracellular vesicle trafficking and recycling. This manipulation leaves endocytosis from the plasma membrane intact (De Camilli *et al.*, 1995; Le and Nabi, 2003), thus enabling us to study channel recycling and channel endocytosis in relative isolation of one another. Incubation of HEK-K cells at 16° C for 1 hour reduced steady-state surface Kv1.2 by approximately 50% relative to control cells maintained at 37° C

(Figure 12E; n=27, **p<0.001). This profound drop in surface Kv1.2 levels indicated that recycling does play a key role in the maintenance of surface Kv1.2. In striking contrast, temperature block of recycling did not impair the ability of filipin to potently increase the level of surface Kv1.2 (Figure 12A, Figure 12B; n=12, *p=0.019, **p<0.001). This finding confirms that, although recycling has an important role in maintaining levels of surface Kv1.2, filipin blockade of cholesterol-dependent steady-state Kv1.2 endocytosis is sufficient to increase surface channel levels. However, it also raises the question of which branch of this homeostatic pathway is acted upon by ROCK? We therefore used temperature block in combination with ROCK inhibition to address this question. Under these conditions, temperature block completely prevented the Y27632-induced increase of surface Kv1.2 (Figure 12C, Figure 12D; n≥10, *p<0.01, **p<0.001). Based on these results, we surmise that basal ROCK activity in un-stimulated HEK-K cells exerts its effect on steady-state surface Kv1.2 not by reducing cholesterol-dependent channel endocytosis *per se*, but by reducing the recycling of constitutively endocytosed channel back to the plasma membrane.

Dynamin is essential for both steady-state and LPA-induced modulation of Kv1.2 trafficking

Dynamin is a small GTPase with a well-established role in both cholesterol-dependent and clathrin-dependent endocytosis (De Camilli *et al.*, 1995; Sever *et al.*, 2000). It was previously reported that tyrosine kinase-induced endocytosis of Kv1.2 is dynamin dependent (Nesti *et al.*, 2004). We therefore asked whether dynamin is also necessary for RhoA/ROCK-mediated endocytosis of Kv1.2. The small-molecule

inhibitor, dynasore (Macia *et al.*, 2006) was used to block the GTPase activity of dynamin. Increasing concentrations of dynasore applied to un-stimulated HEK-K cells (30 min at 37ºC) elicited a significant, concentration-dependent increase of surface Kv1.2 (Figure 13A; n=3, *$p<0.01$, **$p<0.001$). To ascertain the role of dynamin in stimulus-induced Kv1.2 trafficking, surface Kv1.2 levels were measured in LPA and Y27632-treated cells in the presence or absence of a maximal concentration of dynasore. Application of Y27632 (10 μM, 30 min) in control cells elicited a significant increase in surface channel levels, however it had no effect in cells pre-treated with 80 μM dynasore (Figure 13B; n=6, *$p<0.05$, **$p<0.001$). Similarly, LPA (10 μM, 15 min) caused a significant decrease in surface Kv1.2 levels in control cells, but its effect was abolished in cells pre-treated with dynasore (Figure 13B; n=6, *$p<0.05$, **$p<0.001$). We conclude that dynamin is necessary for both steady-state homeostasis of surface Kv1.2, and clathrin-dependent, LPA-induced Kv1.2 endocytosis.

ROCK modulates Kv1.2 trafficking through LIM kinase/cofilin

Previous studies have indicated a role for actin remodeling by the Arp2/3 protein complex in Kv1.2 trafficking (Hattan *et al.*, 2002; Williams *et al.*, 2007; Connors *et al.*, 2008). This was intriguing since cofilin, an actin-severing protein, also interacts with Arp2/3 to remodel actin (Ichetovkin *et al.*, 2002). Cofilin's actin-severing activity is regulated through phosphorylation-dependent inhibition by LIM kinase (LIMK), which itself can be activated by ROCK (Bernard, 2007). We therefore assessed the role of LIMK in Kv1.2 trafficking by over-expressing a dominant negative LIMK (D460A) that lacks kinase activity (Matsui *et al.*, 2002). In un-stimulated HEK-K cells, steady-state

levels of surface Kv1.2 are significantly increased in the presence of LIMK (D460A) (Figure 14A; n=78, **p<0.001). When LPA was applied (10 μM, 15 min) in the presence of LIMK (D460A), loss of surface Kv1.2 was blocked (Figure 14B, Figure 14C; n=21, ^p<0.05, *p<0.01, **p<0.001). The Y27632-induced increase of surface Kv1.2 was not significantly affected by over-expression of LIMK (D460A) (Figure 14D; n=21, p=0.143). In addition, transfection of increasing amounts of DNA encoding an active form of cofilin (S3A) that cannot be phosphorylated and deactivated by LIMK (Arber *et al.*, 1998) results in a concentration-dependent increase of steady-state surface Kv1.2 (Figure 15; n=12, *p<0.01, **p<0.001). Over-expression of active cofilin was designed to mimic the presence of LIMK (D460A). These results suggest that the LIMK/cofilin pathway is partially responsible for controlling steady-state channel endocytosis, but not channel recycling, as evidenced by a persistent Y27632-induced increase of surface Kv1.2 in the presence of LIMK (D460A). Additionally and importantly, the LIMK/cofilin pathway is essential for RhoA-mediated Kv1.2 suppression via LPA receptor activation.

Discussion

Potassium channel regulation is a fundamental means for controlling cellular excitability. In this chapter we report that RhoA signals through ROCK to regulate potassium ion channel trafficking. We show that the Kv1.2 potassium channel is subject to two independent forms of trafficking at the plasma membrane, one modulates the homeostasis of Kv1.2 at the cell surface and the other results in acute Kv1.2 endocytosis

following G protein-coupled receptor activation. Intriguingly, we found that RhoA/ROCK has a central role in both forms, but that it affects distinct stages of trafficking for each. Acute endocytosis of Kv1.2 is clathrin-dependent and RhoA/ROCK is required for internalization of the channel from the plasma membrane. In contrast, RhoA/ROCK also appears to modulate steady-state surface homeostasis, which involves cholesterol-dependent trafficking, not at the internalization stage, but later at the point of Kv1.2 recycling back to the plasma membrane. This study therefore not only unites two previous studies, one showing that RhoA has a central role in the tyrosine kinase-dependent suppression of Kv1.2 ionic current (Huang *et al.*, 1993) and the other identifying endocytosis as a key mechanism for the suppression of Kv1.2 (Nesti *et al.*, 2004), it also provides new insight into the complex and still poorly understood role of RhoA in regulating membrane protein trafficking in general.

Using both pharmacological inhibition and over-expression of dominant negative RhoA we showed that RhoA activity inversely correlates to the level of Kv1.2 at the cell surface. Application of C3 exoenzyme or over-expression of a dominant negative form of RhoA both caused significant and dose-dependent increases in Kv1.2 present at the cell surface (Figure 1). These findings are supported by immuonofluoresence data showing co-localization of RhoA at the cell periphery in un-stimulated cells (Figure 2) since such peripheral localization is expected for active, but not inactive RhoA (Takaishi *et al.*, 1995). In addition to regulating steady-state levels of Kv1.2 at the cell surface, RhoA is required for the acute regulation of the channel. Our finding that Kv1.2 surface levels and ionic current are negatively regulated by activation of G protein-coupled LPA

receptors is consistent with previous studies reporting similar effects with muscarinic acetylcholine receptors (Cachero *et al.*, 1998; Hattan *et al.*, 2002; Nesti *et al.*, 2004; Williams *et al.*, 2007) (Figure 3). LPA receptors can couple to $G_{12/13}$ to activate RhoA (Moolenaar, 1995; Bian *et al.*, 2006); that they do so here is confirmed by the ability of C3 exoenzyme to inhibit the LPA-induced endocytosis of Kv1.2 (Figure 4A, Figure 4B). We note that C3 exoenzyme blockade of LPA-induced endocytosis is not complete. This could result from LPA triggering parallel pathways to modulate Kv1.2, only one of which involves RhoA (Bian *et al.*, 2006). Alternatively, inhibition of RhoA by C3 exoenzyme may not have been complete under the experimental conditions used. We view the latter as more likely since inhibition of the RhoA downstream effector ROCK with Y27632 completely inhibited LPA-induced channel endocytosis (Figure 6, Figure 7). This result suggested that RhoA is the primary conduit for LPA effects on Kv1.2.

Although several studies have examined the relationship between Kv1.2 function and its endocytosis (Nesti *et al.*, 2004; Williams *et al.*, 2007; Connors *et al.*, 2008), the mechanisms by which Kv1.2 undergoes endocytosis remained unclear. An earlier report (Williams *et al.*, 2007) as well as the data shown in Figures 1-7 indicate that Kv1.2 in the plasma membrane is subject to both steady-state and acute regulation. Here we report that steady-state regulation of Kv1.2 involves cholesterol-dependent channel internalization, and that acute, receptor-induced endocytosis proceeds through a separate, clathrin-dependent mechanism. These studies used a combination of clathrin knock-down and cholesterol sequestration to functionally isolate these two pathways. In Figure 11 we show that application of the sterol-binding agent, filipin, caused an accumulation

of Kv1.2 at the cell surface. It is notable that filipin does not block LPA-induced channel endocytosis despite having blocked cholesterol-dependent endocytosis (Figure 11B, Figure 11C). This result could be explained if more than one pool of Kv1.2 exists. A pool of Kv1.2 subject to cholesterol-dependent endocytosis may be isolated from the clathrin endocytosis machinery, possibly by being sequestered in lipid rafts. Disruption of those rafts with filipin could have potentially made additional channel available for endocytosis via the clathrin-dependent pathway activated by the LPA receptor. Localization of Kv1.2 to cholesterol-rich domains is plausible given that such localization has been demonstrated for the closely related channels Kv1.3 and Kv1.5 (Martens *et al.*, 2001; Martinez-Marmol *et al.*, 2008; Vicente *et al.*, 2008). We also note that clathrin-dependent endocytosis can be affected by depletion of cholesterol (Lajoie and Nabi, 2007), and caution must be exercised when attributing the effects observed with cholesterol sequestering drugs to a clathrin-independent pathway. In Figure 10A we show that RNAi knockdown of clathrin protein did not alter the level of steady-state surface Kv1.2 but completely abolished LPA-induced channel endocytosis. Clathrin knockdown in these experiments was functionally effective since it significantly reduced Tf receptor endocytosis (Figure 9). Therefore, in this experimental system, filipin and clathrin RNAi are effective means of functionally isolating cholesterol and clathrin dependent endocytosis.

The contribution of RhoA/ROCK to clathrin-dependent endocytosis is well established (Muro *et al.*, 2003; Nishimura *et al.*, 2003; Samaniego *et al.*, 2007). The role of RhoA/ROCK in cholesterol-dependent endocytosis is less well studied but has also

been described (Mayor and Pagano, 2007). We show that although RhoA/ROCK affects both pathways as it pertains to Kv1.2 trafficking, the mechanisms by which it does so are distinct. Using low temperature to inhibit intracellular trafficking we found that, compared to 37ºC control, surface Kv1.2 levels are significantly reduced but remained sensitive to cholesterol sequestration with filipin (Figure 12A, Figure 12B, Figure 12E). This finding is consistent with the expected selective block of channel recycling but not of constitutive channel endocytosis at low temperature. Remarkably, although it did not inhibit filipin's ability to increase surface Kv1.2 levels, temperature block of channel recycling completely abolished the Y27632-induced increase in surface Kv1.2 levels (Figure 12C, Figure 12D). This result suggested that basal ROCK functions as a negative regulator of Kv1.2 recycling to the plasma membrane. Collectively, these findings indicate that steady state Kv1.2 levels are governed by a homeostatic balance between cholesterol-dependent channel endocytosis and subsequent recycling back to the plasma membrane, and that RhoA/ROCK signaling affects only the recycling component of this pathway.

Clathrin-dependent endocytosis is, generally, dynamin-dependent. In contrast, clathrin-independent modes of endocytosis can proceed with or without a requirement for dynamin (Mayor and Pagano, 2007). In a previous study, Kv1.2 endocytosis via muscarinic acetylcholine receptor stimulation was reported to be dynamin-dependent (Nesti *et al.*, 2004). Here we show that inhibition of the GTPase activity of dynamin with the small-molecule inhibitor dynasore (Macia *et al.*, 2006) blocks both constitutive and LPA-induced Kv1.2 endocytosis (Figure 13). Since receptor-induced and steady-state

Kv1.2 trafficking occur through distinct mechanisms, it is likely that dynamin has different functional roles in each case. We believe that the most likely mechanism for dynamin in LPA-induced channel endocytosis is through its classical role as a pinchase for clathrin-coated vesicles (Danino *et al.*, 2004). Dynamin's roles in other forms of trafficking are less clear, although mounting evidence indicates that it has a key role in intracellular vesicle trafficking by modulating actin dynamics in coordination with cortactin (Williams *et al.*, 2007). A previous study identified distinct domains within cortactin that are required for the independent regulation of steady-state or receptor induced Kv1.2 trafficking (Williams *et al.*, 2007). Collectively, these findings raise the intriguing possibility that RhoA, cortactin and dynamin act in concert to regulate channel trafficking through their effects on actin dynamics.

The idea that RhoA/ROCK affects channel trafficking through an actin-dependent mechanism is shown more directly with the finding that ROCK modulates Kv1.2 trafficking through LIM kinase/cofilin (Figure 14, Figure 15). Over-expression of a dominant negative form of LIMK or an active form of cofilin elicits an increase in steady-state surface channel levels (Figure 14A, Figure 15). Dominant negative LIMK significantly reduces LPA-induced endocytosis of Kv1.2 (Figure 14B, Figure 14C). Strikingly, dominant negative LIMK had no significant effect on the elevation of surface Kv1.2 levels that occurs following ROCK inhibition with Y27632 (Figure 14D). We surmise that LIMK affects the internalization phase of the steady-state and receptor-induced pathways for Kv1.2 endocytosis, but does not modulate ROCK-dependent Kv1.2 recycling. This is consistent with other reports of a requirement for LIMK activity in the

internalization of beta-adrenergic (Volovyk *et al.*, 2006) and EGF (Nishimura *et al.*, 2004) receptors. This is not unexpected since LIMK can be activated by multiple upstream proteins in addition to ROCK, such as PAK (Edwards *et al.*, 1999; Dan *et al.*, 2001; Sumi *et al.*, 2001b). Involvement of LIM kinase/cofilin in both the steady-state and receptor-induced trafficking of Kv1.2 is particularly informative in view of a similar dual role for the actin regulating protein cortactin (Williams *et al.*, 2007). This convergence at the level of Kv1.2 trafficking, as well as the reported ability of cofilin and cortactin to act in concert to regulate actin dynamics (Yamaguchi and Condeelis, 2007; Lai *et al.*, 2008), suggests that Kv1.2 trafficking through both cholesterol and clathrin-dependent pathways involves a complex interaction of multiple actin-regulatory systems.

Phosphorylation of proteins is particularly important for the regulated trafficking of Kv1.2. In this chapter we demonstrated that ROCK is critical for both steady-state Kv1.2 homeostasis and for LPA-induced Kv1.2 endocytosis. ROCK is a serine/threonine kinase whose activity is enhanced by interacting with active RhoA (Ishizaki *et al.*, 1996; Matsui *et al.*, 1996). When activated, ROCK goes on to modulate many cytoskeleton-based processes in the cell by phosphorylating downstream proteins such as LIM kinase (LIMK) (Kaibuchi *et al.*, 1999; Riento and Ridley, 2003), which we demonstrated is also critical for steady-state and stimulus-induced Kv1.2 trafficking. In the absence of ROCK phosphorylation, the N-terminus of LIMK performs an auto-inhibitory function towards the catalytic domain (Edwards and Gill, 1999). ROCK-mediated phosphorylation of LIMK occurs in a specific region of its catalytic domain called the activation segment which is an enzyme-specific region between highly conserved sequence motifs DFG and

APE (Taylor and Radzio-Andzelm, 1994). Activation and relief of LIMK auto-inhibition occurs upon its phosphorylation by ROCK on threonine 508 (LIMK1) or threonine 505 (LIMK2) within the activation segment (Ohashi *et al.*, 2000; Sumi *et al.*, 2001a). A downstream target of LIMK is cofilin, an actin-binding protein that promotes the turnover and dynamic polymerization of actin filaments (Arber *et al.*, 1998; Ichetovkin *et al.*, 2002; Bernard, 2007). We have also touched upon the role of cofilin for Kv1.2 trafficking in this chapter. LIMK phosphorylates cofilin on serine 3 which leads to its inactivation and subsequent accumulation of filamentous actin (Arber *et al.*, 1998). Inactivation of cofilin is a function of phosphorylation-dependent inhibition of the ability of cofilin to bind filamentous actin (Blanchoin *et al.*, 2000; Chen *et al.*, 2002; Maciver and Hussey, 2002).

The discovery that RhoA/ROCK is necessary for both the constitutive and stimulus-induced regulation of the same protein, Kv1.2, is significant because it broadens the overall view of how RhoA can regulate membrane protein trafficking. The finding that RhoA/ROCK can affect two modes of Kv1.2 trafficking also suggests a mechanism by which RhoA can differentially regulate membrane potential in response to distinct regulatory signals. In one instance, low levels of RhoA/ROCK activity couple to a cholesterol-dependent Kv1.2 trafficking system, thereby affecting steady-state resting membrane potential. High levels of RhoA/ROCK, in contrast, affect a parallel but distinct mechanism for Kv1.2 endocytosis requiring clathrin.

Initially, the central hypothesis of this thesis may have seemed to take only a small step forward in furthering our understanding of how Kv1.2 is regulated because it

aimed to connect RhoA-mediated Kv1.2 ionic current regulation and CCh-induced, tyrosine phosphorylation-dependent Kv1.2 endocytosis by demonstrating that RhoA activation induces channel internalization. However, in the course of this work we also uncovered new cell biological mechanisms that operate to control Kv1.2 function, providing additional context through which previously published pathways that regulate the channel can be better understood and further highlighting the interconnectedness of Kv1.2 regulatory pathways.

Signals that modulate the actin cytoskeleton have become a major focus of this research since the discovery that RhoA, a well-known actin regulatory protein, is involved in CCh-induced, tyrosine phosphorylation-dependent Kv1.2 ionic current suppression (Cachero *et al.*, 1998). The paper that first implicated endocytosis as a mechanism of Kv1.2 regulation showed that an intact actin cytoskeleton was required to maintain steady-state homeostasis of the channel (Nesti *et al.*, 2004). Others have demonstrated that the actin-binding protein cortactin is essential for both steady-state Kv1.2 homeostasis and for CCh-induced endocytosis (Hattan *et al.*, 2002; Williams *et al.*, 2007). Distinct functional domains within cortactin confer the ability of the protein to modulate these two processes independently (Williams *et al.*, 2007). Additionally, previous data (Nesti *et al.*, Unpublished data) suggested that clathrin-dependent endocytosis was the mechanism through which CCh induces Kv1.2 internalization.

In this chapter, we show that RhoA/ROCK, like cortactin, has dual roles in regulating Kv1.2 at the cell surface. Our demonstration that LIM kinase (LIMK) and cofilin are involved downstream of ROCK in Kv1.2 trafficking further strengthens the

link between cytoskeletal regulation and control of cellular excitability. Moreover, cofilin and cortactin are known to coordinately modulate the actin cytoskeleton through Arp2/3 (Ichetovkin *et al.*, 2002), further strengthening the connections between individual Kv1.2 regulatory pathways. We have presented strong evidence that clathrin-dependent endocytosis is responsible for stimulus-induced Kv1.2 internalization and have shown that other forms of endocytosis also control channel trafficking. Overall, the data contained within this chapter advances our knowledge of how Kv1.2 trafficking occurs and it also supports and strengthens assertions from previous published studies. We propose a model (Figure 16) in which Kv1.2 surface expression, and thus activity, is modulated by the combined effects of multiple actin-regulating proteins, including dynamin, RhoA, ROCK, LIMK, and cofilin acting through distinct trafficking pathways.

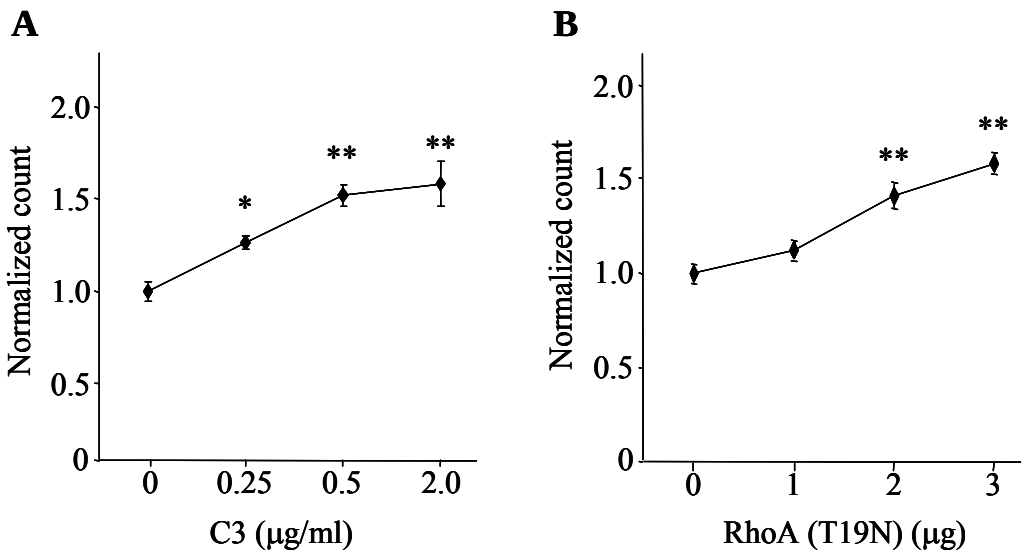


Figure 1. RhoA Activity Can Modulate Levels of Surface Kv1.2

(A) Application of increasing concentrations of C3 exoenzyme (6 h) to HEK-K cells significantly increases the level of steady-state surface Kv1.2 as detected by flow cytometry (n=4, *p<0.05, **p<0.01). (B) Transient transfection of increasing amounts of DNA encoding a dominant negative form of RhoA (T19N) significantly increases the level of steady-state surface Kv1.2 in a concentration-dependent manner (n=18, **p<0.001).

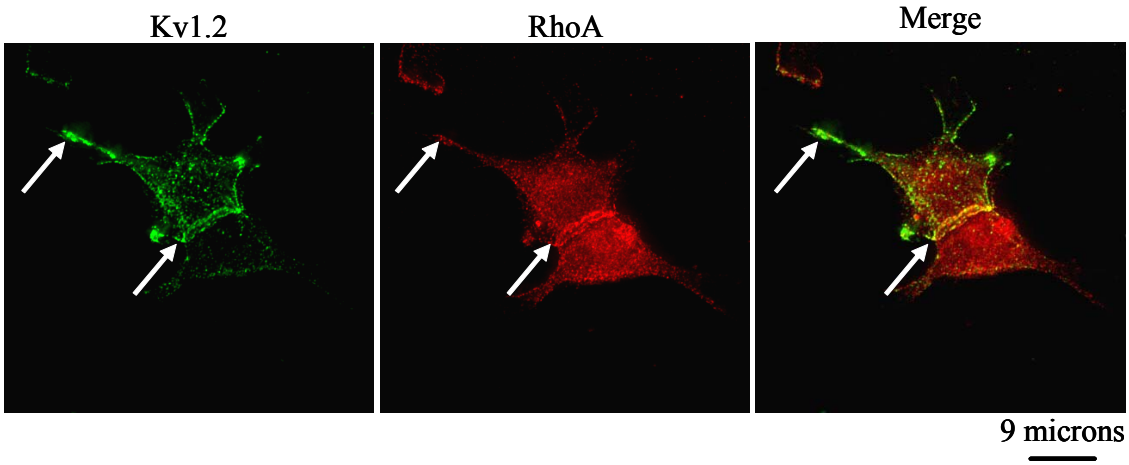


Figure 2. Surface Kv1.2 Co-localizes with RhoA in Un-stimulated HEK-K Cells

Under steady-state conditions surface Kv1.2 exists in regions of the cell enriched with RhoA. In HEK-K cells surface Kv1.2 was labeled with α-Kv1.2e (green) and total RhoA was labeled with α-RhoAm (red). Specific areas of overlap are indicated (arrows).

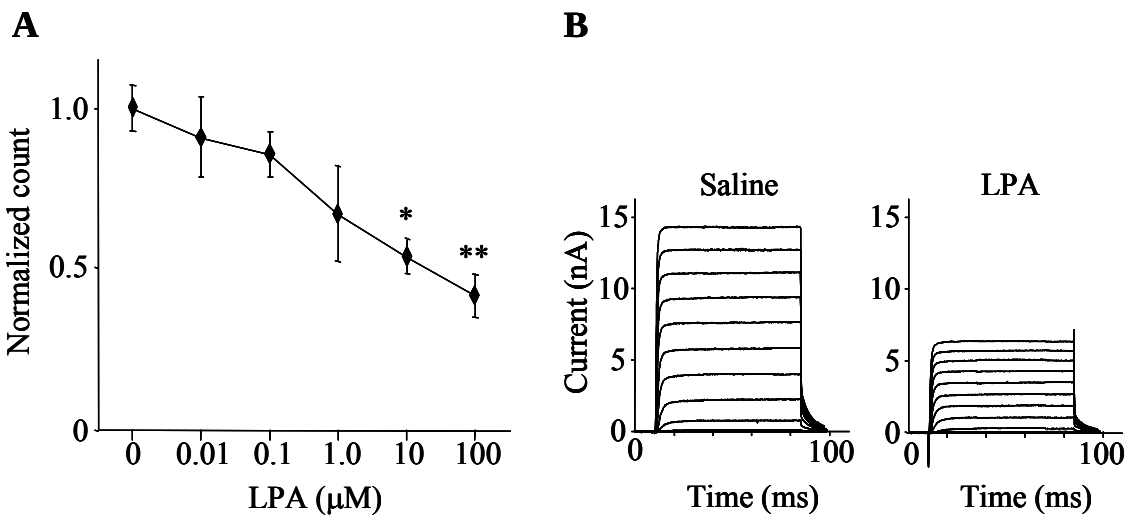


Figure 3. RhoA Activity and Surface Kv1.2 are Inversely Correlated

(A) Application of increasing concentrations of lysophosphatidic acid (LPA) (15 min) significantly reduces the level of Kv1.2 at the cell surface in a concentration-dependent manner as detected by flow cytometry (n=5, *$p<0.05$, **$p<0.01$). (B) Average whole-cell currents evoked in HEK-K cells with pulses from -70 to +50 mv in increments of 10 mv in the presence of saline or LPA (10 μM, 15 min). LPA application significantly reduces mean whole-cell currents. Current traces for each condition represent the average of at least 11 cells (n=11, $p<0.01$).

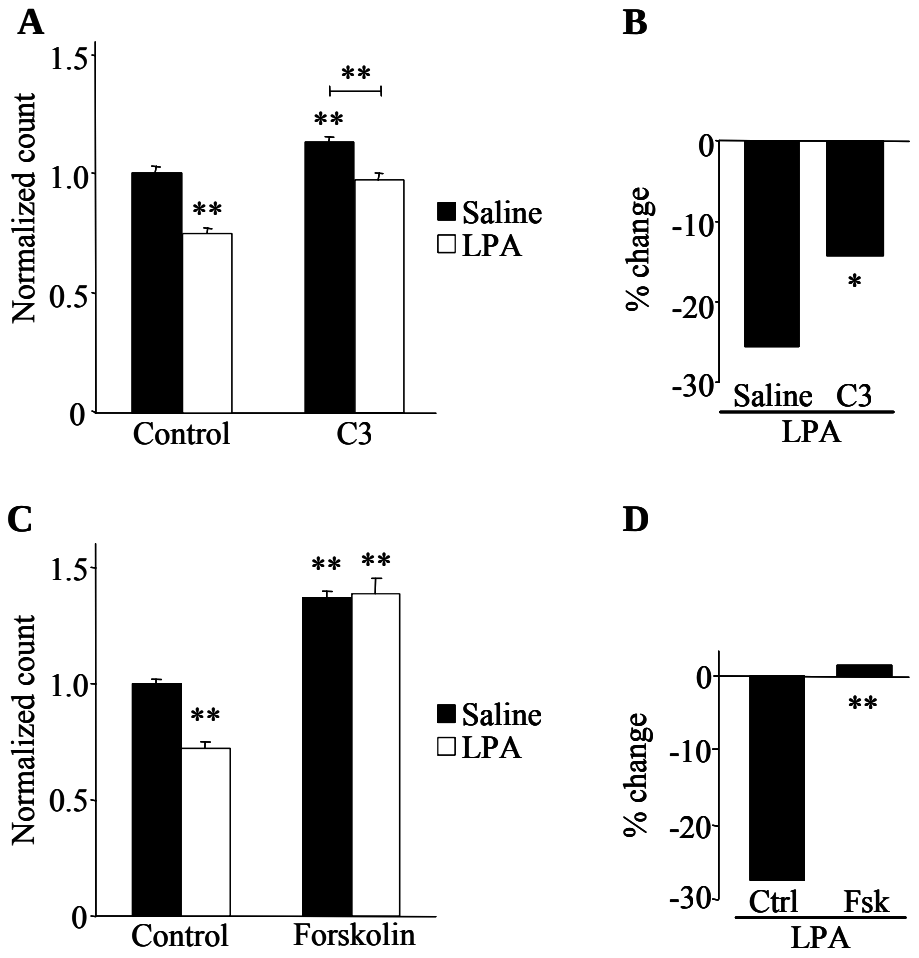


Figure 4. RhoA Inhibitors Reduce LPA-induced Kv1.2 Suppression

(A) Pre-treatment of HEK-K cells with C3 exoenzyme (0.5 μg/ml, 6 h) significantly increases steady-state surface Kv1.2 although LPA-induced suppression of Kv1.2 still occurs as detected by flow cytometry (n=31, **p<0.01). (B) LPA-induced suppression of Kv1.2 is significantly reduced in the presence of C3 exoenzyme (n=31, *p<0.05). (C) Application of the adenylate cyclase activator forskolin (10μM, 10 min) significantly increases the level of Kv1.2 at the cell surface and strongly inhibits LPA-induced suppression of the channel.(n=15, **p<0.001). (D) LPA-induced suppression of Kv1.2 is significantly reduced in the presence of forskolin (n=15, **p<0.001).

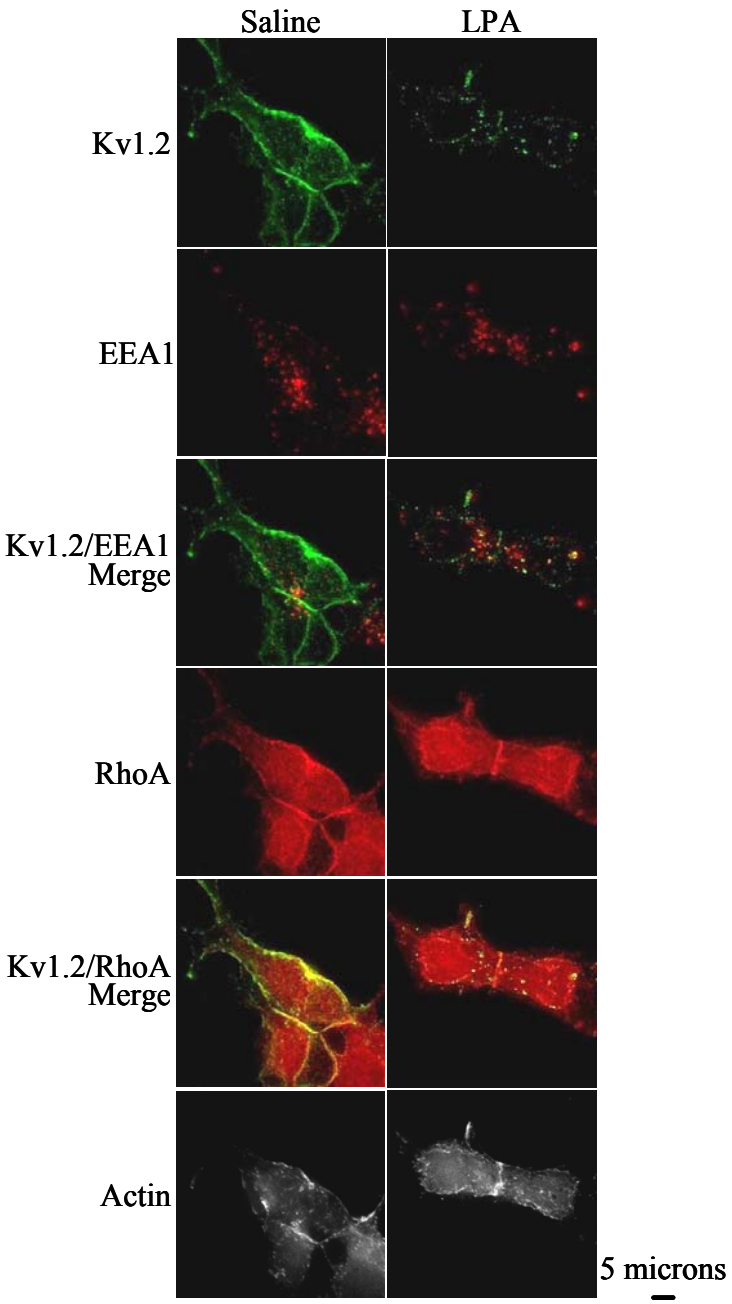


Figure 5. Activation of RhoA Triggers Kv1.2 Endocytosis

In HEK-K cells, LPA application (10 μM, 15 min) results in translocation of Kv1.2 (green) from the edge of the cell to intracellular puncta that contain EEA1 or are adjacent to EEA1-containing structures (right column). EEA1 was labeled with α-EEA1m (red). Overlap of surface Kv1.2 and total RhoA (red) is highly reduced in these intracellular puncta (right column).

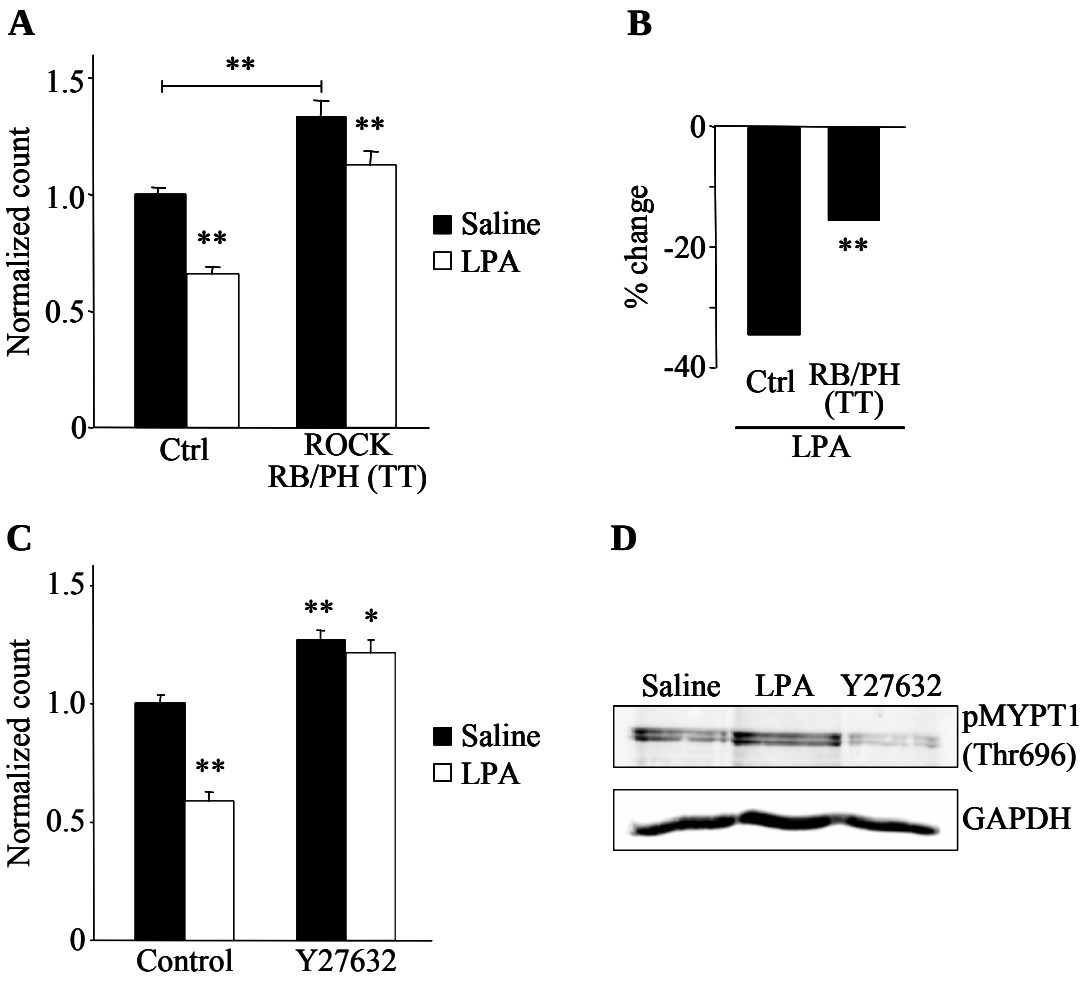


Figure 6. LPA-induced Kv1.2 Endocytosis Requires ROCK

(A) Over-expression of a dominant negative form of ROCK (RB/PH (TT)) in HEK-K cells significantly increases steady-state levels of surface Kv1.2. LPA-induced suppression still occurs as detected by flow cytometry (n=69, **p<0.001). (B) LPA-induced suppression of Kv1.2 is significantly reduced in the presence of dominant negative ROCK (n=69, **p<0.001). (C) Pre-treatment with the ROCK inhibitor Y27632 (10 μM, 30 min) prevents LPA-induced endocytosis of Kv1.2. Y27632, when applied alone, significantly increases surface Kv1.2 levels above that of saline control (n=40, *p<0.01, **p<0.001). (D) Application of LPA (10 μM, 15 min) stimulates ROCK activity while application of Y27632 inhibits basal ROCK activity as measured by immunoblot of phosphorylated myosin phosphatase 1 (pMYPT1). pMYPT1 was detected with α-pMYPT1p. GAPDH was detected with α-GAPDHm as a loading control.

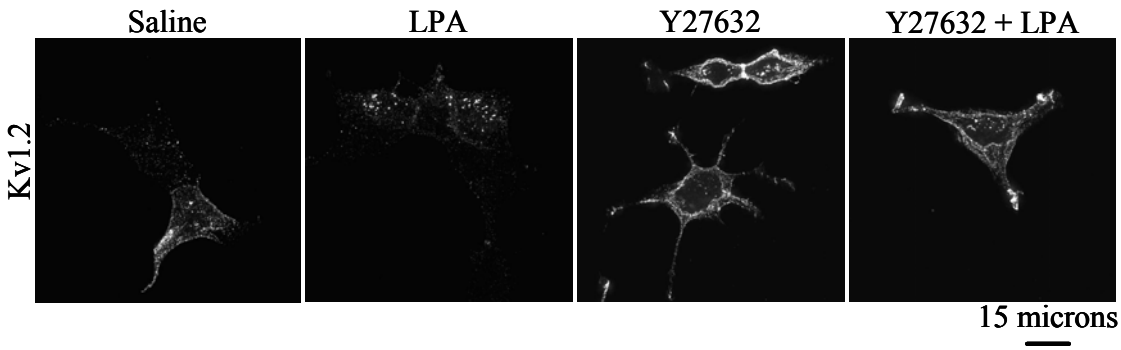


Figure 7. ROCK Inhibition Prevents LPA-induced Kv1.2 Endocytosis

In HEK-K cells, pre-treatment with Y27632 (10 μM, 30 min) blocks the translocation of Kv1.2 from the edge of cells to intracellular puncta upon stimulation with LPA (10 μM, 15 min). Surface Kv1.2 was labeled with α-Kv1.2e before stimulation with control saline or LPA.

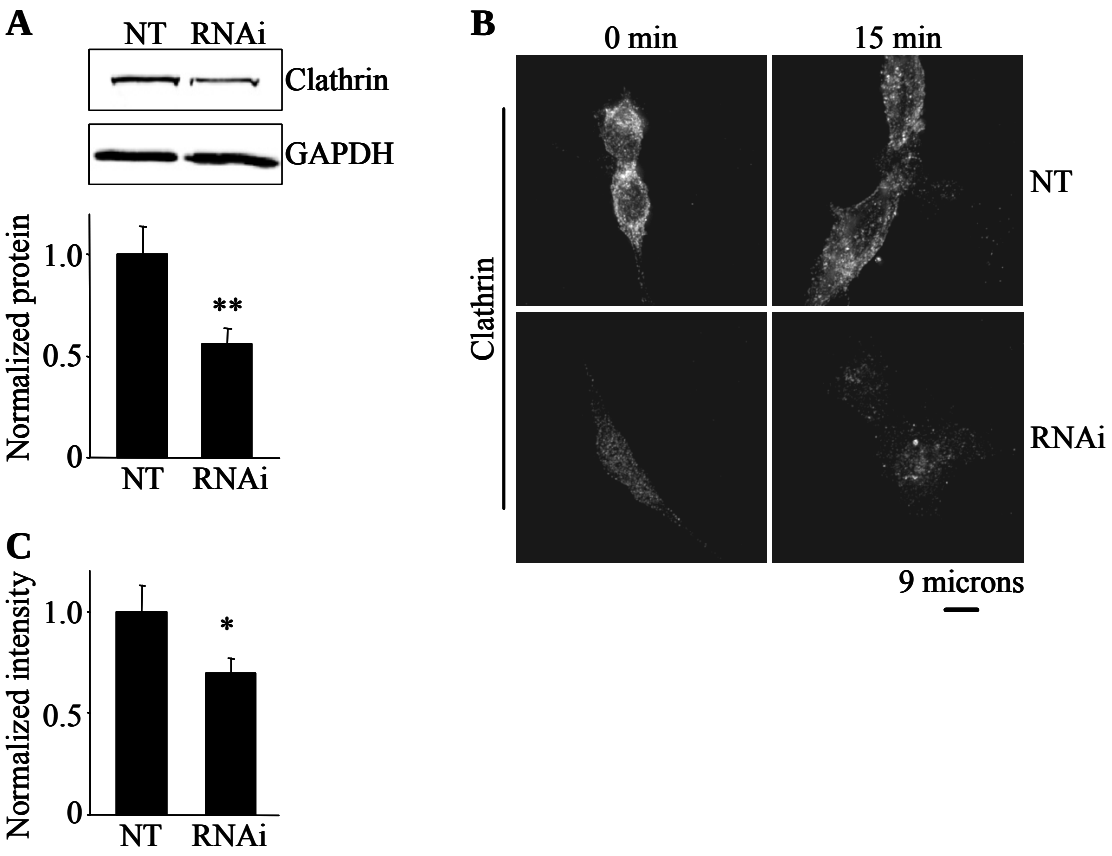


Figure 8. Clathrin RNAi Reduces Clathrin Protein in HEK-K Cells

(A) Transfection of HEK-K cells with RNAi directed against the heavy chain of human clathrin depletes clathrin protein by 50% compared to non-targeting RNAi control. Clathrin heavy chain protein was detected with α-CHCm. GAPDH was detected with α-GAPDHm as a loading control (n=8; **p=0.008). (B) Immunofluorescence of single optical slices through the center of HEK-K cells illustrates that clathrin RNAi reduces clathrin signal intensity compared to non-targeting control both at 0 min and 15 min incubation at 37ºC. Clathrin heavy chain was detected with α-CHCm. (C) Quantitation of immunofluorescence images reveals that clathrin RNAi reduces clathrin signal intensity by approximately 30% compared to non-targeting control (n=37; *p=0.035).

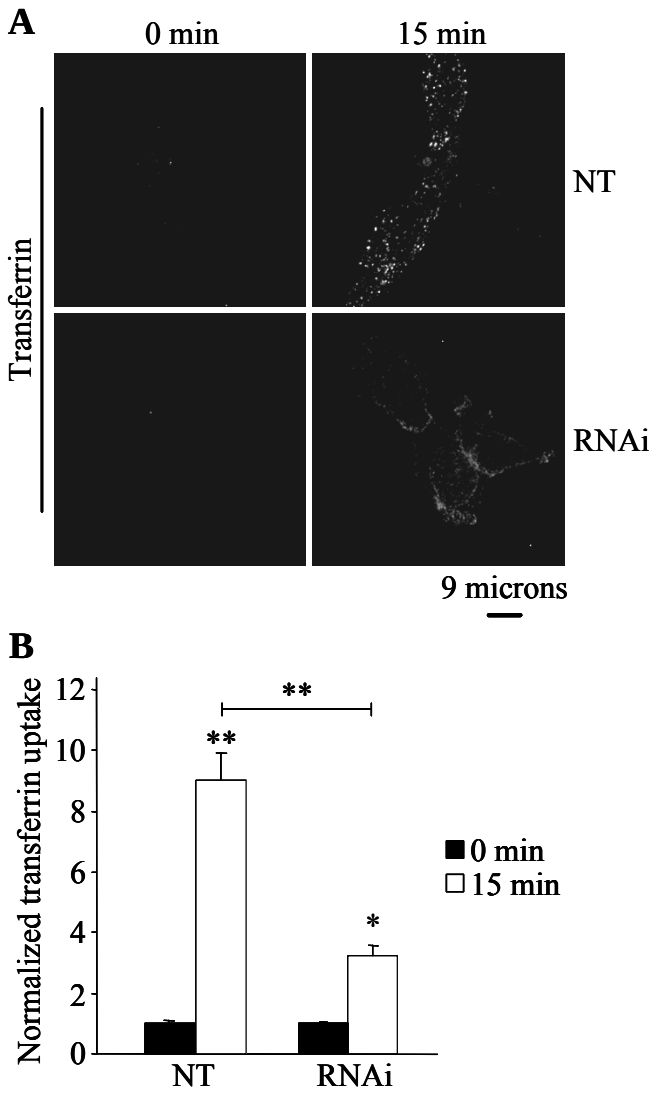


Figure 9. Clathrin RNAi Impairs Transferrin Receptor Internalization

(A) Immunofluorescence of single optical slices through the center of HEK-K cells illustrates that clathrin RNAi reduced the number of internalized Alexa Fluor 488-conjugated transferrin-containing puncta compared to non-targeting RNAi control after 15 min incubation at 37ºC. (B) Quantitation of immunofluorescence images reveals that transferrin uptake is reduced 3-fold in cells with clathrin RNAi compared to cells with non-targeting RNAi control (n=17; *$p<0.05$, **$p<0.001$).

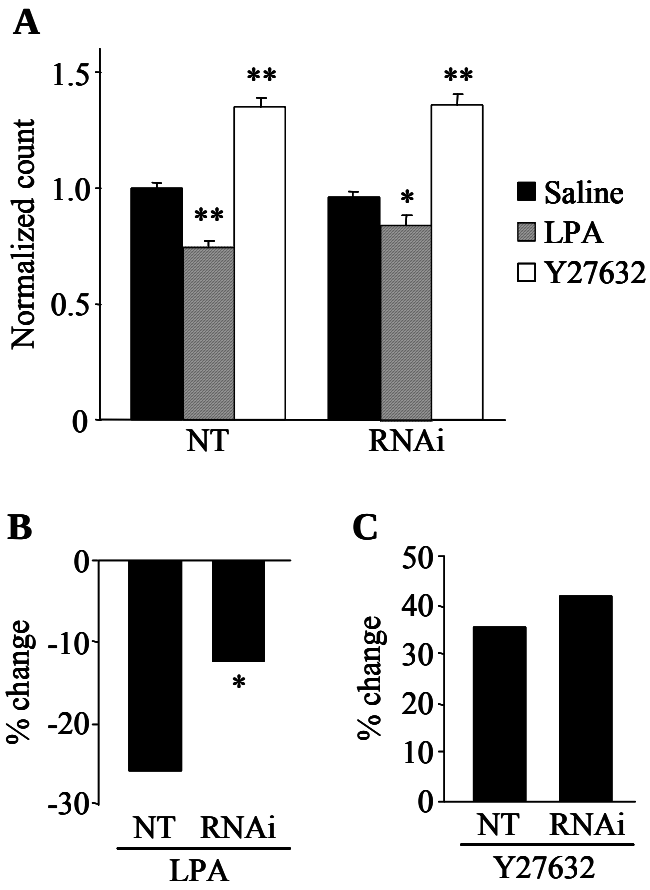


Figure 10. LPA-induced Kv1.2 Endocytosis is Clathrin-dependent

(A) Flow cytometric analysis of HEK-K cells reveals that clathrin RNAi reduces LPA-induced endocytosis of Kv1.2 while having no effect on steady-state surface Kv1.2 levels. Treatment with Y27632 (10 μM, 30 min) significantly increases surface Kv1.2 in the absence or presence of clathrin RNAi (n=33; *p<0.05, **p<0.001). (B) LPA-induced Kv1.2 endocytosis is reduced by approximately 50% in the presence of clathrin RNAi (n=33; *p<0.05). (C) The effect of Y27632 is not significantly altered by the presence of clathrin RNAi (n=33; p=0.493).

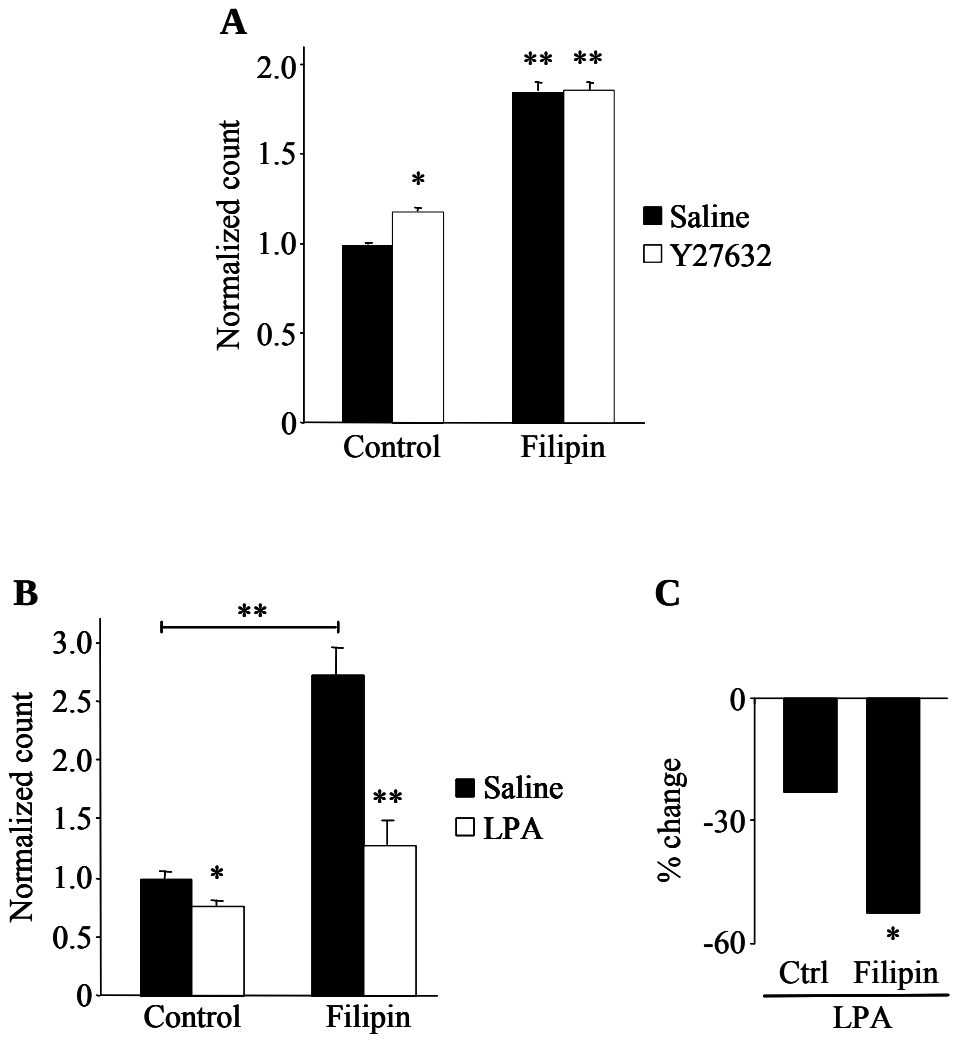


Figure 11. Constitutive Cholesterol-dependent Endocytosis is Critical for Kv1.2 Homeostasis

(A) Application of the sterol-binding agent filipin (7.7 μM, 1 h) to HEK-K cells significantly increases steady-state surface Kv1.2 as detected by flow cytometry. Subsequent treatment with Y27632 (10 μM, 30 min) does not elicit a further increase (n=32; *p<0.01, **p<0.001). (B) Filipin application does not prevent LPA-induced Kv1.2 endocytosis upon application of LPA (10 μM, 15 min) (n=5; *p<0.05, **p<0.001). (C) LPA-induced Kv1.2 endocytosis is significantly increased in the presence of filipin (n=5; *p<0.05).

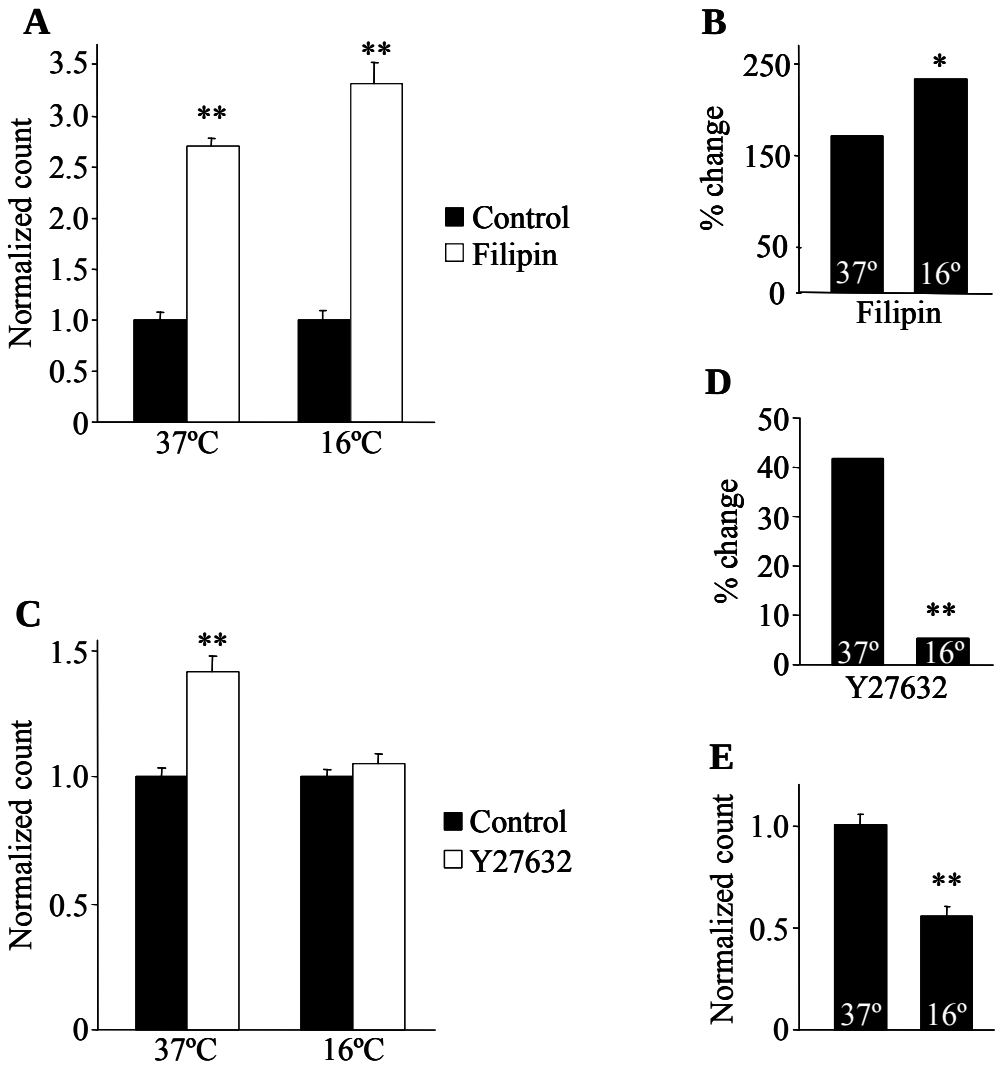


Figure 12. Constitutive Recycling is Critical for Kv1.2 Homeostasis

(A) Application of filipin (7.7 μM, 1 h) to HEK-K cells significantly increases surface Kv1.2 at both 37ºC and 16ºC as detected by flow cytometry (n=12; **p<0.001). (B) Application of filipin at 16ºC results in a significantly larger increase of surface Kv1.2 compared to 37ºC control (n=12; *p=0.019). (C) Y27632 (10 μM, 10 min) does not elicit an increase of steady-state surface Kv1.2 at 16ºC (n≥10; **p<0.001). (D) Application of Y27632 at 16ºC results in virtually no increase of surface Kv1.2 compared to 37ºC control (n≥10, **p<0.01). (E) Incubation at 16ºC results in significantly reduced levels of steady-state surface Kv1.2 compared to 37ºC control (n=27, **p<0.001).

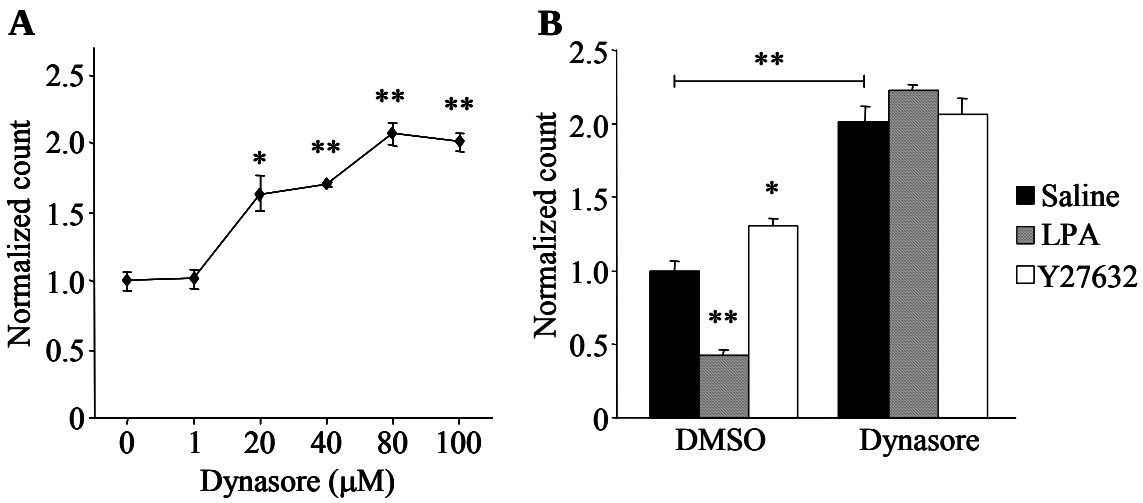


Figure 13. Dynamin is Essential for Steady-state Homeostasis and LPA-induced Kv1.2 Endocytosis

(A) Treatment of un-stimulated HEK-K cells with increasing concentrations of dynasore (30 min at 37ºC) results in a significant, concentration-dependent increase in steady-state surface Kv1.2 as detected by flow cytometry (n=3; *$p<0.01$, **$p<0.001$). (B) LPA (10 μM, 15 min) and Y27632 (10 μM, 30 min) have no effect in the presence of dynasore (n=6; *$p<0.05$, **$p<0.001$).

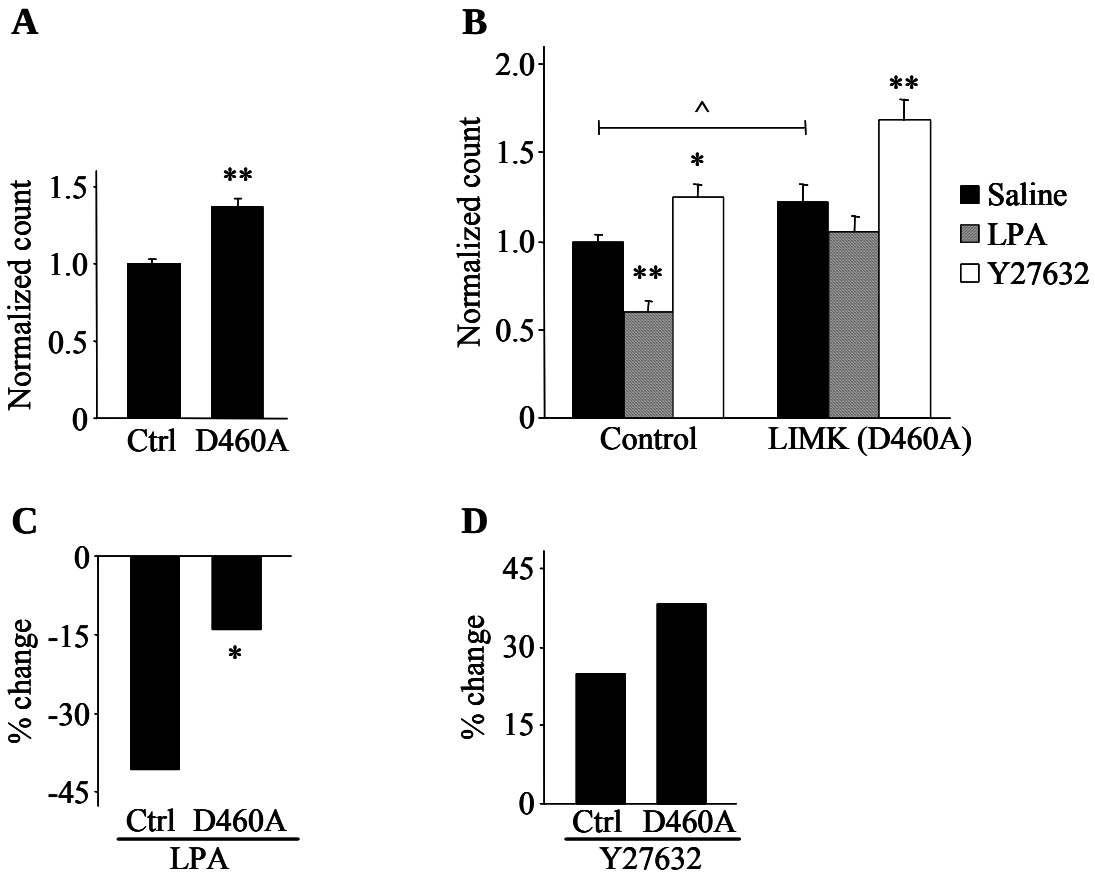


Figure 14. LIM Kinase Modulates Kv1.2 Trafficking Downstream of ROCK

(A) Over-expression of a dominant negative form of LIM kinase 1 (D460A) significantly increases steady-state surface Kv1.2 in HEK-K cells as detected by flow cytometry (n=78; **p<0.001). (B) LIM kinase 1 (D460A) inhibits LPA-induced endocytosis of Kv1.2. Y27632 (10 μM, 30 min) elicits an increase in surface Kv1.2 in the absence or presence of LIM kinase 1 (D460A) (n=21; ^p<0.05, *p<0.01, **p<0.001). (C) In the presence of LIM kinase 1 (D460A), LPA-induced Kv1.2 endocytosis is significantly reduced (n=21; *p≤0.05). (D) The Y27632 effect was larger in the presence of LIM kinase 1 (D460A) but this was not significant (n=21; p=0.143).

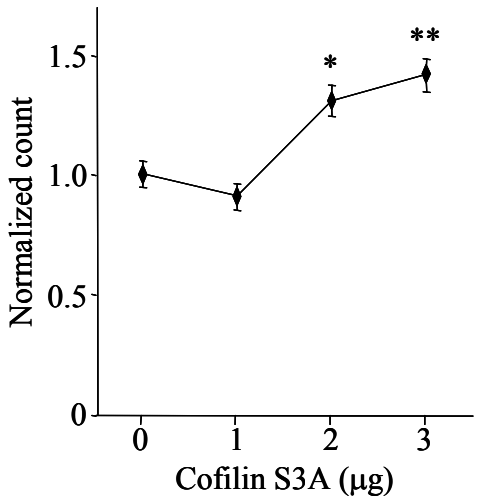


Figure 15. Cofilin Modulates Kv1.2 Trafficking Downstream of LIM Kinase

Transient transfection of HEK-K cells with increasing amounts of DNA encoding a constitutively active form of cofilin (S3A) significantly increases steady-state surface Kv1.2 in a concentration-dependent manner as detected by flow cytometry (n=12, *$p<0.01$, **$p<0.001$).

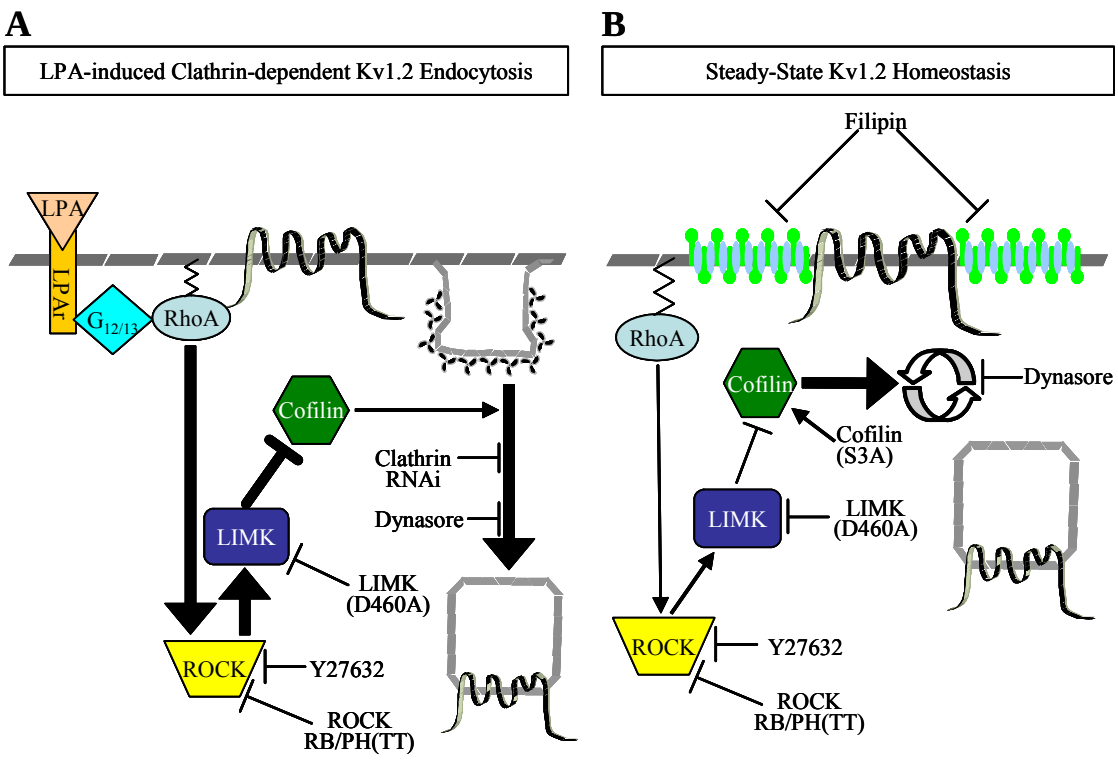


Figure 16. Proposed Model for Kv1.2 Regulation by RhoA/Rho-kinase

(A) LPA activates the RhoA/ROCK/LIM kinase pathway to trigger clathrin-dependent Kv1.2 endocytosis. Dynamin is essential for both steady-state and LPA-induced Kv1.2 trafficking. (B) Steady-state Kv1.2 homeostasis is maintained by a balance of constitutive, cholesterol-dependent endocytosis and recycling. Basal RhoA acts through ROCK, LIM kinase, and cofilin to control constitutive Kv1.2 recycling.

CHAPTER 3: SUPPLEMENTAL DATA AND FUTURE DIRECTIONS

Introduction

In the previous chapter many questions were answered about how RhoA signaling relates to Kv1.2 regulation. Many additional questions were raised during the course of those studies. This chapter is designed to address some of them and provide new directions for continued research into the regulation of cellular excitability by RhoA.

Previous studies demonstrated that tyrosine kinase-dependent phosphorylation is a potent mechanism of channel suppression (Huang *et al.*, 1993; Holmes *et al.*, 1996). For Kv1.2 tyrosine phosphorylation via the M1 muscarinic acetylcholine receptor (M1 mAChR) was shown to require trans-activation of the EGF receptor tyrosine kinase (Tsai *et al.*, 1997). It was later shown that RhoA activity was necessary downstream of carbachol (CCh) stimulation of M1 mAChR for suppression of Kv1.2 ionic current (Cachero *et al.*, 1998). A subsequent study identified endocytosis as a physical mechanism for Kv1.2 suppression (Nesti *et al.*, 2004). What remained was to determine if a link exists between RhoA signaling and tyrosine kinase-dependent modulation of Kv1.2. To that end, the contribution of RhoA and ROCK to CCh-induced Kv1.2 endocytosis was evaluated.

Chapter 2 also established a role for basal RhoA/ROCK signaling in the control of constitutive outward trafficking of Kv1.2. We hypothesized this signaling controls recycling of constitutively endocytosed channel but another possibility exists; the outward trafficking of newly synthesized Kv1.2 from the Golgi apparatus. In support of

this hypothesis Yang and colleagues have shown in kidney epithelial cells that ROCK is required for outward trafficking of Na^+/H^+ antiporter (NHE) from a sub-apical pool to the apical cell surface in response to increased pH load (Yang *et al.*, 2007). In this chapter we test this hypothesis; the effect of basal ROCK inhibition on steady-state surface Kv1.2 was evaluated under conditions of impaired Golgi protein trafficking.

The previous chapter also demonstrated that a cholesterol-dependent endocytosis pathway is a critical part of steady-state Kv1.2 homeostasis, but did not elaborate on the specifics of the pathway beyond its requirement for dynamin. To address this question, chapter 3 includes a more in-depth assessment of the effectiveness of the sterol-binding agent filipin to perturb constitutive channel endocytosis and an investigation into the contribution of caveolae to constitutive Kv1.2 endocytosis. Other labs have demonstrated that Kv1.5 channels associate with caveolin, that this association regulates channel trafficking to cholesterol-rich membrane regions, and that this serves as a means to modulate channel function (Martens *et al.*, 2001; McEwen *et al.*, 2008). Caveolin-dependent endocytosis has also been shown to require dynamin, therefore it was a natural candidate for study (Minshall *et al.*, 2000; Pelkmans *et al.*, 2001; Le *et al.*, 2002).

As mentioned throughout this thesis, RhoA signals through a variety of downstream effectors. Chapter 2 explored how RhoA signals through ROCK to control Kv1.2 trafficking without investigating how or if other RhoA effectors may be involved. In chapter 3, the contribution of another RhoA effector, protein kinase N, is evaluated. Protein kinase N (PKN), like ROCK, is a serine/threonine kinase (Amano *et al.*, 1996b).

PKN was an attractive candidate for study here because of work conducted in yeast where it was shown to regulate endocytosis via phosphorylation of proteins that come together to form an endocytic complex. Complex formation is inhibited upon PKN-mediated protein phosphorylation (Cope *et al.*, 1999; Zeng and Cai, 1999; Zeng *et al.*, 2001), making PKN a negative regulator of endocytosis in yeast. PKN has also been shown to regulate intermediate filament polymerization. Phosphorylation by PKN inhibits polymerization of neurofilamin, vimentin, and glial fibrillary acidic protein (GFAP) (Mukai *et al.*, 1996; Matsuzawa *et al.*, 1997). Phosphorylation of the microtubule-associated protein tau by PKN has also been demonstrated, which reduces the stabilization and assembly of microtubules, which has important implications for neuron function (Taniguchi *et al.*, 2001). As was done with ROCK, studies in chapter 3 were designed to assess the role of PKN in both steady-state and stimulus-induced Kv1.2 trafficking.

Materials and Methods

Materials

Rabbit polyclonal antibody directed against the first extracellular loop of Kv1.2 (α-Kv1.2e) was developed in conjunction with Biosource International (Camarillo, CA). GM 130 polyclonal (α-GM 130) antibody was purchased from EMD Biosciences (San Diego, CA). AlexaFluor-conjugated secondary antibodies and phalloidin were purchased from Invitrogen (Carlsbad, CA). Y27632 was purchased from EMD Biosciences (San Diego, CA), C3 exoenzyme was purchased from Cytoskeleton Inc. (Denver, CO),

HA1077 was purchased from Millipore (Billerica, MA), Lysophosphatidic acid (LPA), Carbachol, and Brefeldin A were purchased from Sigma-Aldrich (St. Louis, MO). Caveolin-1 (S80E) and PKN (K644E) were generously provided by Dr. J. Pessin (SUNY Stony Brook) and Dr. Y. Ono (Kobe University).

Cell culture and transfection

Human embryonic kidney 293 cells stably expressing Kv1.2-α, Kvβ2 and the M1 muscarinic acetylcholine receptor (HEK-K) were utilized and cultured as previously reported (Nesti *et al.*, 2004). All experimental plasmid constructs were co-transfected into HEK-K cells with pEGFP-N1 using calcium phosphate. Confluent cultures were plated to a density of 3.3 x 10^4 cells per cm^2 onto tissue culture plates or glass cover slips (Corning Glass Works, Corning, NY) coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO) (0.1 mg/ml) or plasma fibronectin (0.015 mg/ml) for flow cytometry and immunofluoresence 12-16 h in serum-free media before use.

Detection of surface Kv1.2

For flow cytometric detection of surface Kv1.2, HEK-K cells were treated with saline, vehicle control solution, or an appropriate stimulus, then 154 mM sodium azide as in (Nesti *et al.*, 2004). After azide, the cells were lifted and placed in a test tube where surface Kv1.2 was labeled with 0.33 μg/ml α-Kv1.2e. Antibody binding was detected with fluorescently conjugated anti-rabbit IgG (0.1 μg/ml). Fluorescence was detected by flow cytometry as described below.

Flow cytometry

A single laser flow cytometer (Easy Cyte PCA-96; Guava Technologies, Hayward, CA) was used. In all HEK-K cells, α-Kv1.2e primary antibody was detected by labeling with a goat α-rabbit secondary antibody conjugated to PE/Cy^{TM}5 with an excitation peak of 488nm and an emission peak of 667nm (Southern Biotechnology, Birmingham, AL). Surface Kv1.2 in transfected cells was taken as the distribution of cells emitting at 667 nm (indicating surface Kv1.2) in cells also emitting at 508 nm (indicating GFP expression). Background staining was quantitated in cells using goat α-rabbit-PE/Cy^{TM}5 without prior application of α-Kv1.2e.

Immunofluorescence

Confluent cultures were plated as for flow cytometry and pre-treated as indicated at 37ºC. α-Kv1.2e was applied to live cells at 0.33 μg/ml for 10 min at 37ºC. Cells were fixed with 4% paraformaldehyde (Polysciences, Warrington, PA) in PHEM 6.1 buffer (60 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA; pH to 6.1 with KOH) at room temperature. Cells were extracted with ice cold acetone. Coverslips were incubated in blocking buffer (PHEM 6.9, 3% goat serum, 0.1% fish skin gelatin) at room temperature. Other primary antibodies were then applied (rabbit α-GM 130 = 0.7 μg/ml) diluted in blocking buffer. Alexa Fluor-conjugated secondary antibodies were applied (4 μg/ml in blocking buffer) (Invitrogen, Carlsbad, CA) at room temperature. F-actin was detected with Alexa Fluor 488 phalloidin (Invitrogen) (4 U/ml). Coverslips were mounted on glass slides with ProLong Gold mounting medium (Molecular Probes) according to the

provided protocol. Images were obtained with the DeltaVision reconstruction microscopy system (Applied Precision).

Statistical analysis

Descriptive statistics are provided in figures as line or bar graphs indicating the sample mean with error bars indicating the standard error of the mean (SEM). Detection of statistical difference between two independent measurements was by one-way t-test. Comparison of percent changes between pairs of independent measurements was by two-way ANOVA. Post-hoc power analysis was used to gauge experimental sensitivity and sample sizes necessary to achieve 95% power. Sample populations were considered to be significant at $p \leq 0.05$.

Results

Tyrosine kinase-dependent Kv1.2 endocytosis requires RhoA and ROCK

The studies of Kv1.2 in this thesis built from work previously conducted that demonstrated the channel is endocytosed and electrophysiologically suppressed downstream of tyrosine phosphorylation at specific residues and that phosphorylation occurs following M1 mAChR stimulation with CCh (Huang *et al.*, 1993; Hattan *et al.*, 2002; Nesti *et al.*, 2004). It was CCh-induced Kv1.2 suppression that was initially blocked by inhibiting RhoA using C3 exoenzyme (Cachero *et al.*, 1998). In chapter 2 we used LPA to link RhoA to Kv1.2 endocytosis. We did not, however, directly test the hypothesis that M1 mAChRs also elicit Kv1.2 endocytosis through RhoA. In this chapter we test that hypothesis.

Treatment of HEK-K cells with C3 exoenzyme (0.5 μg/ml, 6 h) elicited a significant increase of steady-state surface Kv1.2. Subsequent stimulation of these cells with CCh (10 μM, 10 min) still significantly reduced surface Kv1.2 levels (Figure 1A; n=17, *p<0.05, **p<0.001). The magnitude of the CCh-induced Kv1.2 endocytosis was reduced by over 50% in the presence of C3 exoenzyme although this reduction was not significant by 2-way ANOVA (Figure 1B; n=17, p=0.23). Based on the variability of this pilot study, a power analysis illustrated that a sample size of over 40 would be required to detect a difference in CCh-induced suppression as large the one as shown in Figure 1B with 95% confidence (Figure 1C). Inhibition of ROCK by application of Y27632 (10 μM, 30 min) also resulted in a significant increase of steady-state surface Kv1.2. CCh stimulation in the presence of Y27632 did not significantly suppress the channel (Figure 2A, Figure 2B; n=18, *p<0.05, **p<0.001). Based on the variability of this pilot study, a power analysis illustrated that a sample size of over 80 would be required to detect a difference in CCh-induced channel suppression as large as the one shown in Figure 2B with 95% confidence (Figure 2C). Although these are only preliminary results, the data support the hypothesis that inhibition of RhoA and ROCK reduce CCh-induced Kv1.2 endocytosis and suggests that RhoA signaling is part of a larger overall network of signals that regulate Kv1.2.

Basal ROCK modulates steady-state Kv1.2 at the plasma membrane

The elevation of steady-state surface Kv1.2 by inhibition of basal ROCK can be the result of increased recycling of constitutively endocytosed channel, as hypothesized in chapter 2, or of increased outward trafficking of newly synthesized Kv1.2 from the

Golgi apparatus to the plasma membrane. To evaluate this possibility HEK-K cells were subjected to brefeldin A (BFA) to disrupt the integrity of the Golgi apparatus (Yoo *et al.*, 2005). Application of BFA (5 μM, 1 h) disrupted the Golgi apparatus (Figure 3) as evidenced by dispersal and more punctate staining of the Golgi marker GM 130 in BFA-treated cells compared to methanol-treated control. BFA treatment significantly reduced steady-state levels of surface Kv1.2 suggesting that, to some extent, outward trafficking of newly synthesized channel does contribute to Kv1.2 homeostasis. However, despite having a compromised Golgi apparatus, application of Y27632 (10μM, 30 min) to BFA-treated cells still elicits a significant increase of surface Kv1.2 (Figure 4A; n=15, *$p<0.05$, **$p<0.01$). The magnitude of the Y27632-induced increase is the same in the absence or presence of BFA (Figure 4B; n=15, $p=0.54$). This result supports the previous hypothesis from chapter 2 that basal ROCK negatively regulates constitutive Kv1.2 recycling at the plasma membrane independently of constitutive, cholesterol-dependent endocytosis.

Caveolin influences steady-state Kv1.2 homeostasis

It has been shown that manipulating membrane cholesterol can modulate the distribution and trafficking of other Kv channels such as Kv1.3, Kv1.4, and Kv2.1 (O'Connell and Tamkun, 2005). Earlier in this dissertation we demonstrated the essential nature of plasma membrane cholesterol for surface expression of Kv1.2 (Chapter 2; Figures 11, 12). This was done, in part, by utilizing the sterol-binding agent filipin to disrupt membrane cholesterol. Figure 5 shows that application of increasing concentrations of filipin (1 h) to un-stimulated HEK-K cells elicits a concentration-

dependent increase of steady-state surface Kv1.2, with a maximal effect occurring between 5.0 and 10 μM (n=5, **p<0.001).

To gain a more complete appreciation of different cholesterol-dependent mechanisms through which constitutive endocytosis of Kv1.2 may occur, we chose to evaluate the contribution of one well-known route of internalization, namely caveolae. To test the idea that caveolin may be important for Kv1.2 trafficking in our system, a dominant negative form of caveolin 1, (S80E) was over-expressed. This point mutation has been shown to act as a phospho-mimetic which disrupts caveolae organization and subsequent endocytosis (Shigematsu *et al.*, 2003). Caveolin 1 (S80E) over-expression produces a significant increase in steady-state surface Kv1.2, suggesting that some caveolin-dependent endocytosis is occurring in our HEK-K cells and is partially contributing to overall Kv1.2 homeostasis (Figure 6A; n=24, *p<0.01, **p<0.001). Despite the presence of caveolin 1 (S80E), Y27632 treatment (10 μM, 30 min) elicited an additional increase in surface Kv1.2 (Figure 6A; n=24, *p<0.01, **p<0.001). The magnitude of the Y27632-induced increase of surface Kv1.2 was elevated by over 50% in the presence of caveolin 1 (S80A) (Figure 6B; n=24, p=0.12) but was not significant by 2-way ANOVA. Based on the variability of these experiments, a power analysis illustrated that a sample size of over 70 would be required to detect a difference in Y27632-induced channel elevation as large as the one shown in Figure 6B with 95% confidence (Figure 6C). This result lends additional support to the assertion that basal ROCK signaling negatively regulates constitutive Kv1.2 recycling at the plasma

membrane independently of constitutive, cholesterol-dependent endocytosis which, this data suggests, partially involves caveolae.

Protein kinase N can control Kv1.2 trafficking downstream of RhoA

We began investigating protein kinase N (PKN) in our HEK-K cell system using pharmacological inhibitors. As tends to be the case, specific inhibitors are seldom as specific as they initially are deemed and experiments have shown that the ROCK inhibitor Y27632, which we use extensively throughout our studies, has some ability to inhibit PKN. Another inhibitor, HA1077, is designed to inhibit PKN but has some ability to inhibit ROCK (Davies *et al.*, 2000). When applied separately (10 μM, 30 min) both HA1077 and Y27632 significantly increase steady-state surface Kv1.2. When applied together, the individual increases are additive (Figure 7A; n=53; **p<0.001), suggesting that ROCK and PKN act in parallel to regulate steady-state surface Kv1.2. In an experiment designed to confirm that the HA1077 effect was specific to PKN, increasing amounts of a dominant negative form of PKN that lacks catalytic activity, PKN (K644E), were transfected into HEK-K cells. This resulted in a concentration-dependent increase of steady-state surface Kv1.2 (Figure 7B; n=10; **p<0.01), suggesting in conjunction with HA1077 data, that PKN is at least partially active in un-stimulated HEK-K cells and can modulate steady-state surface Kv1.2.

Because of its demonstrated ability to modulate steady-state Kv1.2, we next hypothesized that, like ROCK, PKN contributes to LPA-induced, clathrin-dependent endocytosis of Kv1.2. To test this hypothesis, endocytosis of Kv1.2 after LPA application (10 μM, 15 min) was measured in the presence or absence of over-expressed

PKN (K644E). Under these conditions PKN (K644E) significantly increased steady-state surface Kv1.2, although LPA-induced endocytosis of the channel still occurred (Figure 8A; n=69, **p<0.001). The magnitude of the LPA-induced endocytosis is reduced by approximately 33% in the presence of dominant negative PKN but was not significant by 2-way ANOVA (Figure 8B; n=69, p=0.98). Based on the variability of these experiments, a power analysis illustrated that a sample size of over 100 would be required to detect a difference in LPA-induced channel endocytosis as large as the one shown in Figure 6B with 95% confidence (Figure 8C). Alternatively, over-expression of an active form of PKN, PKN (AF3), which lacks an N-terminal auto-inhibitory domain, does not reduce LPA-induced endocytosis of Kv1.2. Rather, the endocytosis of the channel is significantly increased in the presence of over-expressed PKN (AF3) (Figure 9A, Figure 9B; n=11, *p<0.01, **p<0.001). Curiously, PKN (AF3) dramatically increases steady-state surface Kv1.2 as well (Figure 9A; n=11, **p<0.001). Taken together, these results suggest that PKN can be an important regulator of Kv1.2 downstream of RhoA and provides the rationale to investigate the potential of RhoA effectors other than ROCK to regulate cellular excitability.

Discussion

Regulation of cellular excitability is a complex process that involves many overlapping networks of signals. In this chapter we performed experiments designed to answer specific questions about how RhoA mediates cellular excitability by controlling

Kv1.2 trafficking. We also began exploring avenues of RhoA downstream signaling that were not touched upon in chapter 2 of this thesis.

We previously demonstrated that Kv1.2 undergoes both steady-state and acute forms of regulation that involve independent trafficking pathways, both with a requirement for RhoA/ROCK. Following those experiments, we became interested in another RhoA downstream effector, protein kinase N (PKN) based on the results of a study that indicated certain ROCK inhibitors, like Y27632, also have some ability to inhibit PKN (Davies *et al.*, 2000). In Figure 7A we show that application of the PKN inhibitor HA1077 elicits a significant increase of steady-state surface Kv1.2, similar to that obtained by the application of Y27632. Concurrent application of the two inhibitors results in an even larger increase of surface Kv1.2, suggesting that both ROCK and PKN can modulate steady-state surface Kv1.2. Over-expression of increasing amounts of DNA encoding dominant negative PKN (K644E) results in a concentration-dependent increase of steady-state surface Kv1.2 (Figure 7B) further suggesting that PKN alone has the ability to modulate Kv1.2 trafficking. These data suggest the possibility that PKN, like ROCK can modulate the recycling phase of steady-state Kv1.2 homeostasis. To more fully explore how PKN can modulate steady-state Kv1.2, future studies using the HA1077 inhibitor can be conducted. This includes time-course and dose-response studies, in conjunction with Y27632 to ascertain the maximal results these pharmacological agents can elicit and if their effects truly are independent. Other studies utilizing low temperature in conjunction with HA1077 and PKN (K644E) over-

expression can more fully answer these questions as well by separating constitutive endocytosis from constitutive recycling.

That PKN modulates Kv1.2 trafficking is not surprising as PKN has been previously reported to control the kinetics of intracellular EGF receptor trafficking (Gampel *et al.*, 1999). Indeed, a role for PKN has emerged through studies in yeast that demonstrate a requirement for PKN in maintaining dynamic actin polymerization in concert with the Arp2/3 complex, allowing for proper vesicle internalization and subsequent trafficking (Duncan and Payne, 2005). Specifically, PKN in yeast has been shown to phosphorylate and inhibit Pan1p, a protein that is instrumental for recruiting endocytic proteins like clathrin to sites of internalization (Huang *et al.*, 2003). Moreover, Pan1p can bind F-actin and activate the Arp2/3 complex (Toshima *et al.*, 2005), characteristics very similar to those of cortactin, a protein previously reported to be required for proper steady-state and acute Kv1.2 regulation (Hattan *et al.*, 2002; Williams *et al.*, 2007). This sets up the intriguing possibility that PKN can negatively regulate proteins that directly couple the actin cytoskeleton to sites of endocytosis.

In Figures 8 and 9, we show that PKN can also affect acute trafficking of Kv1.2. Over-expression of dominant negative (K644E) and active (AF3) forms of PKN have opposing effects on LPA-induced endocytosis of Kv1.2. There is a significant increase of both steady-state surface Kv1.2 and LPA-induced channel endocytosis in the presence of active PKN. Since PKN functions as a negative regulator of endocytic protein recruitment (Huang *et al.*, 2003; Duncan and Payne, 2005; Toshima *et al.*, 2005) it seems reasonable to posit that active PKN should perturb constitutive endocytosis and elevate

steady-state surface Kv1.2. This assertion suggests that PKN can modulate the internalization phase of Kv1.2 homeostasis, perhaps also explaining the additive effects of treating HEK-K cells with Y27632 and HA1077. We were surprised however, by the enhanced LPA-induced Kv1.2 endocytosis in the presence of PKN (AF3), as this result seems to contradict what the literature says about PKN endocytic function. Caution must be exercised when interpreting the results of over-expressing PKN proteins as dominant negative PKN over-expression has been shown to perturb other signaling pathways downstream of RhoA (Gampel *et al.*, 1999). Therefore the effects of PKN (K644E) or (AF3) on LPA-induced Kv1.2 endocytosis may not be totally accurate. An additional series of experiments that could prove very informative again utilize low temperature, as was done in chapter 2, to more fully explore whether over-expressing PKN (K644E) or (AF3) modulates constitutive Kv1.2 endocytosis, recycling, or if the effects in Figures 8 and 9 are an over-expression phenomenon.

In the previous chapter we demonstrated that constitutive, cholesterol-dependent steady-state Kv1.2 homeostasis could be effectively separated from acute, clathrin-dependent Kv1.2 endocytosis. Furthermore, we demonstrated that for steady-state Kv1.2 homeostasis, the cholesterol-dependent internalization phase and the recycling phase can be manipulated independently from one another. Our previous conclusion regarding the role of RhoA/ROCK in steady-state Kv1.2 homeostasis was that ROCK functions as a negative regulator of the recycling phase. We confirm this conclusion by answering a new question; does ROCK modulate delivery of newly synthesized Kv1.2 to the cell surface? Using brefeldin A (BFA) to disrupt the Golgi apparatus (Figure 3) (Yoo *et al.*,

2005) had no effect on the ability of ROCK inhibition to elicit a significant increase of surface Kv1.2 despite the fact that BFA lowered the initial steady-state level of channel at the cell surface (Figure 4). These results are interesting, especially when combined with those obtained in a study that reported the opposite effect of cyclic-AMP signaling on steady-state homeostasis of Kv1.2 (Connors *et al.*, 2008). In this report, it was increased cyclic-AMP signaling led to elevated levels of surface Kv1.2, suggesting that ROCK and cyclic-AMP work from opposite ends to coordinately regulate steady-state Kv1.2 homeostasis. This would not be a surprising occurrence as it has been demonstrated that activation of protein kinase A (PKA) downstream of cyclic-AMP can negatively regulate RhoA and ROCK signaling (Dong *et al.*, 1998; Forget *et al.*, 2002).

In Figure 6 we show that over-expression of a dominant negative caveolin 1 (S80E) has an effect on steady-state Kv1.2 homeostasis. This resulted in increased steady-state surface Kv1.2. In the presence of caveolin 1 (S80E), the Y27632-induced surface Kv1.2 increase trended toward being elevated. It is important to note that the results shown in Figure 6 represent the outcome of an initial pilot study. With that in mind, a power analysis on these preliminary results was performed with the goal of estimating the sample size needed to detect changes in the Y27632-mediated elevation of surface Kv1.2 with 95% confidence. Caveolae are known to be involved in the regulation of other *Shaker* family channels such as Kv1.3 and Kv1.5 (Martens *et al.*, 2001; Eldstrom *et al.*, 2006; Vicente *et al.*, 2008) so their involvement with Kv1.2 is not unexpected. It is notable, however, that Kv1.3 and Kv1.5 are not exclusively found in caveolae (Martinez-Marmol *et al.*, 2008). Based on the results in Figure 6, the same

could be said for Kv1.2 because the effects of caveolin-1 (S80E) over-expression are not nearly as pronounced as those of filipin treatment, suggesting that steady-state Kv1.2 homeostasis is cholesterol- but not necessarily caveolae-dependent. To pursue the involvement of caveolae further, one could also apply filipin to cells over-expressing caveolin 1 (S80E). If caveolae are partially involved in Kv1.2 homeostasis, one would not expect caveolin 1 (S80E) to elicit an additional increase of surface channel beyond filipin treatment alone. In addition, the ability of Kv1.2 to undergo clathrin-dependent endocytosis supports the existence of pools of Kv1.2 that do not necessarily reside in relatively cholesterol-rich regions of the plasma membrane. A recent study has implicated caveolin 1 as a regulator of Rho/ROCK activity in fibroblasts (Grande-Garcia *et al.*, 2007). This report goes on to say that in the absence of caveolin 1, Src kinase activity is increased, which then can elevate p190RhoGAP activity resulting in reduced RhoA/ROCK signaling (Grande-Garcia *et al.*, 2007). These data could potentially explain the increased Y27632 effect obtained in the presence of caveolin 1 (S80E) (Figure 6). Putting these results into the larger context of Kv1.2 regulation sets up the possibility that caveolin 1 may be involved in both the constitutive, cholesterol-dependent internalization phase and the RhoA/ROCK sensitive recycling phase of steady-state Kv1.2 homeostasis.

We also report that RhoA/ROCK is involved in Kv1.2 endocytosis triggered by stimulation of the M1 muscarinic acetylcholine receptor (M1 mAChR) with carbachol (CCh). It was through M1 mAChR signaling that endocytosis of Kv1.2 was first demonstrated (Nesti *et al.*, 2004). Inhibition of RhoA activity with C3 exoenzyme led to

reduced Kv1.2 endocytosis in the presence of CCh (Figure 1) as did inhibition of ROCK activity with Y27632 (Figure 2). Importantly, both application of C3 exoenzyme and Y27632 also resulted in significant increases of steady-state surface Kv1.2, as was demonstrated in chapter 2. These findings indicate that RhoA/ROCK modulates Kv1.2 trafficking downstream of multiple G protein-coupled receptors, not just those activated by LPA. It is known however, that both LPA and M1 mACh receptors can signal via $G_{12/13}$ to activate RhoA (Moolenaar, 1995; Fromm *et al.*, 1997; Bian *et al.*, 2006). We also note that C3 exoenzyme blockade of CCh-induced endocytosis is not complete. This could result from CCh triggering parallel pathways to modulate Kv1.2, only one of which involves RhoA (Tsai *et al.*, 1997). Alternatively, inhibition of RhoA by C3 exoenzyme may not have been complete under the experimental conditions used. We view the latter as more likely since inhibition of ROCK with Y27632 did not allow for significant CCh-induced channel endocytosis (Figure 2). Importantly, the results shown in Figures 1 and 2 represent the outcomes of initial pilot studies. We conducted power analyses using these preliminary results to estimate the sample size needed to detect changes in CCh-induced Kv1.2 endocytosis with 95% confidence. These results begin to put RhoA signaling into the context of a larger body of research that has shown modulation of Kv1.2 function occurs through tyrosine kinase-dependent trafficking of the channel that relies on signals impinging on the actin cytoskeleton (Huang *et al.*, 1993; Hattan *et al.*, 2002; Nesti *et al.*, 2004; Williams *et al.*, 2007).

Because regulation of cellular excitability is critical for proper cardiovascular and neuronal function, there will necessarily be a complex network of signals whose

combined effects intricately modulate excitability in those systems. This work has successfully elucidated how RhoA and its downstream effectors can modulate cellular excitability by controlling the steady-state homeostasis and acute trafficking of Kv1.2. The experiments presented in chapter 3 of this thesis are significant because they confirm and strengthen assertions made in chapter 2, such as that basal ROCK activity modulates the recycling phase of steady-state Kv1.2 homeostasis. They also investigate the involvement of more specific or alternative signaling components in the overall scheme of RhoA-mediated Kv1.2 trafficking, broadening our understanding of the redundancy and interconnectedness of the mechanisms through which cellular excitability is regulated.

The investigations in chapters two and three of this thesis were carried out in a model cell system (HEK-K cells) that is amenable to various forms of cellular and biochemical analysis. Utilization of this model system was crucial because the results thereby derived form the basis of future studies investigating Kv1.2 trafficking and regulation in a more physiological setting. An example of such a setting is neurons of the central nervous system.

Hippocampal neurons are of particular interest because Kv1.2 is heavily endogenously expressed there (Tsaur *et al.*, 1992; Sheng *et al.*, 1994; Veh *et al.*, 1995; Grosse *et al.*, 2000). Kv1.2 localizes predominantly to the dendrites of the hilar and pyramidal neurons of the CA3 and CA1 regions with very little channel present in the cell bodies of these neurons (Sheng *et al.*, 1994). There is also significant Kv1.2 localization in the molecular layer of the dentate gyrus which, studies have shown,

overlaps with Kv1.4 potentially indicating the presence of heteromeric Kv1 channels there (Sheng *et al.*, 1992; Sheng *et al.*, 1993).

The hippocampus is also amenable to many different types of experimentation including neuron dissociation and culture, slice culture, electrophysiology, immunocytochemistry, and biochemical analyses. Indeed, it has been shown that the developmental timecourse of Kv1 channel expression in hippocampal cultures is very similar to that of the intact tissue (Grosse *et al.*, 2000), supporting the use of *ex-vivo* hippocampal cultures for studying ion channel regulation. Additionally, it has been shown that long-term potentiation, a model for learning and memory, can be elicited in the hippocampus via muscarinic acetylcholine receptor stimulation. This G protein-coupled receptor activation correlates to increased responsiveness of these neurons to afferent stimulation along with increased activity of tyrosine and serine/threonine kinases (Segal and Auerbach, 1997). This, coupled with the knowledge that Kv1.2 ionic current is suppressed in a tyrosine phosphorylation-dependent manner downstream of muscarinic G protein-coupled receptor stimulation (Huang *et al.*, 1993) makes investigating the RhoA/ROCK-dependent trafficking of Kv1.2 in the hippocampus all the more interesting.

RhoA/ROCK has already been implicated in long-term spatial memory. Studies by Dash and colleagues have shown that intra-hippocampal infusion of LPA to activate RhoA in live rats leads to enhanced performance in behavioral tests designed to measure formation of long-term spatial memory. Additionally, intra-hippocampal infusion of the ROCK inhibitor Y27632 impairs long-term spatial memory (Dash *et al.*, 2004). Given our conclusions about RhoA/ROCK-mediated Kv1.2 trafficking in HEK-K cells, the

results of Dash and colleagues suggest that RhoA/ROCK-mediated memory formation in the hippocampus may be the result, at least in part, of altered ion channel trafficking. Conducting a series of imaging experiments on the hippocampi of rats in the presence of LPA or Y27632 would allow us to assess the relative levels of surface Kv1.2 and electrophysiological measurements on the same tissues would allow for a determination of celluar excitability. We expect there to be less surface Kv1.2 and enhanced cellular excitability (reduced Kv1.2 current) in the presence of LPA and greater surface Kv1.2 and reduced cellular excitability (increased Kv1.2 current) in the presence of Y27632 commensurate with our data indicating that RhoA activation elicits Kv1.2 endocytosis and inhibition of basal ROCK relieves downregulation of constitutive channel recycling to the plasma membrane.

Downstream of ROCK, we implicated LIM kinase (LIMK) as a crucial component regulating Kv1.2 trafficking. It too has been implicated in hippocampal function. LIMK1 null mice have dramatically altered hippocampal dendritic spine morphology as well as impaired spatial learning and enhanced long-term potentiation (Meng *et al.*, 2002). This correlated to a reduction of phosphorylated cofilin and an overall reduction of synapse surface area (Meng *et al.*, 2002). It is in the dendrites of hippocampal neurons that Kv1.2 can be found (Sheng *et al.*, 1994), therefore it seems reasonable to hypothesize that these physiological LIMK-mediated effects would impact the function of Kv1.2 there. Based on our HEK-K cell results, we expect the absence of LIMK1 to increase the levels of surface Kv1.2 as LIMK appears to function downstream of ROCK as a negative regulator of constitutive Kv1.2 recycling. However, LIMK1 null

mice also have reduced synapse surface area which would seem to limit the space available for Kv1.2 channels to modulate synaptic transmission. Therefore, application of LIMK RNAi or anti-sense oligonucleotides via microinjection or permeabilization of hippocampal slices may be a more suitable technique for investigating the role of LIMK in Kv1.2 trafficking in neurons. A combination of imaging and electrophysiology studies will shed light on the localization and function of Kv1.2 under conditions of altered LIMK signaling.

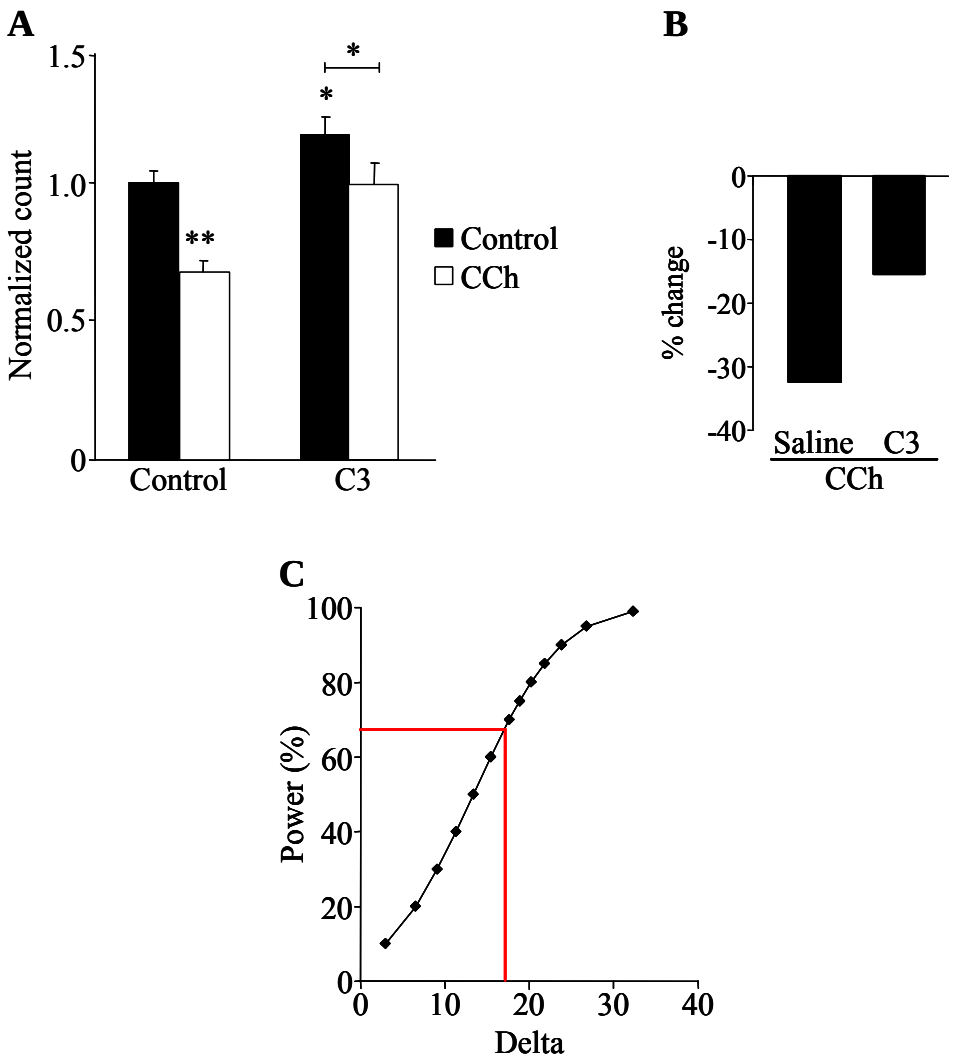


Figure 1. Effect of RhoA Inhibition on Carbachol-induced Kv1.2 Endocytosis

(A) Pre-treatment of HEK-K cells with C3 exoenzyme (0.5 μg/ml, 6 h) increases steady-state surface Kv1.2 and reduces CCh-induced Kv1.2 endocytosis as detected by flow cytometry (n=17, *p<0.05, **p<0.001). (B) The trend is toward reduced CCh-induced endocytosis of Kv1.2 in the presence of C3 exoenzyme but the reduction is not significant (n=17, p=0.23). (C) This experiment has approximately 67% power to detect a difference in CCh-induced Kv1.2 endocytosis as large as the one shown in (B). Based on this analysis, a sample size of over 40 is necessary to achieve 95% power.

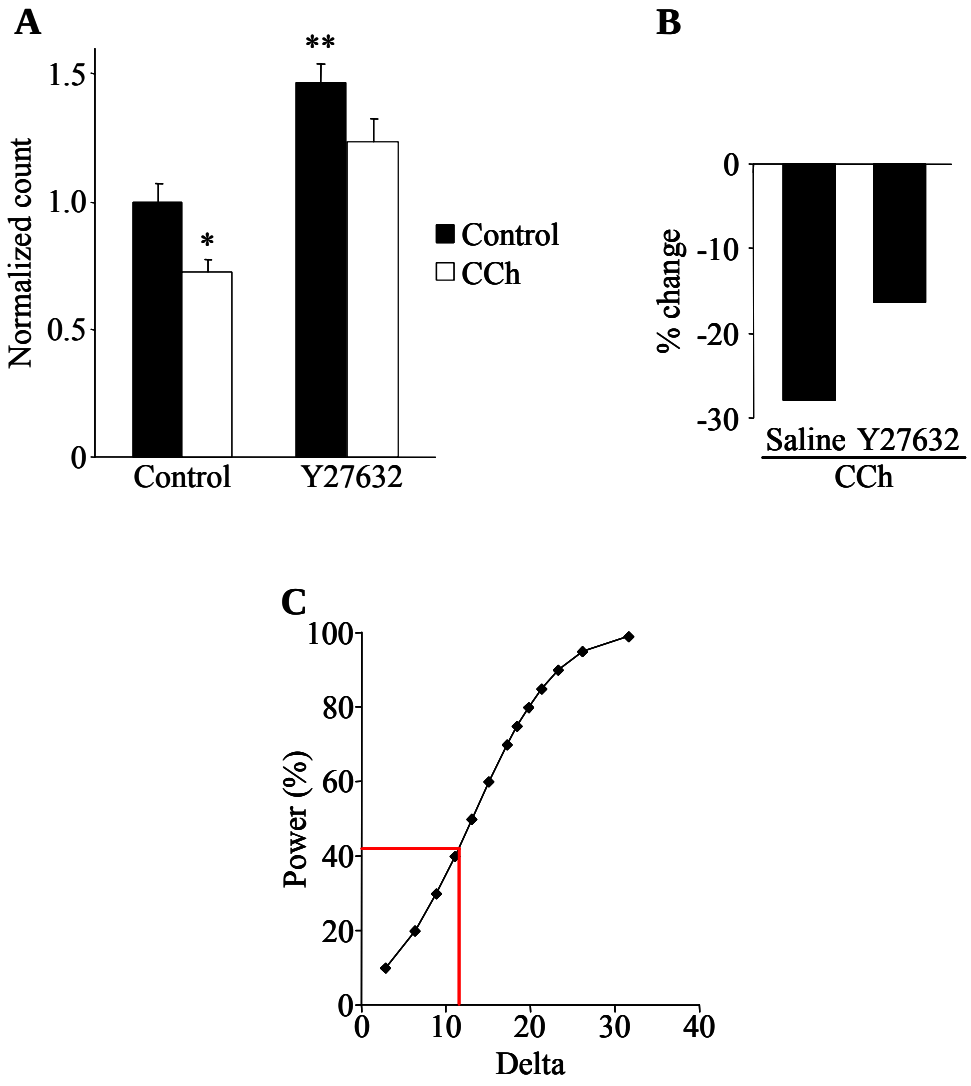


Figure 2. Effect of ROCK Inhibition on Carbachol-induced Kv1.2 Endocytosis

(A) Application of Y27632 (10 μM, 30 min) significantly increases steady-state surface Kv1.2 and reduces CCh-induced Kv1.2 endocytosis as detected by flow cytometry (n=18; *$p<0.05$, **$p<0.001$). (B) The trend is toward reduced CCh-induced endocytosis of Kv1.2 in the presence of Y27632 but the reduction is not significant (n=18, $p=0.74$). (C) This experiment has approximately 42% power to detect a difference in CCh-induced Kv1.2 endocytosis as large as the one shown in (B). Based on this analysis, a sample size of over 80 is necessary to achieve 95% power.

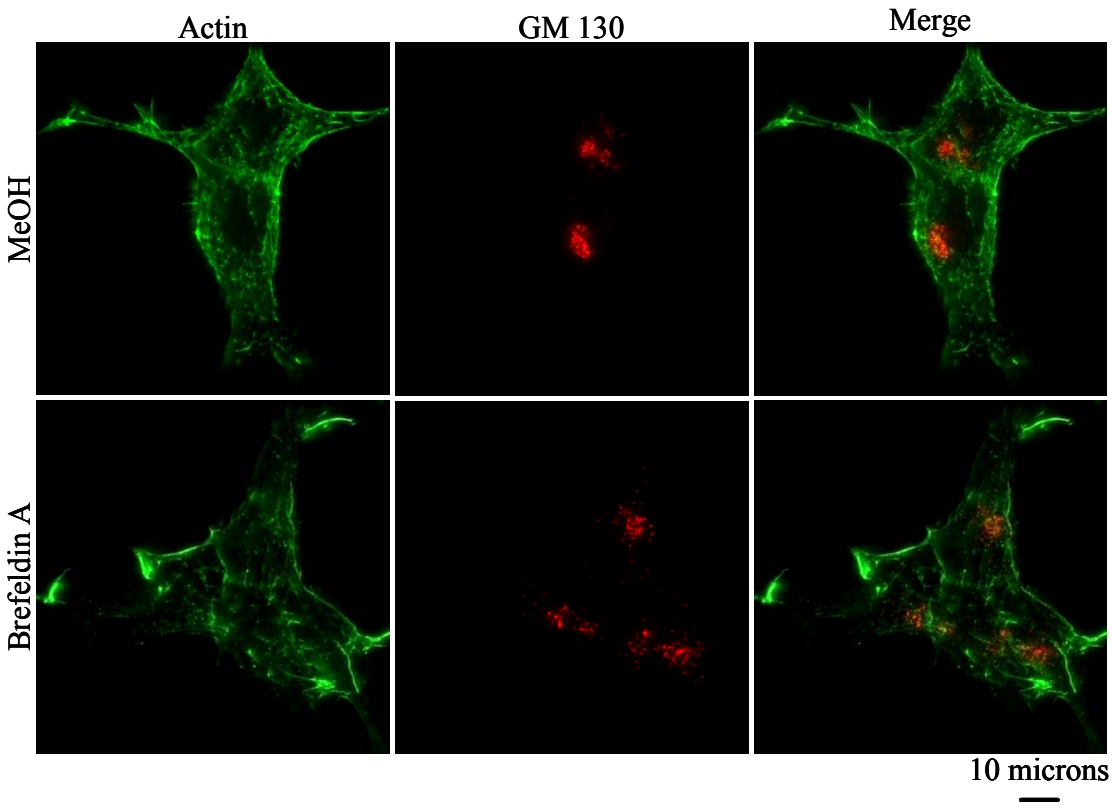


Figure 3. Brefeldin A Disrupts the Golgi Apparatus in HEK-K Cells

Application of brefeldin A (5µM, 1 h) redistributes staining of the Golgi marker GM 130 into a more punctate distribution compared to to methanol control (red). GM 130 was labeled with a rabbit polyclonal antibody. Actin (green) was labeled with phalloidin.

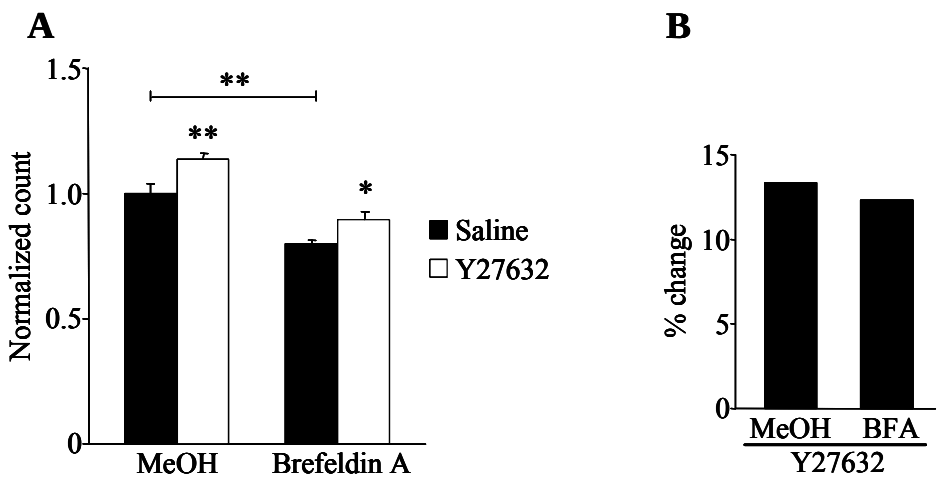


Figure 4. ROCK Negatively Regulates Constitutive Kv1.2 Recycling

(A) Pre-treatment of HEK-K cells with brefeldin A (5 μM, 1 h) significantly reduces steady-state levels of surface Kv1.2 but does not prevent Y27632 (10 μM, 30 min) from eliciting a significant increase of surface Kv1.2 as detected by flow cytometry (n=15; *p<0.05, **p<0.01). (B) The Y27632-induced increase of surface Kv1.2 in the absence or presence of brefeldin A is not significantly different (n=15; p=0.54).

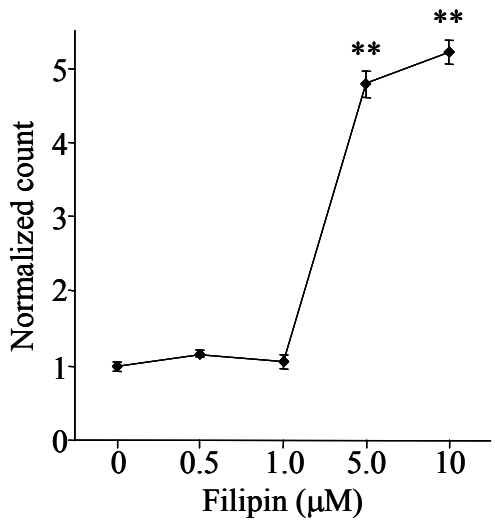


Figure 5. Filipin Dose-response Experiment

Application of increasing concentrations of the sterol-binding agent filipin (1 h) to unstimulated HEK-K cells results in increased levels of steady-state surface Kv1.2 as detected by flow cytometry (n=5, **p<0.001).

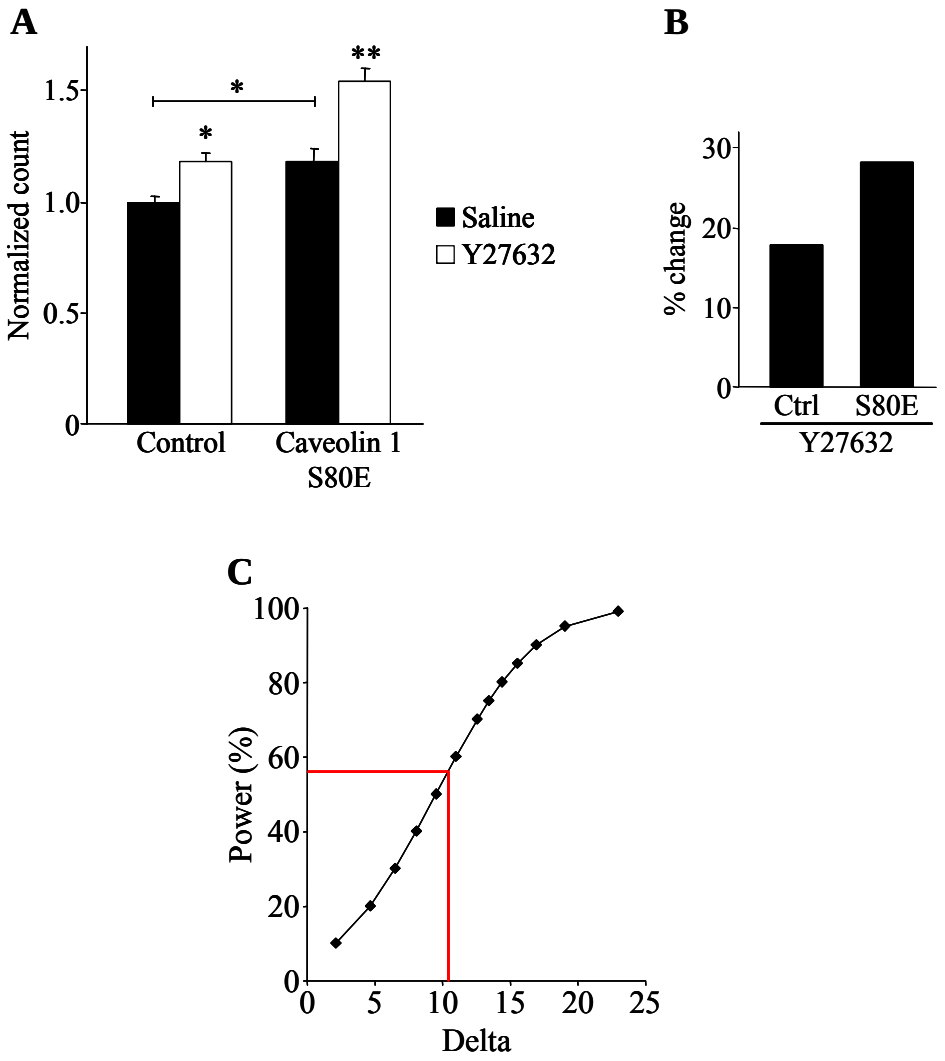


Figure 6. Caveolin Influences Steady-state Kv1.2 Homeostasis

(A) Over-expression of a dominant negative form of caveolin 1 (S80E) elicits a significant increase of steady-state surface Kv1.2. Application of Y27632 (10μM, 30 min) in the absence or presence of caveolin 1 (S80E) elicits a significant increase of surface Kv1.2 as detected by flow cytometry (n=24; *p<0.01, **p<0.001). (B) The trend is toward an enhanced Y27632 effect in the presence of caveolin 1 (S80E) but the increase is not significant (n=24, p=0.12). (C) This experiment has approximately 56% power to detect a difference in the Y27632 effect as large as the one shown in (B). Based on this analysis, a sample size of over 70 is necessary to achieve 95% power.

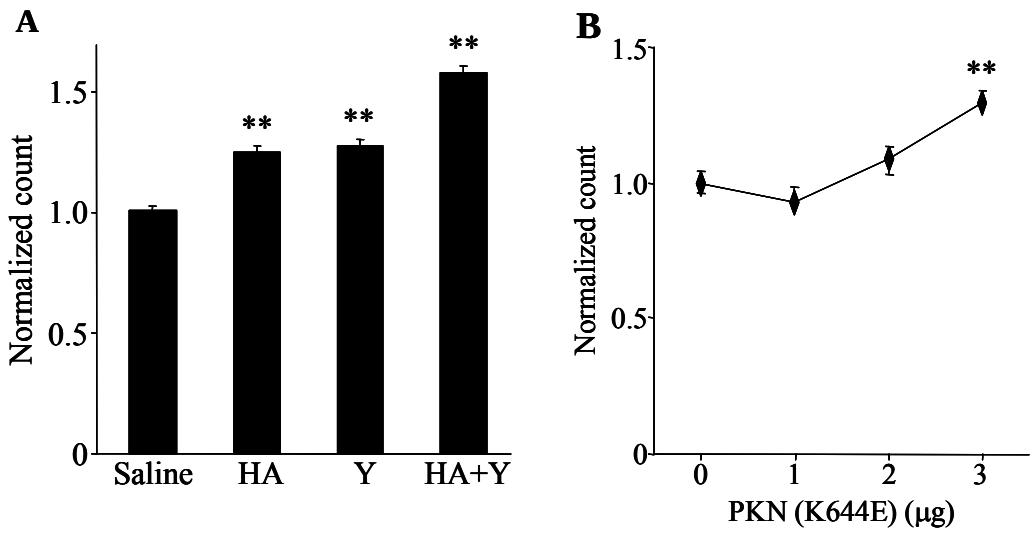


Figure 7. Protein Kinase N Modulates Steady-state Kv1.2 Homeostasis Downstream of RhoA

(A) Application of the PKN inhibitor HA1077 (10 μM, 30 min) to HEK-K cells significantly increases steady-state surface Kv1.2 independent of the effects of the ROCK inhibitor Y27632 (10 μM, 30 min) as detected by flow cytometry (n=53; **p<0.001). (B) Transient transfection of increasing amounts of DNA encoding a dominant negative form of PKN (K644E) significantly increases steady-state surface Kv1.2 in a concentration-dependent manner (n=10; **p<0.01).

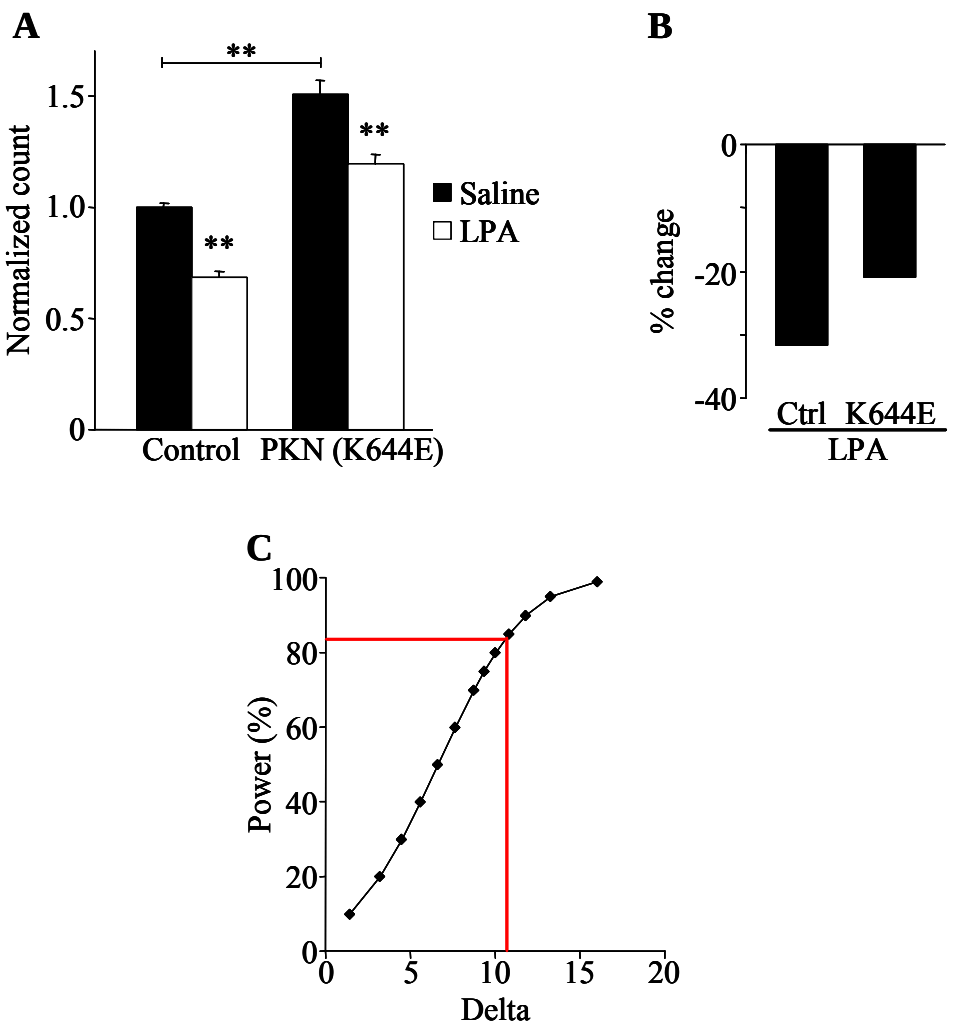


Figure 8. Effect of Dominant Negative Protein Kinase N on LPA-induced Kv1.2 Endocytosis

(A) Over-expression of dominant negative PKN (K644E) in HEK-K cells reveals significantly increased steady-state surface Kv1.2 although LPA-induced endocytosis still occurs as detected by flow cytometry (n=69; **p<0.001). (B) The trend is toward reduced LPA-induced endocytosis of Kv1.2 in the presence of PKN (K644E) but the reduction is not significant (n=69; p=0.98). (C) This experiment has approximately 83% power to detect a difference in LPA-induced Kv1.2 endocytosis as large as the one shown in (B). Based on this analysis, a sample size of over 100 is necessary to achieve 95% power.

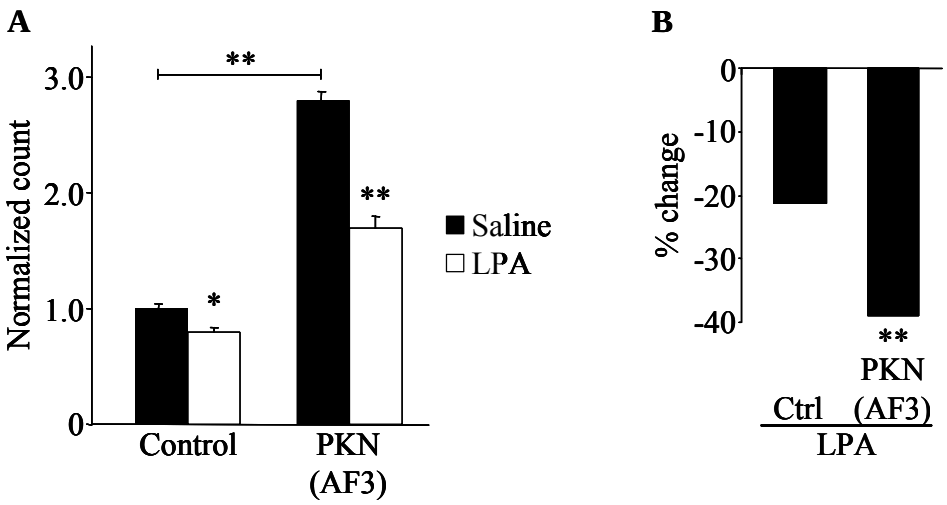


Figure 9. Active Protein Kinase-N Enhances LPA-induced Kv1.2 Endocytosis

(A) Over-expression of constitutively active PKN (AF3) in HEK-K cells results in significantly higher levels of steady-state surface Kv1.2 with LPA-induced Kv1.2 endocytosis occuring regardless of PKN (AF3) over-expression (n=11; *$p<0.01$, **$p<0.001$). (B) LPA-induced Kv1.2 endocytosis is significantly increased in the presence of PKN (AF3) (n=11; **$p<0.001$).

Permissions

Chapter 1: figure 1

Attribution-Noncommercial-Share Alike 3.0 United States

You are free:
To Share — to copy, distribute, display, and perform the work

To Remix — to make derivative works

Under the following conditions:

Attribution. You must attribute the work in the manner specified by the author or licensor (but not in any way that suggests that they endorse you or your use of the work).

Noncommercial. You may not use this work for commercial purposes.

Share Alike. If you alter, transform, or build upon this work, you may distribute the resulting work only under the same or similar license to this one.

For any reuse or distribution, you must make clear to others the license terms of this work. The best way to do this is with a link to this web page.

Any of the above conditions can be waived if you get permission from the copyright holder.

Apart from the remix rights granted under this license, nothing in this license impairs or restricts the author's moral rights.

http://images.google.com/imgres?imgurl=http://www.gregalo.com/action_potential.jpg&imgrefurl=http://www.gregalo.com/neuralnets.html&usg=__qs-rEpYySrgtLSXCPwwt0ClPQrY=&h=732&w=580&sz=90&hl=en&start=2&tbnid=TVTovPO4SNkfiM:&tbnh=141&tbnw=112&prev=/images%3Fq%3Daction%2Bpotential%26gbv%3D2%26hl%3Den%26sa%3DG

Chapter 1: figure 2

From: Roderick MacKinnon [mackinn@mail.rockefeller.edu]
Sent: Mon 11/24/08 12:16 PM
To: Stirling, Lee D
Subject: Re: Image Usage Inquiry
Dear Lee,

You have my permission to use the figures. It is sufficient to cite the papers. Thank you for asking. Good luck!
Sincerely,
Rod MacKinnon

On Nov 24, 2008, at 11:56 AM, Stirling, Lee D wrote:

Dr. MacKinnon,

My name is Lee Stirling. I am writing to respectfully ask for your permission to include images from two of your publications in my Master of Science dissertation. Specifically, the images I am referring to are; figure 1a, 1b from your paper published in 2003 in FEBS Letters entitled: Potassium Channels; and figure 2 from your paper published in 2005 in Science entitled: Crystal structure of a mammalian voltage-dependent Shaker family K+ channel. These are wonderful images illustrating the structure and function of Shaker family potassium channels. Is there any particular way in which I should reference these images other than citing the papers of which they are a part? I thank you in advance for your reply.
Sincerely and respectfully,

Lee Stirling

Department of Pharmacology
University of Vermont
College of Medicine
E-317 Given Building
89 Beaumont Avenue
Burlington, VT 05405-0068
(802) 656-4264

Order Details
MOLECULAR BIOLOGY OF THE CELL
Order detail ID: 24319534
ISBN/ISSN: 9780815332183
Publication Year: 2002
Publisher: GARLAND PUBLISHING, INCORPORATED
Rightsholder: Garland Science - Books
Author/Editor: Bruce Alberts
Permission Status: Granted
Comment: You have permission from our end but you MUST also obtain permission from D.A. Doyle et al., Science 280:69-77,1998 which the figure is adapted from.
Requested use: Dissertation
Republication title: DUAL ROLES FOR RHOA/RHO-KINASE IN THE REGULATED TRAFFICKING OF A VOLTAGE-SENSITIVE POTASSIUM CHANNEL
Republishing organization: THE UNIVERSITY OF VERMONT
Organization status: Not for profit
Republication date: 12/10/2008
Circulation/Distribution: 5
Type of content: Figure, diagram, or table
Description of requested content: K+ specificity of the selectivity filter in a K+ channel
Page range(s): 637
Requested content's publication date: 01/01/2002
Your reference: THESIS, CHAPTER 1

This is a License Agreement between Anthony D Morielli and The American Association for the Advancement of Science ("The American Association for the Advancement of Science"). The license consists of your order details, the terms and conditions provided by The American Association for the Advancement of Science, and the payment terms and conditions.
License Number: 2090321205136
License date: Dec 15, 2008
Licensed content publisher: The American Association for the Advancement of Science
Licensed content publication: Science
Licensed content title: The Structure of the Potassium Channel: Molecular Basis of K Conduction and Selectivity
Licensed content author: Declan A. Doyle, JoJo Morais Cabral, Richard A. Pfuetzner, Anling Kuo, Jacqueline M. Gulbis, Steven L. Cohen, Brian T. Chait, Roderick MacKinnon
Licensed content date: Apr 3, 1998
Type of Use: Thesis
Requestor type: Original Author
Format: Print and electronic
Portion: Figure
Order reference number:
Title of your thesis/dissertation: Dual Roles for RhoA/Rho-kinase In The Regulated Trafficking Of A Voltage-sensitive Potassium Channel
Expected completion date: Dec 2008
Estimated size(pages): 112

Chapter 1: figure 3

This is a License Agreement between Anthony D Morielli ("You") and The American Association for the Advancement of Science ("The American Association for the Advancement of Science"). The license consists of your order details, the terms and conditions provided by The American Association for the Advancement of Science, and the payment terms and conditions.
License Number: 2090340452918
License date: Dec 15, 2008
Licensed content publisher: The American Association for the Advancement of Science
Licensed content publication: Science
Licensed content title: Crystal Structure of a Mammalian Voltage-Dependent Shaker Family K Channel
Licensed content author: Stephen B. Long, Ernest B. Campbell, Roderick MacKinnon
Licensed content date: Aug 5, 2005
Type of Use: Thesis
Requestor type: Original Author
Format: Print and electronic
Portion: Figure
Title of your thesis/dissertation: Dual Roles for RhoA/Rho-kinase In The Regulated Trafficking Of A Voltage-sensitive Potassium Channel
Expected completion date: Dec 2008
Estimated size(pages): 112

From: Bezanilla, Francisco [fbezanilla@peds.bsd.uchicago.edu]
Sent: Mon 11/24/08 11:14 AM To: Stirling, Lee D
Subject: RE: Usage of Image
Dear Lee,
Of course, you can use it. Just quote the web site URL. That would be enough.
Sincerely,
F. Bezanilla

From: Stirling, Lee D [mailto:Lee.Stirling@med.uvm.edu]
Sent: Monday, November 24, 2008 9:47 AM
To: Bezanilla, Francisco
Subject: Usage of Image

Dr. Bezanilla,
My name is Lee Stirling. I am writing to respectfully ask for your permission to include an image from your neuroscience website in my Master of Science dissertation. Specifically, the image I am asking about is the subunit structure of the Shaker K+ channel (http://nerve.bsd.uchicago.edu/KHTML/sub_struct.htm). I have found this image to be one of the best depictions of the trans-membrane portion of Shaker channels. Is there any particular way in which I should reference this image other than using the web address? I thank you in advance for your reply.
Sincerely and respectfully,
Lee Stirling

Department of Pharmacology
UVM College of Medicine
E-317 Given Building
89 Beaumont Avenue
Burlington, VT 05405-0068
(802) 656-4264

Chapter 1: figure 4

This is a License Agreement between Anthony D Morielli ("You") and Nature Publishing Group ("Nature Publishing Group"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.
License Number: 2090331506187
License date: Dec 15, 2008
Licensed content publisher: Nature Publishing Group
Licensed content publication: Nature
Licensed content title: Rho GTPases in cell biology
Licensed content author: Sandrine Etienne-Manneville, Alan Hall
Volume number:
Issue number:
Pages:
Year of publication: 2002
Portion used: Figures / tables
Requestor type: Student
Type of Use: Thesis / Dissertation
Billing Type: Invoice
Company: Anthony D Morielli
Billing Address: University of Vermont E-317 Given Building
89 Beaumont Ave. Burlington, VT 05405
United States

Chapter 1: figure 5

From: Editorial Office [editorial@portlandpress.com] Sent: Fri 12/05/08 07:02 AM
To: Stirling, Lee D
Subject: BST080032 Image Usage Inquiry
Dear Mr Stirling

Thank you for your permissions enquiry. I am pleased to inform you that permission to reproduce the requested material has been granted subject to our terms and conditions (see attached). If you require any further assistance, please feel free to contact me.
Regards,

Lucy White
Pre-Press Specialist

From: Stirling, Lee D [mailto:Lee.Stirling@med.uvm.edu]
Sent: 25 November 2008 19:02
To: Editorial Office
Subject: Image Usage Inquiry

To Whom it May Concern,

My name is Lee Stirling. I am writing to respectfully ask for permission to include an image from one of your publications in my Master of Science dissertation which illustrates ion channel trafficking controlled by RhoA signaling. Specifically, the image I am referring to is figure 1a from the paper published in 2005 in Biochemistry Society Transactions, volume 33(4), pages 649-651 entitled: Function and regulation of RhoE. I have found this image to be one of the best depictions of signaling downstream of RhoA. Is there any particular way in which I should reference this image other than citing the paper? I thank you in advance for your reply.
Sincerely and respectfully,

Lee Stirling

Department of Pharmacology
UVM College of Medicine
E-317 Given Building
89 Beaumont Avenue
Burlington, VT 05405-0068
(802) 656-4264

Cumulative Bibliography

Adamson, P., Marshall, C.J., Hall, A., and Tilbrook, P.A. (1992). Post-translational modifications of p21rho proteins. The Journal of biological chemistry *267*, 20033-20038.

Adda, S., Fleischmann, B.K., Freedman, B.D., Yu, M., Hay, D.W., and Kotlikoff, M.I. (1996). Expression and function of voltage-dependent potassium channel genes in human airway smooth muscle. The Journal of biological chemistry *271*, 13239-13243.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). Molecular Biology of the Cell. Garland Science: New York.

Amano, M., Chihara, K., Nakamura, N., Kaneko, T., Matsuura, Y., and Kaibuchi, K. (1999). The COOH terminus of Rho-kinase negatively regulates rho-kinase activity. The Journal of biological chemistry *274*, 32418-32424.

Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., and Kaibuchi, K. (1996a). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). The Journal of biological chemistry *271*, 20246-20249.

Amano, M., Mukai, H., Ono, Y., Chihara, K., Matsui, T., Hamajima, Y., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996b). Identification of a putative target for Rho as the serine-threonine kinase protein kinase N. Science (New York, N.Y *271*, 648-650.

Aoki, H., Izumo, S., and Sadoshima, J. (1998). Angiotensin II activates RhoA in cardiac myocytes: a critical role of RhoA in angiotensin II-induced premyofibril formation. Circ Res *82*, 666-676.

Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O., and Caroni, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. Nature *393*, 805-809.

Aspenstrom, P. (1999). Effectors for the Rho GTPases. Current opinion in cell biology *11*, 95-102.

Bernard, O. (2007). Lim kinases, regulators of actin dynamics. The international journal of biochemistry & cell biology *39*, 1071-1076.

Berneche, S., and Roux, B. (2001). Energetics of ion conduction through the K+ channel. Nature *414*, 73-77.

Bian, D., Mahanivong, C., Yu, J., Frisch, S.M., Pan, Z.K., Ye, R.D., and Huang, S. (2006). The G12/13-RhoA signaling pathway contributes to efficient lysophosphatidic acid-stimulated cell migration. Oncogene *25*, 2234-2244.

Bishop, A.L., and Hall, A. (2000). Rho GTPases and their effector proteins. The Biochemical journal *348 Pt 2*, 241-255.

Blanchoin, L., Robinson, R.C., Choe, S., and Pollard, T.D. (2000). Phosphorylation of Acanthamoeba actophorin (ADF/cofilin) blocks interaction with actin without a change in atomic structure. Journal of molecular biology *295*, 203-211.

Brew, H.M., Gittelman, J.X., Silverstein, R.S., Hanks, T.D., Demas, V.P., Robinson, L.C., Robbins, C.A., McKee-Johnson, J., Chiu, S.Y., Messing, A., and Tempel, B.L. (2007). Seizures and reduced life span in mice lacking the potassium channel subunit Kv1.2, but hypoexcitability and enlarged Kv1 currents in auditory neurons. Journal of neurophysiology *98*, 1501-1525.

Cachero, T.G., Morielli, A.D., and Peralta, E.G. (1998). The small GTP-binding protein RhoA regulates a delayed rectifier potassium channel. Cell *93*, 1077-1085.

Chakrabarti, P. (1993). Anion binding sites in protein structures. Journal of molecular biology *234*, 463-482.

Chandy, K.G. (1991). Simplified gene nomenclature. Nature *352*, 26.

Chang, W.J., Rothberg, K.G., Kamen, B.A., and Anderson, R.G. (1992). Lowering the cholesterol content of MA104 cells inhibits receptor-mediated transport of folate. The Journal of cell biology *118*, 63-69.

Chen, C.Y., Wong, E.I., Vidali, L., Estavillo, A., Hepler, P.K., Wu, H.M., and Cheung, A.Y. (2002). The regulation of actin organization by actin-depolymerizing factor in elongating pollen tubes. The Plant cell *14*, 2175-2190.

Cherfils, J., and Chardin, P. (1999). GEFs: structural basis for their activation of small GTP-binding proteins. Trends in biochemical sciences *24*, 306-311.

Chihara, K., Amano, M., Nakamura, N., Yano, T., Shibata, M., Tokui, T., Ichikawa, H., Ikebe, R., Ikebe, M., and Kaibuchi, K. (1997). Cytoskeletal rearrangements and transcriptional activation of c-fos serum response element by Rho-kinase. The Journal of biological chemistry *272*, 25121-25127.

Choe, S. (2002). Potassium channel structures. Nat Rev Neurosci *3*, 115-121.

Connors, E.C., Ballif, B.A., and Morielli, A.D. (2008). Homeostatic regulation of Kv1.2 potassium channel trafficking by cyclic AMP. The Journal of biological chemistry *283*, 3445-3453.

Cope, M.J., Yang, S., Shang, C., and Drubin, D.G. (1999). Novel protein kinases Ark1p and Prk1p associate with and regulate the cortical actin cytoskeleton in budding yeast. The Journal of cell biology *144*, 1203-1218.

Copley, R.R., and Barton, G.J. (1994). A structural analysis of phosphate and sulphate binding sites in proteins. Estimation of propensities for binding and conservation of phosphate binding sites. Journal of molecular biology *242*, 321-329.

Cox, A.D., and Der, C.J. (1992). Protein prenylation: more than just glue? Current opinion in cell biology *4*, 1008-1016.

Cox, D., Chang, P., Zhang, Q., Reddy, P.G., Bokoch, G.M., and Greenberg, S. (1997). Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. The Journal of experimental medicine *186*, 1487-1494.

Cox, R.H., Folander, K., and Swanson, R. (2001). Differential expression of voltage-gated K(+) channel genes in arteries from spontaneously hypertensive and Wistar-Kyoto rats. Hypertension *37*, 1315-1322.

Dada, L.A., Novoa, E., Lecuona, E., Sun, H., and Sznajder, J.I. (2007). Role of the small GTPase RhoA in the hypoxia-induced decrease of plasma membrane Na,K-ATPase in A549 cells. Journal of cell science *120*, 2214-2222.

Dan, C., Kelly, A., Bernard, O., and Minden, A. (2001). Cytoskeletal changes regulated by the PAK4 serine/threonine kinase are mediated by LIM kinase 1 and cofilin. The Journal of biological chemistry *276*, 32115-32121.

Danino, D., Moon, K.H., and Hinshaw, J.E. (2004). Rapid constriction of lipid bilayers by the mechanochemical enzyme dynamin. Journal of structural biology *147*, 259-267.
Dash, P.K., Orsi, S.A., Moody, M., and Moore, A.N. (2004). A role for hippocampal Rho-ROCK pathway in long-term spatial memory. Biochemical and biophysical research communications *322*, 893-898.

Davies, S.P., Reddy, H., Caivano, M., and Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. The Biochemical journal *351*, 95-105.

De Camilli, P., Takei, K., and McPherson, P.S. (1995). The function of dynamin in endocytosis. Current opinion in neurobiology *5*, 559-565.

Dodson, P.D., Billups, B., Rusznak, Z., Szucs, G., Barker, M.C., and Forsythe, I.D. (2003). Presynaptic rat Kv1.2 channels suppress synaptic terminal hyperexcitability following action potential invasion. The Journal of physiology *550*, 27-33.

Dong, J.M., Leung, T., Manser, E., and Lim, L. (1998). cAMP-induced morphological changes are counteracted by the activated RhoA small GTPase and the Rho kinase ROKalpha. The Journal of biological chemistry *273*, 22554-22562.

Donovan, F.M., Pike, C.J., Cotman, C.W., and Cunningham, D.D. (1997). Thrombin induces apoptosis in cultured neurons and astrocytes via a pathway requiring tyrosine kinase and RhoA activities. J Neurosci *17*, 5316-5326.

Doyle, D.A., Morais Cabral, J., Pfuetzner, R.A., Kuo, A., Gulbis, J.M., Cohen, S.L., Chait, B.T., and MacKinnon, R. (1998). The structure of the potassium channel: molecular basis of K+ conduction and selectivity. Science (New York, N.Y *280*, 69-77.

Drechsel, D.N., Hyman, A.A., Hall, A., and Glotzer, M. (1997). A requirement for Rho and Cdc42 during cytokinesis in Xenopus embryos. Curr Biol *7*, 12-23.

Duncan, M.C., and Payne, G.S. (2005). An endocytic Prk-ing brake. Nature cell biology *7*, 210-212.

Dunn, W.A., and Hubbard, A.L. (1984). Receptor-mediated endocytosis of epidermal growth factor by hepatocytes in the perfused rat liver: ligand and receptor dynamics. The Journal of cell biology *98*, 2148-2159.

Edwards, D.C., and Gill, G.N. (1999). Structural features of LIM kinase that control effects on the actin cytoskeleton. The Journal of biological chemistry *274*, 11352-11361.

Edwards, D.C., Sanders, L.C., Bokoch, G.M., and Gill, G.N. (1999). Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nature cell biology *1*, 253-259.

Eldstrom, J., Van Wagoner, D.R., Moore, E.D., and Fedida, D. (2006). Localization of Kv1.5 channels in rat and canine myocyte sarcolemma. FEBS letters *580*, 6039-6046.

Etienne-Manneville, S., and Hall, A. (2002). Rho GTPases in cell biology. Nature *420*, 629-635.

Fadool, D.A., Holmes, T.C., Berman, K., Dagan, D., and Levitan, I.B. (1997). Tyrosine phosphorylation modulates current amplitude and kinetics of a neuronal voltage-gated potassium channel. J Neurophysiol *78*, 1563-1573.

Feng, J., Ito, M., Ichikawa, K., Isaka, N., Nishikawa, M., Hartshorne, D.J., and Nakano, T. (1999). Inhibitory phosphorylation site for Rho-associated kinase on smooth muscle myosin phosphatase. The Journal of biological chemistry *274*, 37385-37390.

Fleming, I.N., Elliott, C.M., and Exton, J.H. (1996). Differential translocation of rho family GTPases by lysophosphatidic acid, endothelin-1, and platelet-derived growth factor. The Journal of biological chemistry *271*, 33067-33073.

Flynn, P., Mellor, H., Palmer, R., Panayotou, G., and Parker, P.J. (1998). Multiple interactions of PRK1 with RhoA. Functional assignment of the Hr1 repeat motif. The Journal of biological chemistry *273*, 2698-2705.

Forget, M.A., Desrosiers, R.R., Gingras, D., and Beliveau, R. (2002). Phosphorylation states of Cdc42 and RhoA regulate their interactions with Rho GDP dissociation inhibitor and their extraction from biological membranes. The Biochemical journal *361*, 243-254.

Fromm, C., Coso, O.A., Montaner, S., Xu, N., and Gutkind, J.S. (1997). The small GTP-binding protein Rho links G protein-coupled receptors and Galpha12 to the serum response element and to cellular transformation. Proceedings of the National Academy of Sciences of the United States of America *94*, 10098-10103.

Fukata, Y., Oshiro, N., Kinoshita, N., Kawano, Y., Matsuoka, Y., Bennett, V., Matsuura, Y., and Kaibuchi, K. (1999). Phosphorylation of adducin by Rho-kinase plays a crucial role in cell motility. The Journal of cell biology *145*, 347-361.

Gampel, A., Parker, P.J., and Mellor, H. (1999). Regulation of epidermal growth factor receptor traffic by the small GTPase rhoB. Curr Biol *9*, 955-958.

Gong, M.C., Fujihara, H., Somlyo, A.V., and Somlyo, A.P. (1997). Translocation of rhoA associated with Ca2+ sensitization of smooth muscle. The Journal of biological chemistry *272*, 10704-10709.

Gosser, Y.Q., Nomanbhoy, T.K., Aghazadeh, B., Manor, D., Combs, C., Cerione, R.A., and Rosen, M.K. (1997). C-terminal binding domain of Rho GDP-dissociation inhibitor directs N-terminal inhibitory peptide to GTPases. Nature *387*, 814-819.

Grande-Garcia, A., Echarri, A., de Rooij, J., Alderson, N.B., Waterman-Storer, C.M., Valdivielso, J.M., and del Pozo, M.A. (2007). Caveolin-1 regulates cell polarization and directional migration through Src kinase and Rho GTPases. The Journal of cell biology *177*, 683-694.

Grosse, G., Draguhn, A., Hohne, L., Tapp, R., Veh, R.W., and Ahnert-Hilger, G. (2000). Expression of Kv1 potassium channels in mouse hippocampal primary cultures: development and activity-dependent regulation. J Neurosci *20*, 1869-1882.

Grottesi, A., Sands, Z.A., and Sansom, M.S. (2005). Potassium channels: complete and undistorted. Curr Biol *15*, R771-774.

Haghdoust, H., Janahmadi, M., and Behzadi, G. (2007). Physiological role of dendrotoxin-sensitive K+ channels in the rat cerebellar Purkinje neurons. Physiological research / Academia Scientiarum Bohemoslovaca *56*, 807-813.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. Science (New York, N.Y *279*, 509-514.

Hart, M.J., Eva, A., Zangrilli, D., Aaronson, S.A., Evans, T., Cerione, R.A., and Zheng, Y. (1994). Cellular transformation and guanine nucleotide exchange activity are catalyzed by a common domain on the dbl oncogene product. The Journal of biological chemistry *269*, 62-65.

Hart, M.J., Jiang, X., Kozasa, T., Roscoe, W., Singer, W.D., Gilman, A.G., Sternweis, P.C., and Bollag, G. (1998). Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Galpha13. Science (New York, N.Y *280*, 2112-2114.

Hattan, D., Nesti, E., Cachero, T.G., and Morielli, A.D. (2002). Tyrosine phosphorylation of Kv1.2 modulates its interaction with the actin-binding protein cortactin. The Journal of biological chemistry *277*, 38596-38606.

Hattori, T., Shimokawa, H., Higashi, M., Hiroki, J., Mukai, Y., Tsutsui, H., Kaibuchi, K., and Takeshita, A. (2004). Long-term inhibition of Rho-kinase suppresses left ventricular remodeling after myocardial infarction in mice. Circulation *109*, 2234-2239.

Hilal-Dandan, R., Means, C.K., Gustafsson, A.B., Morissette, M.R., Adams, J.W., Brunton, L.L., and Heller Brown, J. (2004). Lysophosphatidic acid induces hypertrophy of neonatal cardiac myocytes via activation of Gi and Rho. J Mol Cell Cardiol *36*, 481-493.

Hinrichsen, L., Harborth, J., Andrees, L., Weber, K., and Ungewickell, E.J. (2003). Effect of clathrin heavy chain- and alpha-adaptin-specific small inhibitory RNAs on endocytic accessory proteins and receptor trafficking in HeLa cells. The Journal of biological chemistry *278*, 45160-45170.

Hoffman, G.R., Nassar, N., and Cerione, R.A. (2000). Structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI. Cell *100*, 345-356.

Hol, W.G. (1985). The role of the alpha-helix dipole in protein function and structure. Progress in biophysics and molecular biology *45*, 149-195.

Holmes, T.C., Fadool, D.A., and Levitan, I.B. (1996). Tyrosine phosphorylation of the Kv1.3 potassium channel. J Neurosci *16*, 1581-1590.

Hong, Z., Weir, E.K., Nelson, D.P., and Olschewski, A. (2004). Subacute hypoxia decreases voltage-activated potassium channel expression and function in pulmonary artery myocytes. American journal of respiratory cell and molecular biology *31*, 337-343.

Huang, B., Zeng, G., Ng, A.Y., and Cai, M. (2003). Identification of novel recognition motifs and regulatory targets for the yeast actin-regulating kinase Prk1p. Molecular biology of the cell *14*, 4871-4884.

Huang, C., Ni, Y., Wang, T., Gao, Y., Haudenschild, C.C., and Zhan, X. (1997). Down-regulation of the filamentous actin cross-linking activity of cortactin by Src-mediated tyrosine phosphorylation. The Journal of biological chemistry *272*, 13911-13915.

Huang, X.Y., Morielli, A.D., and Peralta, E.G. (1993). Tyrosine kinase-dependent suppression of a potassium channel by the G protein-coupled m1 muscarinic acetylcholine receptor. Cell *75*, 1145-1156.

Huang, X.Y., Morielli, A.D., and Peralta, E.G. (1994). Molecular basis of cardiac potassium channel stimulation by protein kinase A. Proceedings of the National Academy of Sciences of the United States of America *91*, 624-628.

Ichetovkin, I., Grant, W., and Condeelis, J. (2002). Cofilin produces newly polymerized actin filaments that are preferred for dendritic nucleation by the Arp2/3 complex. Curr Biol *12*, 79-84.

Ihara, K., Muraguchi, S., Kato, M., Shimizu, T., Shirakawa, M., Kuroda, S., Kaibuchi, K., and Hakoshima, T. (1998). Crystal structure of human RhoA in a dominantly active form complexed with a GTP analogue. The Journal of biological chemistry *273*, 9656-9666.

Imamura, F., Horai, T., Mukai, M., Shinkai, K., Sawada, M., and Akedo, H. (1993). Induction of in vitro tumor cell invasion of cellular monolayers by lysophosphatidic acid or phospholipase D. Biochemical and biophysical research communications *193*, 497-503.

Ishikawa, K., Tanaka, M., Black, J.A., and Waxman, S.G. (1999). Changes in expression of voltage-gated potassium channels in dorsal root ganglion neurons following axotomy. Muscle & nerve *22*, 502-507.

Ishizaki, T., Maekawa, M., Fujisawa, K., Okawa, K., Iwamatsu, A., Fujita, A., Watanabe, N., Saito, Y., Kakizuka, A., Morii, N., and Narumiya, S. (1996). The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. The EMBO journal *15*, 1885-1893.

Jan, L.Y., and Jan, Y.N. (1997). Cloned potassium channels from eukaryotes and prokaryotes. Annu Rev Neurosci *20*, 91-123.

Johnson, L.N., and O'Reilly, M. (1996). Control by phosphorylation. Current opinion in structural biology *6*, 762-769.

Kaibuchi, K., Kuroda, S., and Amano, M. (1999). Regulation of the cytoskeleton and cell adhesion by the Rho family GTPases in mammalian cells. Annu Rev Biochem *68*, 459-486.

Kamb, A., Iverson, L.E., and Tanouye, M.A. (1987). Molecular characterization of Shaker, a Drosophila gene that encodes a potassium channel. Cell *50*, 405-413.

Kandel, E.R., Schwartz, J.H., and Jessell, T.M. (2000). Principles of Neural Science. McGraw-Hill.

Kaneko, T., Maeda, A., Takefuji, M., Aoyama, H., Nakayama, M., Kawabata, S., Kawano, Y., Iwamatsu, A., Amano, M., and Kaibuchi, K. (2005). Rho mediates endocytosis of epidermal growth factor receptor through phosphorylation of endophilin A1 by Rho-kinase. Genes Cells *10*, 973-987.

Keep, N.H., Barnes, M., Barsukov, I., Badii, R., Lian, L.Y., Segal, A.W., Moody, P.C., and Roberts, G.C. (1997). A modulator of rho family G proteins, rhoGDI, binds these G proteins via an immunoglobulin-like domain and a flexible N-terminal arm. Structure *5*, 623-633.

Keller, J., Schmidt, M., Hussein, B., Rumenapp, U., and Jakobs, K.H. (1997). Muscarinic receptor-stimulated cytosol-membrane translocation of RhoA. FEBS letters *403*, 299-302.

Kim, D.S., Choi, J.O., Rim, H.D., and Cho, H.J. (2002). Downregulation of voltage-gated potassium channel alpha gene expression in dorsal root ganglia following chronic constriction injury of the rat sciatic nerve. Brain research *105*, 146-152.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science (New York, N.Y *273*, 245-248.

Kjoller, L., and Hall, A. (1999). Signaling to Rho GTPases. Exp Cell Res *253*, 166-179.

Kobayashi, N., Horinaka, S., Mita, S., Nakano, S., Honda, T., Yoshida, K., Kobayashi, T., and Matsuoka, H. (2002). Critical role of Rho-kinase pathway for cardiac performance and remodeling in failing rat hearts. Cardiovasc Res *55*, 757-767.

Kozasa, T., Jiang, X., Hart, M.J., Sternweis, P.M., Singer, W.D., Gilman, A.G., Bollag, G., and Sternweis, P.C. (1998). p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13. Science (New York, N.Y *280*, 2109-2111.

Kwik, J., Boyle, S., Fooksman, D., Margolis, L., Sheetz, M.P., and Edidin, M. (2003). Membrane cholesterol, lateral mobility, and the phosphatidylinositol 4,5-bisphosphate-dependent organization of cell actin. Proceedings of the National Academy of Sciences of the United States of America *100*, 13964-13969.

Lai, F.P., Szczodrak, M., Block, J., Faix, J., Breitsprecher, D., Mannherz, H.G., Stradal, T.E., Dunn, G.A., Small, J.V., and Rottner, K. (2008). Arp2/3 complex interactions and actin network turnover in lamellipodia. The EMBO journal *27*, 982-992.

Lajoie, P., and Nabi, I.R. (2007). Regulation of raft-dependent endocytosis. Journal of cellular and molecular medicine *11*, 644-653.

Lamarche, N., and Hall, A. (1994). GAPs for rho-related GTPases. Trends Genet *10*, 436-440.

Lamaze, C., Chuang, T.H., Terlecky, L.J., Bokoch, G.M., and Schmid, S.L. (1996). Regulation of receptor-mediated endocytosis by Rho and Rac. Nature *382*, 177-179.

Lambe, E.K., and Aghajanian, G.K. (2001). The role of Kv1.2-containing potassium channels in serotonin-induced glutamate release from thalamocortical terminals in rat frontal cortex. J Neurosci *21*, 9955-9963.

Laube, G., Roper, J., Pitt, J.C., Sewing, S., Kistner, U., Garner, C.C., Pongs, O., and Veh, R.W. (1996). Ultrastructural localization of Shaker-related potassium channel subunits and synapse-associated protein 90 to septate-like junctions in rat cerebellar Pinceaux. Brain research *42*, 51-61.

Le, P.U., Guay, G., Altschuler, Y., and Nabi, I.R. (2002). Caveolin-1 is a negative regulator of caveolae-mediated endocytosis to the endoplasmic reticulum. The Journal of biological chemistry *277*, 3371-3379.

Le, P.U., and Nabi, I.R. (2003). Distinct caveolae-mediated endocytic pathways target the Golgi apparatus and the endoplasmic reticulum. Journal of cell science *116*, 1059-1071.

Leterrier, C., Bonnard, D., Carrel, D., Rossier, J., and Lenkei, Z. (2004). Constitutive endocytic cycle of the CB1 cannabinoid receptor. The Journal of biological chemistry *279*, 36013-36021.

Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J.M., Plowman, G.D., Rudy, B., and Schlessinger, J. (1995). Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. Nature *376*, 737-745.

Long, S.B., Campbell, E.B., and Mackinnon, R. (2005). Crystal structure of a mammalian voltage-dependent Shaker family K+ channel. Science (New York, N.Y *309*, 897-903.

Longenecker, K., Read, P., Derewenda, U., Dauter, Z., Liu, X., Garrard, S., Walker, L., Somlyo, A.V., Nakamoto, R.K., Somlyo, A.P., and Derewenda, Z.S. (1999). How RhoGDI binds Rho. Acta crystallographica *55*, 1503-1515.

Lozinskaya, I.M., and Cox, R.H. (1997). Effects of age on Ca2+ currents in small mesenteric artery myocytes from Wistar-Kyoto and spontaneously hypertensive rats. Hypertension *29*, 1329-1336.

Luo, L., Jan, L.Y., and Jan, Y.N. (1997). Rho family GTP-binding proteins in growth cone signalling. Current opinion in neurobiology *7*, 81-86.

Luykenaar, K.D., Brett, S.E., Wu, B.N., Wiehler, W.B., and Welsh, D.G. (2004). Pyrimidine nucleotides suppress KDR currents and depolarize rat cerebral arteries by activating Rho kinase. Am J Physiol Heart Circ Physiol *286*, H1088-1100.

Macia, E., Ehrlich, M., Massol, R., Boucrot, E., Brunner, C., and Kirchhausen, T. (2006). Dynasore, a cell-permeable inhibitor of dynamin. Developmental cell *10*, 839-850.

Maciver, S.K., and Hussey, P.J. (2002). The ADF/cofilin family: actin-remodeling proteins. Genome biology *3*, reviews3007.

MacKinnon, R. (1995). Pore loops: an emerging theme in ion channel structure. Neuron *14*, 889-892.

MacKinnon, R. (2003). Potassium channels. FEBS letters *555*, 62-65.

Maesaki, R., Ihara, K., Shimizu, T., Kuroda, S., Kaibuchi, K., and Hakoshima, T. (1999). The structural basis of Rho effector recognition revealed by the crystal structure of human RhoA complexed with the effector domain of PKN/PRK1. Mol Cell *4*, 793-803.

Martens, J.R., Sakamoto, N., Sullivan, S.A., Grobaski, T.D., and Tamkun, M.M. (2001). Isoform-specific localization of voltage-gated K+ channels to distinct lipid raft populations. Targeting of Kv1.5 to caveolae. The Journal of biological chemistry *276*, 8409-8414.

Martinez-Marmol, R., Villalonga, N., Sole, L., Vicente, R., Tamkun, M.M., Soler, C., and Felipe, A. (2008). Multiple Kv1.5 targeting to membrane surface microdomains. Journal of cellular physiology *217*, 667-673.

Mason, H.S., Latten, M.J., Godoy, L.D., Horowitz, B., and Kenyon, J.L. (2002). Modulation of Kv1.5 currents by protein kinase A, tyrosine kinase, and protein tyrosine phosphatase requires an intact cytoskeleton. Molecular pharmacology *61*, 285-293.

Matsui, S., Matsumoto, S., Adachi, R., Kusui, K., Hirayama, A., Watanabe, H., Ohashi, K., Mizuno, K., Yamaguchi, T., Kasahara, T., and Suzuki, K. (2002). LIM kinase 1 modulates opsonized zymosan-triggered activation of macrophage-like U937 cells. Possible involvement of phosphorylation of cofilin and reorganization of actin cytoskeleton. The Journal of biological chemistry *277*, 544-549.

Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996). Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. The EMBO journal *15*, 2208-2216.

Matsuzawa, K., Kosako, H., Inagaki, N., Shibata, H., Mukai, H., Ono, Y., Amano, M., Kaibuchi, K., Matsuura, Y., Azuma, I., and Inagaki, M. (1997). Domain-specific phosphorylation of vimentin and glial fibrillary acidic protein by PKN. Biochemical and biophysical research communications *234*, 621-625.

Mayor, S., and Pagano, R.E. (2007). Pathways of clathrin-independent endocytosis. Nature reviews *8*, 603-612.

McEwen, D.P., Li, Q., Jackson, S., Jenkins, P.M., and Martens, J.R. (2008). Caveolin regulates kv1.5 trafficking to cholesterol-rich membrane microdomains. Molecular pharmacology *73*, 678-685.

Meng, Y., Zhang, Y., Tregoubov, V., Janus, C., Cruz, L., Jackson, M., Lu, W.Y., MacDonald, J.F., Wang, J.Y., Falls, D.L., and Jia, Z. (2002). Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. Neuron *35*, 121-133.

Minshall, R.D., Tiruppathi, C., Vogel, S.M., Niles, W.D., Gilchrist, A., Hamm, H.E., and Malik, A.B. (2000). Endothelial cell-surface gp60 activates vesicle formation and trafficking via G(i)-coupled Src kinase signaling pathway. The Journal of cell biology *150*, 1057-1070.

Moolenaar, W.H. (1995). Lysophosphatidic acid signalling. Current opinion in cell biology *7*, 203-210.

Morais-Cabral, J.H., Zhou, Y., and MacKinnon, R. (2001). Energetic optimization of ion conduction rate by the K+ selectivity filter. Nature *414*, 37-42.

Moriki, N., Ito, M., Seko, T., Kureishi, Y., Okamoto, R., Nakakuki, T., Kongo, M., Isaka, N., Kaibuchi, K., and Nakano, T. (2004). RhoA activation in vascular smooth muscle cells from stroke-prone spontaneously hypertensive rats. Hypertens Res *27*, 263-270.

Morrison, K.J., Moore, R.H., Carsrud, N.D., Trial, J., Millman, E.E., Tuvim, M., Clark, R.B., Barber, R., Dickey, B.F., and Knoll, B.J. (1996). Repetitive endocytosis and recycling of the beta 2-adrenergic receptor during agonist-induced steady state redistribution. Molecular pharmacology *50*, 692-699.

Mukai, H., Toshimori, M., Shibata, H., Kitagawa, M., Shimakawa, M., Miyahara, M., Sunakawa, H., and Ono, Y. (1996). PKN associates and phosphorylates the head-rod domain of neurofilament protein. The Journal of biological chemistry *271*, 9816-9822.

Muro, S., Wiewrodt, R., Thomas, A., Koniaris, L., Albelda, S.M., Muzykantov, V.R., and Koval, M. (2003). A novel endocytic pathway induced by clustering endothelial ICAM-1 or PECAM-1. Journal of cell science *116*, 1599-1609.

Nakamura, A., Hayashi, K., Ozawa, Y., Fujiwara, K., Okubo, K., Kanda, T., Wakino, S., and Saruta, T. (2003). Vessel- and vasoconstrictor-dependent role of rho/rho-kinase in renal microvascular tone. Journal of vascular research *40*, 244-251.

Narumiya, S., Ishizaki, T., and Watanabe, N. (1997). Rho effectors and reorganization of actin cytoskeleton. FEBS letters *410*, 68-72.

Nesti, E., Everill, B., and Morielli, A.D. (2004). Endocytosis as a mechanism for tyrosine kinase-dependent suppression of a voltage-gated potassium channel. Molecular biology of the cell *15*, 4073-4088.

Niggli, V. (1999). Rho-kinase in human neutrophils: a role in signalling for myosin light chain phosphorylation and cell migration. FEBS letters *445*, 69-72.

Nishida, M., Tanabe, S., Maruyama, Y., Mangmool, S., Urayama, K., Nagamatsu, Y., Takagahara, S., Turner, J.H., Kozasa, T., Kobayashi, H., Sato, Y., Kawanishi, T., Inoue, R., Nagao, T., and Kurose, H. (2005). G alpha 12/13- and reactive oxygen species-dependent activation of c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase by angiotensin receptor stimulation in rat neonatal cardiomyocytes. The Journal of biological chemistry *280*, 18434-18441.

Nishimura, Y., Itoh, K., Yoshioka, K., Tokuda, K., and Himeno, M. (2003). Overexpression of ROCK in human breast cancer cells: evidence that ROCK activity mediates intracellular membrane traffic of lysosomes. Pathol Oncol Res *9*, 83-95.

Nishimura, Y., Yoshioka, K., Bernard, O., Himeno, M., and Itoh, K. (2004). LIM kinase 1: evidence for a role in the regulation of intracellular vesicle trafficking of lysosomes and endosomes in human breast cancer cells. European journal of cell biology *83*, 369-380.

Nobes, C.D., and Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. The Journal of cell biology *144*, 1235-1244.

Noda, M., Yasuda-Fukazawa, C., Moriishi, K., Kato, T., Okuda, T., Kurokawa, K., and Takuwa, Y. (1995). Involvement of rho in GTP gamma S-induced enhancement of phosphorylation of 20 kDa myosin light chain in vascular smooth muscle cells: inhibition of phosphatase activity. FEBS letters *367*, 246-250.

O'Connell, K.M., and Tamkun, M.M. (2005). Targeting of voltage-gated potassium channel isoforms to distinct cell surface microdomains. Journal of cell science *118*, 2155-2166.

Ohashi, K., Nagata, K., Maekawa, M., Ishizaki, T., Narumiya, S., and Mizuno, K. (2000). Rho-associated kinase ROCK activates LIM-kinase 1 by phosphorylation at threonine 508 within the activation loop. The Journal of biological chemistry *275*, 3577-3582.

Olofsson, B. (1999). Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. Cellular signalling *11*, 545-554.

Pan, Z.K., Ye, R.D., Christiansen, S.C., Jagels, M.A., Bokoch, G.M., and Zuraw, B.L. (1998). Role of the Rho GTPase in bradykinin-stimulated nuclear factor-kappaB activation and IL-1beta gene expression in cultured human epithelial cells. J Immunol *160*, 3038-3045.

Pan, Z.K., Zuraw, B.L., Lung, C.C., Prossnitz, E.R., Browning, D.D., and Ye, R.D. (1996). Bradykinin stimulates NF-kappaB activation and interleukin 1beta gene expression in cultured human fibroblasts. J Clin Invest *98*, 2042-2049.

Papazian, D.M., Shao, X.M., Seoh, S.A., Mock, A.F., Huang, Y., and Wainstock, D.H. (1995). Electrostatic interactions of S4 voltage sensor in Shaker K+ channel. Neuron *14*, 1293-1301.

Papazian, D.M., Timpe, L.C., Jan, Y.N., and Jan, L.Y. (1991). Alteration of voltage-dependence of Shaker potassium channel by mutations in the S4 sequence. Nature *349*, 305-310.

Paterson, H.F., Self, A.J., Garrett, M.D., Just, I., Aktories, K., and Hall, A. (1990). Microinjection of recombinant p21rho induces rapid changes in cell morphology. The Journal of cell biology *111*, 1001-1007.

Pearson, R.B., and Kemp, B.E. (1991). Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. Methods Enzymol *200*, 62-81.

Pelkmans, L., Kartenbeck, J., and Helenius, A. (2001). Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. Nature cell biology *3*, 473-483.

Perona, R., Montaner, S., Saniger, L., Sanchez-Perez, I., Bravo, R., and Lacal, J.C. (1997). Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. Genes Dev *11*, 463-475.

Prokopenko, S.N., Saint, R., and Bellen, H.J. (2000). Untying the Gordian knot of cytokinesis. Role of small G proteins and their regulators. The Journal of cell biology *148*, 843-848.

Quilliam, L.A., Lambert, Q.T., Mickelson-Young, L.A., Westwick, J.K., Sparks, A.B., Kay, B.K., Jenkins, N.A., Gilbert, D.J., Copeland, N.G., and Der, C.J. (1996). Isolation of a NCK-associated kinase, PRK2, an SH3-binding protein and potential effector of Rho protein signaling. The Journal of biological chemistry *271*, 28772-28776.

Rameh, L.E., Arvidsson, A., Carraway, K.L., 3rd, Couvillon, A.D., Rathbun, G., Crompton, A., VanRenterghem, B., Czech, M.P., Ravichandran, K.S., Burakoff, S.J., Wang, D.S., Chen, C.S., and Cantley, L.C. (1997). A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. The Journal of biological chemistry *272*, 22059-22066.

Rasband, M.N. (2004). It's "juxta" potassium channel! Journal of neuroscience research *76*, 749-757.

Rasband, M.N., Park, E.W., Vanderah, T.W., Lai, J., Porreca, F., and Trimmer, J.S. (2001). Distinct potassium channels on pain-sensing neurons. Proceedings of the National Academy of Sciences of the United States of America *98*, 13373-13378.

Rasband, M.N., and Trimmer, J.S. (2001). Subunit composition and novel localization of K+ channels in spinal cord. The Journal of comparative neurology *429*, 166-176.

Rhodes, K.J., Keilbaugh, S.A., Barrezueta, N.X., Lopez, K.L., and Trimmer, J.S. (1995). Association and colocalization of K+ channel alpha- and beta-subunit polypeptides in rat brain. J Neurosci *15*, 5360-5371.

Ridley, A.J., and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell *70*, 389-399.

Riento, K., and Ridley, A.J. (2003). Rocks: multifunctional kinases in cell behaviour. Nature reviews *4*, 446-456.

Riobo, N.A., and Manning, D.R. (2005). Receptors coupled to heterotrimeric G proteins of the G12 family. Trends Pharmacol Sci *26*, 146-154.

Rittinger, K., Walker, P.A., Eccleston, J.F., Nurmahomed, K., Owen, D., Laue, E., Gamblin, S.J., and Smerdon, S.J. (1997a). Crystal structure of a small G protein in complex with the GTPase-activating protein rhoGAP. Nature *388*, 693-697.

Rittinger, K., Walker, P.A., Eccleston, J.F., Smerdon, S.J., and Gamblin, S.J. (1997b). Structure at 1.65 A of RhoA and its GTPase-activating protein in complex with a transition-state analogue. Nature *389*, 758-762.

Roberds, S.L., and Tamkun, M.M. (1991). Cloning and tissue-specific expression of five voltage-gated potassium channel cDNAs expressed in rat heart. Proceedings of the National Academy of Sciences of the United States of America *88*, 1798-1802.

Rothberg, K.G., Heuser, J.E., Donzell, W.C., Ying, Y.S., Glenney, J.R., and Anderson, R.G. (1992). Caveolin, a protein component of caveolae membrane coats. Cell *68*, 673-682.

Rotin, D., Kanelis, V., and Schild, L. (2001). Trafficking and cell surface stability of ENaC. American journal of physiology *281*, F391-399.

Rudolph, M.G., Wittinghofer, A., and Vetter, I.R. (1999). Nucleotide binding to the G12V-mutant of Cdc42 investigated by X-ray diffraction and fluorescence spectroscopy: two different nucleotide states in one crystal. Protein Sci *8*, 778-787.

Sahai, E., Alberts, A.S., and Treisman, R. (1998). RhoA effector mutants reveal distinct effector pathways for cytoskeletal reorganization, SRF activation and transformation. The EMBO journal *17*, 1350-1361.

Samaniego, R., Sanchez-Martin, L., Estecha, A., and Sanchez-Mateos, P. (2007). Rho/ROCK and myosin II control the polarized distribution of endocytic clathrin structures at the uropod of moving T lymphocytes. Journal of cell science *120*, 3534-3543.

Satoh, S., Ueda, Y., Koyanagi, M., Kadokami, T., Sugano, M., Yoshikawa, Y., and Makino, N. (2003). Chronic inhibition of Rho kinase blunts the process of left ventricular hypertrophy leading to cardiac contractile dysfunction in hypertension-induced heart failure. J Mol Cell Cardiol *35*, 59-70.

Schnitzer, J.E., Oh, P., Pinney, E., and Allard, J. (1994). Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. The Journal of cell biology *127*, 1217-1232.

Seabra, M.C. (1998). Membrane association and targeting of prenylated Ras-like GTPases. Cellular signalling *10*, 167-172.

Seasholtz, T.M., Majumdar, M., Kaplan, D.D., and Brown, J.H. (1999). Rho and Rho kinase mediate thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration. Circ Res *84*, 1186-1193.

Seasholtz, T.M., Zhang, T., Morissette, M.R., Howes, A.L., Yang, A.H., and Brown, J.H. (2001). Increased expression and activity of RhoA are associated with increased DNA synthesis and reduced p27(Kip1) expression in the vasculature of hypertensive rats. Circ Res *89*, 488-495.

Segal, M., and Auerbach, J.M. (1997). Muscarinic receptors involved in hippocampal plasticity. Life sciences *60*, 1085-1091.

Seko, T., Ito, M., Kureishi, Y., Okamoto, R., Moriki, N., Onishi, K., Isaka, N., Hartshorne, D.J., and Nakano, T. (2003). Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle. Circ Res *92*, 411-418.

Seoh, S.A., Sigg, D., Papazian, D.M., and Bezanilla, F. (1996). Voltage-sensing residues in the S2 and S4 segments of the Shaker K+ channel. Neuron *16*, 1159-1167.

Settleman, J. (1999). Rho GTPases in development. Progress in molecular and subcellular biology *22*, 201-229.

Sever, S., Damke, H., and Schmid, S.L. (2000). Dynamin:GTP controls the formation of constricted coated pits, the rate limiting step in clathrin-mediated endocytosis. The Journal of cell biology *150*, 1137-1148.

Shahrestanifar, M., Fan, X., and Manning, D.R. (1999). Lysophosphatidic acid activates NF-kappaB in fibroblasts. A requirement for multiple inputs. The Journal of biological chemistry *274*, 3828-3833.

Sheng, M., Liao, Y.J., Jan, Y.N., and Jan, L.Y. (1993). Presynaptic A-current based on heteromultimeric K+ channels detected in vivo. Nature *365*, 72-75.

Sheng, M., Tsaur, M.L., Jan, Y.N., and Jan, L.Y. (1992). Subcellular segregation of two A-type K+ channel proteins in rat central neurons. Neuron *9*, 271-284.

Sheng, M., Tsaur, M.L., Jan, Y.N., and Jan, L.Y. (1994). Contrasting subcellular localization of the Kv1.2 K+ channel subunit in different neurons of rat brain. J Neurosci *14*, 2408-2417.

Shi, G., Nakahira, K., Hammond, S., Rhodes, K.J., Schechter, L.E., and Trimmer, J.S. (1996). Beta subunits promote K+ channel surface expression through effects early in biosynthesis. Neuron *16*, 843-852.

Shi, G., and Trimmer, J.S. (1999). Differential asparagine-linked glycosylation of voltage-gated K+ channels in mammalian brain and in transfected cells. J Membr Biol *168*, 265-273.

Shigematsu, S., Watson, R.T., Khan, A.H., and Pessin, J.E. (2003). The adipocyte plasma membrane caveolin functional/structural organization is necessary for the efficient endocytosis of GLUT4. The Journal of biological chemistry *278*, 10683-10690.

Shimkets, R.A., Lifton, R.P., and Canessa, C.M. (1997). The activity of the epithelial sodium channel is regulated by clathrin-mediated endocytosis. The Journal of biological chemistry *272*, 25537-25541.

Smart, E.J., Ying, Y.S., Conrad, P.A., and Anderson, R.G. (1994). Caveolin moves from caveolae to the Golgi apparatus in response to cholesterol oxidation. The Journal of cell biology *127*, 1185-1197.

Solc, C.K., Zagotta, W.N., and Aldrich, R.W. (1987). Single-channel and genetic analyses reveal two distinct A-type potassium channels in Drosophila. Science (New York, N.Y *236*, 1094-1098.

Sorkin, A., and Waters, C.M. (1993). Endocytosis of growth factor receptors. Bioessays *15*, 375-382.

Southan, A.P., and Robertson, B. (1998). Patch-clamp recordings from cerebellar basket cell bodies and their presynaptic terminals reveal an asymmetric distribution of voltage-gated potassium channels. J Neurosci *18*, 948-955.

Subauste, M.C., Von Herrath, M., Benard, V., Chamberlain, C.E., Chuang, T.H., Chu, K., Bokoch, G.M., and Hahn, K.M. (2000). Rho family proteins modulate rapid apoptosis induced by cytotoxic T lymphocytes and Fas. The Journal of biological chemistry *275*, 9725-9733.

Sumi, T., Matsumoto, K., and Nakamura, T. (2001a). Specific activation of LIM kinase 2 via phosphorylation of threonine 505 by ROCK, a Rho-dependent protein kinase. The Journal of biological chemistry *276*, 670-676.

Sumi, T., Matsumoto, K., Shibuya, A., and Nakamura, T. (2001b). Activation of LIM kinases by myotonic dystrophy kinase-related Cdc42-binding kinase alpha. The Journal of biological chemistry *276*, 23092-23096.

Symons, M., and Rusk, N. (2003). Control of vesicular trafficking by Rho GTPases. Curr Biol *13*, R409-418.

Takaishi, K., Sasaki, T., Kameyama, T., Tsukita, S., Tsukita, S., and Takai, Y. (1995). Translocation of activated Rho from the cytoplasm to membrane ruffling area, cell-cell adhesion sites and cleavage furrows. Oncogene *11*, 39-48.

Taniguchi, T., Kawamata, T., Mukai, H., Hasegawa, H., Isagawa, T., Yasuda, M., Hashimoto, T., Terashima, A., Nakai, M., Mori, H., Ono, Y., and Tanaka, C. (2001). Phosphorylation of tau is regulated by PKN. The Journal of biological chemistry *276*, 10025-10031.

Taylor, S.S., and Radzio-Andzelm, E. (1994). Three protein kinase structures define a common motif. Structure *2*, 345-355.

Torsoni, A.S., Fonseca, P.M., Crosara-Alberto, D.P., and Franchini, K.G. (2003). Early activation of p160ROCK by pressure overload in rat heart. Am J Physiol Cell Physiol *284*, C1411-1419.

Toshima, J., Toshima, J.Y., Martin, A.C., and Drubin, D.G. (2005). Phosphoregulation of Arp2/3-dependent actin assembly during receptor-mediated endocytosis. Nature cell biology *7*, 246-254.

Tsai, W., Morielli, A.D., and Peralta, E.G. (1997). The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity. The EMBO journal *16*, 4597-4605.

Tsaur, M.L., Sheng, M., Lowenstein, D.H., Jan, Y.N., and Jan, L.Y. (1992). Differential expression of K+ channel mRNAs in the rat brain and down-regulation in the hippocampus following seizures. Neuron *8*, 1055-1067.

Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M., and Narumiya, S. (1997). Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature *389*, 990-994.

Valiyaveetil, F.I., Leonetti, M., Muir, T.W., and Mackinnon, R. (2006). Ion selectivity in a semisynthetic K+ channel locked in the conductive conformation. Science (New York, N.Y *314*, 1004-1007.

Veh, R.W., Lichtinghagen, R., Sewing, S., Wunder, F., Grumbach, I.M., and Pongs, O. (1995). Immunohistochemical localization of five members of the Kv1 channel subunits: contrasting subcellular locations and neuron-specific co-localizations in rat brain. Eur J Neurosci *7*, 2189-2205.

Vicente, R., Villalonga, N., Calvo, M., Escalada, A., Solsona, C., Soler, C., Tamkun, M.M., and Felipe, A. (2008). Kv1.5 association modifies Kv1.3 traffic and membrane localization. The Journal of biological chemistry *283*, 8756-8764.

Volovyk, Z.M., Wolf, M.J., Prasad, S.V., and Rockman, H.A. (2006). Agonist-stimulated beta-adrenergic receptor internalization requires dynamic cytoskeletal actin turnover. The Journal of biological chemistry *281*, 9773-9780.

Wang, J., Juhaszova, M., Rubin, L.J., and Yuan, X.J. (1997). Hypoxia inhibits gene expression of voltage-gated K+ channel alpha subunits in pulmonary artery smooth muscle cells. The Journal of clinical investigation *100*, 2347-2353.

Wang, J., Weigand, L., Wang, W., Sylvester, J.T., and Shimoda, L.A. (2005). Chronic hypoxia inhibits Kv channel gene expression in rat distal pulmonary artery. Am J Physiol Lung Cell Mol Physiol *288*, L1049-1058.

Wei, Y., Zhang, Y., Derewenda, U., Liu, X., Minor, W., Nakamoto, R.K., Somlyo, A.V., Somlyo, A.P., and Derewenda, Z.S. (1997). Crystal structure of RhoA-GDP and its functional implications. Nat Struct Biol *4*, 699-703.

Williams, M.R., Markey, J.C., Doczi, M.A., and Morielli, A.D. (2007). An essential role for cortactin in the modulation of the potassium channel Kv1.2. Proceedings of the National Academy of Sciences of the United States of America *104*, 17412-17417.

Wissmann, R., Baukrowitz, T., Kalbacher, H., Kalbitzer, H.R., Ruppersberg, J.P., Pongs, O., Antz, C., and Fakler, B. (1999). NMR structure and functional characteristics of the hydrophilic N terminus of the potassium channel beta-subunit Kvbeta1.1. The Journal of biological chemistry *274*, 35521-35525.

Wu, H., and Parsons, J.T. (1993). Cortactin, an 80/85-kilodalton pp60src substrate, is a filamentous actin-binding protein enriched in the cell cortex. The Journal of cell biology *120*, 1417-1426.

Yamaguchi, H., and Condeelis, J. (2007). Regulation of the actin cytoskeleton in cancer cell migration and invasion. Biochimica et biophysica acta *1773*, 642-652.

Yang, E.K., Takimoto, K., Hayashi, Y., de Groat, W.C., and Yoshimura, N. (2004). Altered expression of potassium channel subunit mRNA and alpha-dendrotoxin sensitivity of potassium currents in rat dorsal root ganglion neurons after axotomy. Neuroscience *123*, 867-874.

Yang, X., Huang, H.C., Yin, H., Alpern, R.J., and Preisig, P.A. (2007). RhoA required for acid-induced stress fiber formation and trafficking and activation of NHE3. American journal of physiology *293*, F1054-1064.

Yoo, D., Fang, L., Mason, A., Kim, B.Y., and Welling, P.A. (2005). A phosphorylation-dependent export structure in ROMK (Kir 1.1) channel overrides an endoplasmic reticulum localization signal. The Journal of biological chemistry *280*, 35281-35289.

Yuan, X.J. (1995). Voltage-gated K+ currents regulate resting membrane potential and [Ca2+]i in pulmonary arterial myocytes. Circulation research *77*, 370-378.

Yuan, X.J., Wang, J., Juhaszova, M., Golovina, V.A., and Rubin, L.J. (1998). Molecular basis and function of voltage-gated K+ channels in pulmonary arterial smooth muscle cells. The American journal of physiology *274*, L621-635.

Zeng, G., and Cai, M. (1999). Regulation of the actin cytoskeleton organization in yeast by a novel serine/threonine kinase Prk1p. The Journal of cell biology *144*, 71-82.

Zeng, G., Yu, X., and Cai, M. (2001). Regulation of yeast actin cytoskeleton-regulatory complex Pan1p/Sla1p/End3p by serine/threonine kinase Prk1p. Molecular biology of the cell *12*, 3759-3772.

Zheng, Y., Zangrilli, D., Cerione, R.A., and Eva, A. (1996). The pleckstrin homology domain mediates transformation by oncogenic dbl through specific intracellular targeting. The Journal of biological chemistry *271*, 19017-19020.

Zong, H., Raman, N., Mickelson-Young, L.A., Atkinson, S.J., and Quilliam, L.A. (1999). Loop 6 of RhoA confers specificity for effector binding, stress fiber formation, and cellular transformation. The Journal of biological chemistry *274*, 4551-4560.