UNIVERSITY OF CALGARY

Regulation of a Large Conductance, Calcium-Activated Potassium (BK) Channel by J-Proteins

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF MEDICAL SCIENCE

CALGARY, ALBERTA

APRIL 2011

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UNIVERSITY OF
CALGARY

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Abstract

BK channels are K^+ channels that are widely distributed throughout the nervous system. The activity of presynaptic BK channels governs synaptic transmission by regulating the influx of extracellular calcium through voltage-gated calcium channels. Until now, little is known about the molecular chaperone machinery that participates in the folding and degradation of BK channels. CSPα (cysteine string protein) is a synaptic vesicle-associated molecular chaperone that prevents activity-dependent neuronal degeneration by unknown mechanisms. Using CAD cells that transiently or stably expressed in BK channel, we assessed the influence of CSPα a BK channel expression using Western Blot analyses. This study indicates that CSPα and related J-proteins interact with and changed BK channel expression. We demonstrate that the highly conserved HPD motif within the J-domain is required for BK channel reduction. These results provide the first evidence that CSPα regulates BK channel expression and attribute a key role to CSPα in neurotransmission.

Publications

Manuscripts

Johnson, J. N.*, Ahrendt, E.* and Braun, J. E.A. (2009), "*CSPα: The Neuroprotective J Protein*", Journal Biochemistry and Cell Biology 89: 1-9 (2010), Mini-Review (* authors are equal contributors)

Eva Ahrendt and Janice E. A. Braun; "*Channel Triage, Emerging Insights into the processing and quality control of hERG potassium channels by DnaJA proteins 1, 2 and 4*"; Journal Channels (Austin), Volume 4 Issue 3 2010 Sep 9;4(5)

In Preparation

Regulation of a Large Conductance Ca^{2+}-activated Potassium (BK) Channel by J Proteins

Conference abstract

Eva Ahrendt, Andrew P. Braun, Janice E. A. Braun *„J Protein Chaperones Regulate the Expression of a Large Conductance, Calcium-activated Potassium (BK) Channel"* Poster Presentation at the "53rd Annual Meeting and Conference of the Canadian Society of Biochemistry and Cell Biology: Membrane Proteins in Health and Disease", April 2010 in Banff, Alberta, Canada

Acknowledgements

The completion of this thesis would not have been possible without the help and contribution of several people. Jan Braun gave me the opportunity to work in her lab and has provided countless of hours of advice and training. My committee members, Bob French, Andy Braun and Julie Deans gave me tremendous support and encouragement.

Funding for this thesis was provided by the Alberta Innovates-Health Solutions and The Canadian Institute of Health Research (CIHR) in form of grants to Jan Braun.

Thanks to Andy Braun for advice, training and for providing me the BK channel α-subunit and BKβ1/β4-subunit cDNA constructs.

Thanks to the members of the Braun lab, both past and present - Anna Carnini, Jadah Johnson, Juliane Proft, Cecilia Zhao and Sarah Gibbs - for the insightful discussion and experimental advice and training in the beginning, and for creating a great working environment.

Thanks to members of the Colicos lab who provided the hippocampal neuronal cultures used in this thesis, resources and technical training. In particular, I would like to thank Lucas Scott for training me in fluorescence microscopy.

Thanks to my family for their support and to Diana Radovan, Olesja Hazenbiller and Katharina Fuerderer for their great friendship, support and encouragement.

To my family

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List of Abbreviations

° C	Degrees Celsius
BKα-subunit	Alpha-subunit of BK channel
ABD	Actin-binding domain
APS	Ammonium persulfate
ATP	Adenosine 5' triphosphate
ATPase	Adenosine 5' triphosphatase
Ba^{2+}	Barium ion
BME	Basal medium Eagle
BK channel	'Big' Ca^{2+} activated potassium channel
BSA	Bovine serum albumin
BKβ-subunit	Beta-subunit of BK channel
CAD	CNS-derived catecholaminergic neuronal cell line
cAMP	Cyclic adenosine monophosphate
Ca^{2+}	Calcium ion
CaCl	Calcium chlorid
cDNA	Complementary DNA
C-terminus	COOH-terminus
CFP	Cyan fluorescence protein
CFTR	Cystic fibrosis transmembrane conductance regulator
CHIP	Carboxyl Terminus of Hsc70-Interacting Protein
Cl^-	Chloride ion
CNS	Central nervous system
CSP	Cysteine string protein
CO_2	Carbon dioxide
dbcAMP	Dibutyryl cyclic adenosine monophosphate
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

DTT	Dithiothreitol
EBS	EGTA, β-glycerophosphate and sucrose
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylen glycerol bis (2-aminoethyl ether)-N,N,N'N'-tetra acetic acid
ERAD	Endoplasmic Reticulum Associated Protein Degradation
g	Gram
µg	Microgram
G protein	GTP (guanosine 5' triphosphate)-binding protein
GTP	Guanosine 5' phosphate
Gα	α-subunit of the G-protein
Gβ	β-subunit of the G-protein
Gγ	γ-subunit of the G-protein
h	Hour
HA-tag	Hemagglutinin-tag
HEPES	2-(4-(2-Hydroxyethyl)-1-piperainyl)-ethansulfonic acid
hERG	Human ether-a-go-go related gene
HCl	Hydrogen chloride
H_2O	Water
HPD	Histidine, proline and aspartic acid, proline and aspartic acid
HRP	Horseradish peroxidase
Hsp40	Heat shock protein of 40 kDa
Hsc70	Heat shock cognate protein of 70 kDa, constitutively expressed
Hsp70	Heat shock protein of 70 kDa
Hsp90	Heat shock protein of 90 kDa
IgG	Immunglobulin G
J-domain	Region of a co-chaperone that interacts with Hsc70 and activates its ATPase activity
K_{ATP}	ATP-sensitive potassium channel
K^+	Potassium ion
KCl	Potassium chloride

kDa	Kilodalton
LB	Luria-Bertani medium
KH_2PO_4	Potassium hydroxide phosphate
mA	Milli ampere
MARCK	Myristoylated alanines-rich C kinase substrate
MESNA	Sodium 2-mercaptoethanesulfonate
ml	Milliliter
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesiumdiclorid
min	Minute
mm	Millimeter
mOsm	Milli-osmoles per kg H_2O
mRNA	Messenger ribonucleic acid
mV	Millivolt
N-terminus	NH_2-terminus
Na^+	Sodium ion
NaCl	Sodium chloride
Na_2HPO_4	Sodium hydroxide phosphate
Na_3VO_4	Sodium ortho vandate
nm	Nanometer
NMJ	Neuromuscular junction
OD	Optical density
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline + tween
PMSF	Phenylmethylsulfonyl fluoride
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
Pre-mRNA	Precursor mRNA
PrP^c	Cellular prion protein

pS	Pico siemens
RCK	Regulator for high conductance of K^+
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean
SLO	Slowpoke channel
SNAP25	Synaptosome-associated protein 25 kDa
SNARE	Soluble NSF (N-ethylmaleimide sensitive factor) attachment protein receptor
SGT	Small glutamine-rich tetratricopeptide domain protein
TBS	Tris buffered saline
TE	Tris-EDTA
TEA	Tetraethyammonium
TEMED	N, N, N, N tetramethylethylenediamine
Tris	Tris (hdyroxymethyl) aminoethan
V	Volt
VAMP	Vesicle-associated membrane protein (also known as Synaptobrevin)
U	Units
µl	Microliter
µM	Micromolar

1 INTRODUCTION

1.1 Overview

Membrane ion channels are the fundamental cellular elements that are responsible for the generation and transmission of electrical signals. They are abundant in the brain, where one of their major roles is to govern synaptic transmission. The human body produces a variety of ion channels, with selective permeability for various ions such as Na^+, K^+, Ca^{2+} or Cl^-. These channels show different functional properties, which are important for a myriad of cellular functions. The K^+ selective ion channel family is responsible for a negative resting membrane potential in all excitable cell membranes. The BK channel is abundant in neurons and important for the membrane repolarization after a stimulus. Its function is critical in determining the amplitude and duration of action potentials, firing rates and the overall excitably of neurons. The regulation of BK channel activity, encompassing the mechanisms by which it influences and thus controls K^+ efflux, is essential in neurotransmitter release. The regulation of BK channel activity has been extensively studied, whereas little is known about BK channel biogenesis, trafficking and degradation. Changes in BK channel expression at the neuronal terminal could have many effects on neuronal transmission. Upon an increase or decrease in BK channel expression at the cell surface, the action potential could be either prolonged or shortened and thus could further affect Ca^{2+} homeostasis and neuronal activity and synaptic transmission (Faber and Sah, 2003; Salkoff et al., 2006).

J-proteins are a class of co-chaperones that interact with Hsc70 and thereby activate its ATPase. Hsc70 is a molecular chaperone which participates in a multitude of cellular pathways. It has the ability to bind and sequester unfolded regions of a wide array of substrate proteins. The client binding and release kinetics of Hsc70 are governed by the intrinsic ATPase activity and J-proteins activate the ATPase. All J-proteins contain a highly conserved J-domain, a 70 amino acid region that interacts with Hsc70. The human genome encodes almost fifty J-proteins which have specific cellular and subcellular distributions

and thus have structurally diverse functions. J-proteins recruit client proteins to Hsc70 where the conformation work occurs. CSPα (cysteine string protein) is a unique J-protein because it is abundant in the neuronal tissue, important for neurotransmitter release and it is known to be neuroprotective.

In the research performed for this Master-Thesis, I tested the hypothesis: **"CSPα interacts with and regulates the expression of the BKα subunit of BK channels".** Using biochemical and cell biology techniques, I demonstrated that CSPα reduces BK channel expression. Building on these initial findings, I then set out to provide more definitive information regarding whether related J-proteins or Hsc70 also regulated BK channel protein expression. To begin to address the mechanism underlying CSPα-mediated regulation of BK channel expression, I proceeded to determine which specific amino acids of CSPα are critical for this activity. The CSPα/BK channel association, the influence of BK channel β1- and β4- regulatory subunits, the influence of the proteasome inhibitor lactacystin and the CSPα modulator quercetin were investigated for their influence on CSPα-mediated regulation of BK channel expression. A stable BK channel expressing CAD cell line (CNS-derived catecholaminergic neuronal cell line) was generated and total and cell surface BKα subunit expression was determined. Finally, in preliminary experiments, transient expression of key CSPα constructs identified during the course of this Master's thesis in hippocampal neurons was performed demonstrating co-localization of these constructs with BK channels by immunofluorescence. Ultimately, the hope of this research is to obtain a detailed picture for the role of BK channels in CSPα-mediated neuroprotection. CSPα mediated regulation of BK channel contributes new information to the emerging picture of how chaperones balance the biogenesis and degradation of ion channels.

1.2 BK channels

1.2.1 General overview of BK channels

Membrane ion channels are important to govern synaptic transmission. The activity of K^+-selective ion channels is not only responsible for the negative resting membrane

potential of all cells, it is also critically important for determining the shape and duration of the action potential, and the firing rates and overall excitability of neurons. In neurons, K^+ selective channels are responsible for membrane repolarization following a stimulus, and one such K^+ channel is the BK channel. The BK channel belongs to the SLO (slow poke channel) family of K^+ channels. These channels were originally named 'big' potassium (BK) channels, they are also known as maxi-K^+ or SLO channels. These channels have a large conductance (i.e. 250 pS in symmetrical 140 mM KCl). The term "SLO" arose from early voltage-clamp recordings in the flight muscle of a *Drosophila* mutant with a severely lethargic phenotype (slow poke) and other experiments determined that the calcium-dependent component of the outward K^+ current (BK channel) was absent (Butler et al., 1993; Pallanck and Ganetzky, 1994)

1.2.2 Structure and functional properties of BK channels

The mammalian SLO family of K^+-selective channels includes four genes; each of them encodes one pore-forming α-subunit that forms a homotetrameric BK channel. SLO channel genes and their characteristic properties are shown in Table 1. Whereas the BK channel (SLO1) is activated primarily by cytosolic free Ca^{2+} and membrane depolarization, other SLO channels can be activated by intracellular Na^+ (SLO2) or changes in pH (SLO3).

Table 1: BK channels - classification and blockers

Channel	Alternative Names	Gene and Chromosome (human)	Conductance (K^+)	Blockers	Reference
SLO1	BK BK_{Ca} Maxi-K $K_{Ca}1.1$	KCNMA1 (10q22)	100-270 pS	Paxilline Iberiotoxin Charybdotoxin Tubocurarine Kaliotoxin Penitrem TEA	(Gribkoff et al., 1996a) (Reinhart et al., 1989) (Egan et al., 1993) (Crest et al., 1992) (Gribkoff et al., 1996b)
SLO2.1	Slick BK_{Na} $BK_{Ca}4.2$	KCNT2 (1q31.1)	60-140 pS	Intracellular ATP Ba^{2+} Quinidine	(Santi et al., 2006)
SLO2.2	Slack BK_{Na} $BK_{Ca}4.1$	KCNT1 (9q34.3)	100-180 pS		(Santi et al., 2006)
SLO3	Potassium large conductance pH-sensitive channel $K_{Ca}5.1$	KCNU1 (8q11.2)	70-100 pS		(Schreiber et al., 1998)

In many tissues, BK channel activity is responsible for the predominant outward K^+ current. This channel shows an unusual dual activation process compared to other K^+ channels, i.e. it can be activated through either depolarization of the cell membrane or through elevations in intracellular free Ca^{2+}. It also displays an unusually large single-channel conductance of ~250 pS in symmetrically high K^+. Physiologically, BK channels generate large amounts of ionic current, thereby producing significant membrane hyper-polarizations (Pallotta et al., 1981).

A holo-BK channel consists of four α-subunits arranged symmetrically around a central ion conduction pore. Each α-subunit consists of seven membrane-spanning segments (S0 to S6); this domain is called the ‘core’ domain. A schematic structure of a

typical BK α-subunit is shown in Figure 1. The ion-selective pore is formed by the S5 and S6 segment, and three positively charged residues occur at regular intervals in the S4 segment of voltage sensor. The C-terminus following the S6 segment is located on the cytoplasmic side and is referred as the 'tail' domain. It comprises two-third of the whole protein and contains four hydrophobic regions. The C-terminus consists of two RCK (regulators of conductance of K^+) domains and a Ca^{2+}-bowl adjacent to the second RCK domain. Both RCK domains, along with the Ca^{2+}-bowl, are important for Ca^{2+}-dependent channel gating. The S0 membrane-spanning and the extracellular N-terminal play an important role for the assembly and modulation via the regulatory β-subunit (Kim et al., 2007b).

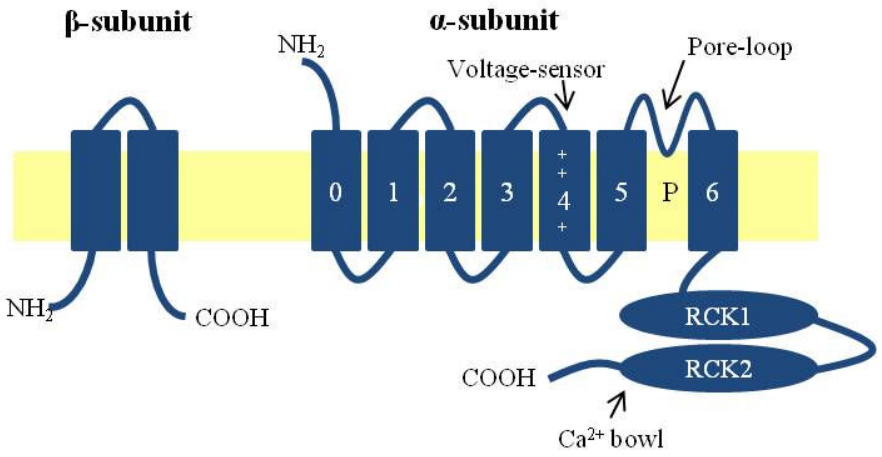

Figure 1: Schematic structure of BK channel.
Schematic BK channel α-subunit and β-subunit structure S0-S6 are transmembrane spanning domains forming an ion selective pore; RCK1 and PCK2 are regulators of the conductance of K^+

The α-subunit of the BK channel is the minimal component of the BK channel. To provide the BK channel with more functional diversity, auxiliary β-subunits interact with the α-subunit. The four β-subunits are structurally distinct from each other and have different effects on BK channels in a variety of tissues. These β-subunits consist of two

membrane-spanning domains (Figure 1) are connected by an extended extracellular loop that contains N-linked glycosylation sites. The N- and C-termini are localized intracellularly. Table 2 lists the different BK channel β-subunits and their tissue distribution. The β-subunits also greatly affect the physiological and pharmacological properties of BK channels (Behrens et al., 2000; Brenner et al., 2000a; Tanaka et al., 2004).

Table 2: BK channel β-subunits and their tissue distribution

Auxiliary subunits	**Tissue and Subcellular distribution**	**Reference**
β1(KCNMB1)	smooth muscles of urinary bladder, the vascular, and the respiratory system, some neurons	(Knaus et al., 1994) (Behrens et al., 2000) (Ledoux et al., 2008) (Tanaka et al., 2004)
β2(KCNMB2)	kidney, pancreas and ovary, testes, small intestine, adrenal chromatin cells and brain	(Behrens et al., 2000) (Brenner et al., 2000a) (Uebele et al., 2000)
β3(KCNMB3)	broad tissue expression pattern, including in different regions of the brain	(Brenner et al., 2000a)
β4(KCNMB4)	neuronal subunit, primarily found in various regions of the brain	(Behrens et al., 2000) (Brenner et al., 2000a)

As mentioned, BK channels can be activated by depolarization through a voltage-sensor domain and also by intracellular Ca^{2+} through a ligand-binding domain. The primary role of the S4 domain is to act as a voltage sensor in the BK channel, which is associated with the pore-forming region. In response to changes in the transmembrane voltage, the S4 domain changes its conformation, thus allowing the channel to open. The ligand-gating mechanisms in BK channels are more complex than their voltage-sensing mechanisms. In such gating mechanisms, the C-terminal RCK domains form an intracellular ring which is composed of four RCK1 and four RCK2 domains. It has been proposed that binding of Ca^{2+} to the gating ring expands the ring, thereby creating tension on the linkers between the gating ring and the S6 segments gates, resulting in opening the channel (Liu et al., 2010;

Yuan et al., 2010). BK channels have at least two high-affinity Ca^{2+} binding sites and one low affinity Ca^{2+}/Mg^{2+} binding site per subunit (Schreiber et al., 1998; Xia et al., 2002).

BK channels have the largest single-channel conductance of all K^+ channels. This high conductance appears to depend upon the inner and outer parts of the conductance pore, which are negatively charged. These negatively charged residues can have an important role in controlling single channel conductance through electrostatic mechanisms acting upon the permeable K^+ ion (Li and Aldrich, 2004). BK channels can be blocked by large organic molecules, e.g. TEA (Tetraethylammonium), which acts on both extra- and intracellular channel sites (Lenaeus et al., 2005).

The BK channel shows functional differences between subtypes and different tissues, respectively, due to alternate splicing of the α-subunit. Some BK channel splice variants show physiologically relevant differences in Ca^{2+} sensitivity, gating kinetics or trafficking efficacy, (Kim et al., 2007a; Shipston, 2001). The vertebrate Slo1 gene has a conserved structure which includes at least 35 exons and 7 alternative pre-mRNA splice sites (Beisel et al., 2007). The majority of alternative splicing sites are in the large C-terminal domain, which comprises two-thirds of each α-subunit. Most of these splicing variants exhibit high levels of constitutive trafficking to the plasma membrane in heterogonous expression systems, but some variants are retained in intracellular compartments (Kim et al., 2007a).

The BK channel can be modulated by protein kinases and phosphatases which regulate phosphorylation sites at the intracellular ‘tail’ domain on the channel protein. BK channels are an important substrate for direct regulation by various serine/threonine kinases, such as PKA (protein kinase A), PKG (protein kinase G), and PKC (protein kinase C), and, as a result, the activity of the channel can be coupled to multiple, diverse signaling cascades. BK channel activity can be either decreased or increased in the presence of specific protein kinases (Ling et al., 2004; Schubert and Nelson, 2001; Weiger et al., 2002).

1.2.3 Physiological role of BK channels in the brain

BK channels are important for the negative-feedback regulation of Ca^{2+} entry in many excitable cell types. BK channels are widely distributed in the mammalian CNS, being located in cell soma, dendrites and presynaptic terminals. Likewise, they are also present in many other cell types, such as exo- and endocrine cells, hair cells and smooth muscle cells. In the presynaptic nerve terminal, BK channels provide a homeostatic (i.e. feedback) mechanism for regulation of synaptic transmission by limiting the influx of extracellular Ca^{2+} through presynaptic voltage-gated Ca^{2+} channels. BK channels in the neuronal cell body are known to contribute to the fast phase of the after-hyperpolarization observed in response to an excitatory stimulus. The after-hyperpolarization potential is a chief determinant of the refractory period and therefore of the maximal firing rate of a neuron. BK channels are activated by entry of Ca^{2+} ions through voltage-sensitive Ca^{2+} channels. Numerous reports demonstrate that BK channels co-localize with Ca^{2+} channels in a variety of neurons: N-type Ca^{2+} channels of pyramidal neurons (Marrion and Tavalin, 1998); L-type Ca^{2+} channels in rat superior cervical ganglia (Davies et al., 1996); P/Q-type Ca^{2+} channels in rat cerebellar Purkinje neurons (Womack and Khodakhah, 2004); T-type channels in rat medial vestibular neurons (Smith et al., 2002). Also in plasma membrane-enriched fractions prepared from rat whole brain, the subunits of several types of Ca^{2+} channels (L-type, P/Q-type, N-type) are reported to co-purify with BK channels (Berkefeld and Fakler, 2008). Overall, these reports indicate that BK channels form physiological complexes with a variety of Ca^{2+} channels that provide the Ca^{2+}, which is necessary for rapid and robust activation of BK channel, and thus repolarization of the membrane. Sun et al. (2004) reported that blocking the N-type channels at the synapse site leads to the significant inhibition of BK channel currents (Sun et al., 2004). This suggests that Ca^{2+} entry through N-type channels has a dual function, i.e. triggering synaptic exocytosis and activating the co-localized BK channel. Presynaptic BK channels act as negative regulators of neurotransmitter release, because they co-localize with Ca^{2+} channels within the same membrane region and are activated by membrane depolarization and cytoplasmic Ca^{2+} and thus are able to determine the release of Ca^{2+} and neurotransmitter (Robitaille et al., 1993).

Mechanisms that control the release of neurotransmitters are important in the CNS, because they control synaptic strength and plasticity, which is fundamental for higher brain function. BK channels have differentially physiological roles in the regulation of the release of various neurotransmitters from distinct brain regions, e.g. neurotransmitter release in excitatory or non-excitatory neurons. BK channels regulate the release of various neurotransmitters; the pharmacological blockage of BK channels failed to change release at GABAergic neurons and catecholaminergic neurotransmitters (dopamine or norepinephrine) but altered the neurotransmitter release at glutamatergic neurons. However, these effects could be also due to expression of different level of BK channels at these synapse (Martire et al., 2010; Sausbier et al., 2004). The activity of BK channels is the focus of elaborate regulation indicating that the precise control of presynaptic BK channels must be important for the control of synaptic strength (i.e. synaptic plasticity) and other brain functions. The main function of the BK channel is to shorten the action potential duration, enhance the rate of repolarization and contribute to rapid after-hyperpolarization, which limits repetitive firing. The activation of the BK channel by the nerve growth factor neurotrophin-3 reduces the amount of neurotransmitter release by shortening the duration of depolarization (Holm et al., 1997). Inhibition of BK channels by drugs like TEA leads to membrane depolarization, enhancement of Ca^{2+} and neurotransmitter release (Gu et al., 2007).

Genetic studies have shown that the BK channel-null mutant of *Drosophila melanogaster* has a behavioral phenotype consistent with a generalized neural and muscle defect. In the nematode *Caenorhabditis elegans,* BK channel-null mutants have been shown to increase neurotransmitter release at the neuromuscular junction. BK channel-null mice display ataxia, high-frequency hearing loss, vascular hypertension, incontinence due to overactive bladder function and erectile dysfunction, consistent with defects in brain and smooth muscle excitability (Meredith et al., 2004; Sausbier et al., 2004). Gain-of-function mutations in human BK channels are found to be responsible for generalized epilepsy and paroxysmal dyskinesia (Du et al., 2005).

1.2.4 Trafficking of BK channels

BK channels are widely expressed in mammalian cells where they play important physiological roles, ranging from control of the blood flow to neuronal excitability and neurotransmitter release. The perturbation of BK channel function may lead to a number of disorders, e.g. hypertension, epilepsy and cerebellar ataxia. Because BK channels are involved in many regulatory physiological mechanisms, it is essential for these channels to be correctly synthesized, folded and trafficked to the cell membrane in order to be functional. BK channel surface expression is associated with many physiological demands, e.g. pregnancy or aging (Song et al., 1999). BK channel activity and function has been studied extensively whereas not much is known is about BK channel folding and trafficking. Over the last decade, various BK channel motifs have been identified to play an important role in BK channel retention or trafficking. Table 3 presents recently identified trafficking motifs which modulate BK channel expression.

Table 3: Trafficking motifs of BK channel and their actions

Domain	Function	Reference
Alternative pre-mRNA splicing sites in large C-terminal domain	– Exhibit high levels of constitutive trafficking to the plasma membrane – Retained in the intracellular compartments and gets modulated in response to hormones, during stress, pregnancy and upon cellular depolarization	(Beisel et al., 2007) (Kim et al., 2007a) (Benkusky et al., 2000) (Lai and McCobb, 2002) (McCobb et al., 2003) (Shipston, 2001)
Hydrophobic endoplasmic reticulum export signals	– Markedly different gating properties and susceptibility to post-transcriptional modification	(Zarei et al., 2004) (Wang et al., 2003)
Splicing at splicing site 7 result in three different extreme C-terminal variants ($Slo1_{VEDEC}$, $Slo1_{EMVYR}$, $Slo1_{QEERL}$)	– Similar gating properties, markedly different patterns of expression on the cell surface – Stimulated by appropriate growth factors, such as transforming growth factor- β1 (TGFβ1) and β-neuregulin-1(NRG1)	(Chiu et al., 2010) (Kim et al., 2007a)
Unique motifs on the C-terminal tail from $Slo1_{VEDEC}$	– Suppress constitutive cell surface expression – Deletions of the unique portions of the $Slo1_{VEDEC}$ C-terminal tail led to progressively greater surface expression	(Chiu et al., 2010) (Ma et al., 2007)
Acidic cluster-like motif between the RCK1 and RCK2	– Transplanting this motif into a non-channel protein led to an increase in surface expression – Acidic motifs have been described as common ER export signal in other channels	(Chen et al., 2010)
Palmitoylation site in the intracellular S0-S1 linker region and C-terminal domain	– Palmitoylation of the S0-S1 linker may be an important factor in controlling BK channel expression and phosphorylation dependent gating	(Jeffries et al., 2010) (Tian et al., 2008)
β-subunits	– Co-expression of β-subunits enhance the steady-state cell surface expression of certain BKα-subunits	(Kim et al., 2007b) (Toro et al., 2006) (Zarei et al., 2007)

Regulation of the trafficking of the Slo1 channel is a physiologically relevant process in the nervous system. Cilliary neurons of the developing chick cilliary ganglion retain BK channels in multiple intracellular compartments before forming synapses with the target tissues. Stimulation with endogenous growth factors at the time of synapse formation triggers the movement of the BK channel into the cell surface membrane (Cameron et al., 2001).

At the present time (as seen in Table 3), several important cellular trafficking motifs in the structure of BK channels have been identified, but it is still unclear which chaperones are involved in the regulation and play an important role in BK channel folding, retention and trafficking, or degradation. Recent publications (Schmidt et al., 2009; Walker et al., 2009; Yan et al., 2010) have demonstrated for the first time that chaperones like Hsp40, Hsc70 or Hsp90 play an important role in channel folding, maturation and trafficking, or degradation.

1.3 Chaperones – J-proteins

Molecular chaperones represent the cellular machinery which is responsible for correct protein folding, stabilization of protein structures and degradation of misfolded proteins. As folding catalysts, they regulate the conformation and activation of client proteins without changing their structure or a component of the final structure. Chaperones are classified into families based on molecular weight: Hsp110, Hsp90, Hsp70, Hsp60, Hsp40 and Hsp25. The focus of this Master-Thesis is on CSPα, a member of the Hsp40 (J-protein) DnaJ protein family.

Hsc70 (the 70 kDa heat shock cognate protein) is a central component of the cellular folding process. It contains an N-terminal ATPase domain and a C-terminal substrate binding domain. The ATPase activity is coupled to a broad range of folding processes that include the folding of newly synthesized proteins, transport of proteins across the membrane, refolding of misfolded proteins, disassembly of protein complexes and conformational changes of components in signal transduction, cell cycle, transcription and apoptosis pathways. A model of Hsc70 is shown in Figure 2. Hsc70 transiently binds

and releases its client protein. The binding and release of target proteins are governed by the ATPase activity of Hsc70. ATP binding to Hsc70 opens the protein substrate binding domain, thereby increasing substrate association and dissociation. In the ATP-bound state, Hsc70 shows low affinity binding for its client proteins. Following hydrolysis of ATP to ADP, the substrate-binding pocket closes and reduces substrate dissociation (i.e. high affinity binding) (Zhao et al., 2008).

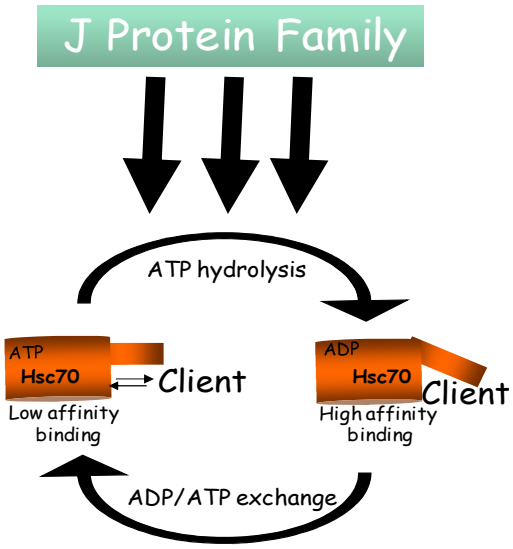

Figure 2: Model of Hsc70 folding machinery (Zhao et al., 2008)

So far, eight human Hsc70 isoforms have been identified, each encoded by a separate gene. Out of these, three isoforms can be rapidly induced in response to a condition of heat shock, while the others are constitutively expressed. The heat-shock response protects cells from stress-induced changes by rapidly up-regulating a number of distinct chaperones (Daugaard et al., 2007).

1.4 The family of J-proteins

J-proteins play an essential role in protein folding. They are found in bacteria, fungi, plants, viruses and animals (Summers et al., 2009). The human genome encodes almost 50 different J-proteins, found in all tissues and cellular organelles. Each member of the

J-protein family has a J-domain. J-proteins are classified into three classes denoted: class I, II and III. The different J-protein classes are listed in Table 4. The J-domain consists of a 70 amino acid region with a highly conserved histidine, proline and aspartic acid (HPD) motif, structured as four helices, the HPD motif being located between helices II and III. The J-domain activates the ATPase activity of Hsc70 and thereby increases substrate binding affinity (Kampinga and Craig, 2010; Zhao et al., 2008).

While the J-domain interacts with and activates Hsc70, the region outside the J-domain displays structural diversity and is thought to underlie the functional diversity of the folding activity of J-proteins. Mechanistically, client proteins are targeted to Hsc70 by J-proteins. J-proteins bind Hsc70 and activate the ATPase. This J-protein/Hsc70 complex may also function as part of a large, multiple-chaperone complex that includes Hsp90 ATPase or other protein cofactors e.g. HIP (Hsp70 interacting protein) and HOP (Hsp70 organizing protein) (Rosales-Hernandez et al., 2009; Sakisaka et al., 2002).

Table 4: Classification of J-proteins

J-protein classification		
Type I	DnaJA	The J-domain is linked by a glycine-rich region to a zinc finger domain, followed by a carboxyl-terminal domain
Type II	DnaJB	The J-domain is linked to a carboxyl-terminal domain by a glycine-rich domain
Type III	DnaJC	They contain a J-domain but no other sequence of homology is present

1.5 CSPα

1.5.1 Structure and isoforms of CSPα

CSPα (cysteine string protein; DnaJC5) is a 34 kDa synaptic vesicle anchored protein that belongs to the class III J-proteins. CSPα was first discovered in *Drosophila melanogaster* (Zinsmaier et al., 1990) and is conserved in invertebrates and mammals. Three isoforms have been identified in the human genome: CSPα, CSPβ and CSPγ (Fernández-Chacón et al., 2004). CSPβ and CSPγ are splice variants of CSPα. The focus of

this Master's thesis is CSPα. CSPα is abundant in neuronal tissue and important for synaptic vesicle function, but it is also expressed in many secretory cells. However the precise function of CSPα still remains unclear. Table 5 shows the tissue and subcellular distribution of CSPα.

Table 5: Tissue and subcellular distribution of CSPα (Johnson et al., 2010)

Cellular and subcellular localization	**Reference**
Retina	(Kohan et al., 1995; Zinsmaier et al., 1990)
Synaptic vesicles	(Mastrogiacomo et al., 1994; Zinsmaier et al., 1994)
Zymogen granules	(Braun and Scheller, 1995; Weng et al., 2009; Zhao et al., 1997)
Chromaffin granules	(Chamberlain and Burgoyne, 1996; Kohan et al., 1995)
Insulin-containing granules	(Brown et al., 1998)
Glucagon-containing granules Somatostatin-containing granules	(Zhang et al., 2002)
Oxynitic mucosa histamine granules Oxynitic mucosa pancreatistatin granules	(Zhao et al., 1997)
Adipocyte plasma membrane	(Chamberlain et al., 2001)
Mammary epithelial vesicle	(Gleave et al., 2001)
Clathrin-coated vesicles	(Blondeau et al., 2004)
Cortical granules	(Smith et al., 2005)
Inner ER membrane of epithetical cells	(Schmidt et al., 2009; Zhang et al., 2002; Zhang et al., 2006)

The CSPα structure (as seen in Figure 3) contains a short N-terminus, followed by a J-domain which is highly conserved throughout the J-protein family. Next is a hydrophobic linker region, followed by a unique cysteine string sequence, containing 13-15 cysteines within a 25 amino-acid stretch. The C-terminus of CSPα shows the highest diversity among the isoforms due to alternative splicing. The C-terminus is also the most diverse region for

the CSPα isoform among species. Compared to other J-proteins, CSPα has a unique structure, except for the J-domain.

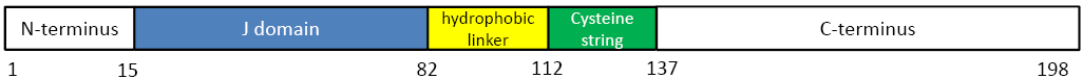

Figure 3: Domain structure of CSPα (Johnson et al., 2010)

Multiple cysteine residues in CSPα are palmitoylated, thereby anchoring CSPα to the cell membrane and secretory vesicles. The hydrophobic linker region and the cysteine region are important for CSPα oligomerization. Oligomerization of CSPα may affect its function (Swayne et al., 2003; Xu et al., 2010). Additionally, the N-terminus contains a serine that can be phosphorylated and thus modulate binding to client proteins, as well as the kinetics of exocytotic release (Evans et al., 2001).

1.5.2 CSPα and its interaction partners

Although CSPα-mediated neuroprotection has been documented (Fernández-Chacón et al., 2004; Zinsmaier et al., 1994), only modest progress has been made in understanding the mechanisms by which CSPα protects neurons. The list in Table 6 presents intriguing candidate proteins which have been proposed as functionally relevant CSPα substrates. CSPα may target either multiple proteins or just a single client protein. However, there is no consensus regarding how the regulation of any of these targets is related to the neuroprotective ability of CSPα.

Table 6: Proteins and molecules which have been reported to interact with CSPα (Johnson et al., 2010)

CSPα interaction partners	Reference
Gα/Gβ	(Gibbs et al., 2009; Magga et al., 2000; Miller et al., 2003; Natochin et al., 2005)
SNAREs	(Chandra et al., 2005; Nie et al., 1999; Seagar et al., 1999; Weng et al., 2009)
Synaptotagmin I	(Evans and Morgan, 2002)
Voltage sensitive Ca^{2+} channel	(Chen et al., 2002; Gundersen and Umbach, 1992; Leveque et al., 1998; Ranjan et al., 1998; Swayne et al., 2006; Umbach and Gundersen, 1997)
Mutant huntingtin	(Miller et al., 2003)
Rab3b	(Sakisaka et al., 2002)
CFTR	(Schmidt et al., 2009; Zhang et al., 2002; Zhang et al., 2006)
MARCKs	(Park et al., 2008)
PrP^C	(Beck et al., 2006)

1.5.3 CSPα and its role in synaptic transmission

Studies of CSPα gene deletion in *Drosophila* have shown that this deletion is semi-lethal - only 4 % of the mutant flies develop to adulthood (Zinsmaier et al., 1994). The flies that do survive to adulthood have a distinctive phenotype; paralytic uncoordinated sluggish movements, spastic jumping, shaking, temperature-sensitive paralysis, correlated with a loss of synaptic transmission. CSPα-null flies showed a 50 % reduction of nerve-evoked neurotransmitter release at 22 °C and a more drastic reduction was seen at temperatures above 29 °C. The surviving adult flies progressively exhibited uncoordinated motor behavior (Umbach et al., 1994). Further studies in *Drosophila* showed that introduction of a J-domain could not rescue CSPα-null mutants for most of their synaptic function; however, it fully restored thermo-tolerance evoked neurotransmitter release. This J-domain add-back completely failed to attenuate the defects in intra-terminal Ca^{2+} levels under rest or during

stimulation, indicating that the J-domain is required for interacting with Hsc70 during the regulation of presynaptic Ca^{2+} homeostasis (Bronk et al., 2005). Overexpression of CSPα did not influence synaptic transmission, but the flies showed a reduced viability and abnormal wing and eye development (Nie et al., 1999).

CSPα-null mice display a normal behavior within the first 2-3 postnatal weeks, followed by widespread neurodegeneration and death within 8 weeks (Fernández-Chacón et al., 2004; Schmitz et al., 2006). Degeneration is reported to be use-dependent (Schmitz et al, 2006). In fact, CSPα is particularly important in high frequency synaptic transmission (García-Junco-Clemente et al., 2010; Schmitz et al., 2006). The underlying pathological cascade of events of degeneration in mice is currently unknown. Collectively, these studies showed that CSPα plays a critical role in the maintenance of synaptic transmission.

1.6 Rationale, Hypothesis and Significance

1.6.1 Rationale

Until recently, chaperones that govern channel folding and processing have been completely unexplored. A new avenue of research has opened up with a handful of reports demonstrating that members of the J-protein chaperone family are key modulators of "cellular channel quality control". Specifically, J-proteins were shown to have a regulatory role in hERG (human ether-a-go-go related gene) (Walker et al., 2009), CFTR (cystic fibrosis transmembrane conductance regulator) (Schmidt et al., 2009) and K_{ATP} channel (ATP-sensitive potassium channel) (Yan et al., 2010), respectively, leading to the notion that J-proteins are responsible for triage decisions regarding whether channels are retained at the cell surface or degraded. So far, not much is known about the molecular chaperones that participate in the folding and degradation of channels.

This study examines the possible role played by synaptic J-proteins in the BK channel expression. Publications over the last decade indicated that CSPα play an important role in stabilizing the neurotransmission machinery, but it remains unclear how CSPα is involved in that. So far, it was able to show that CSPα target different protein at the synaptic terminal, but it was not possible to evaluate how CSPα and its target proteins are neuroprotective and how it is involved in neurotransmission. Here we evaluate whether a possible role for CSPα in targeting BK channel and thus is neuroprotective by changing it expression at the synaptic terminal and thus changes neurotransmission release. The selected J-proteins for this Master's thesis are: CSPα, a synaptic vesicle chaperone with anti-neurodegenerative activity, Hsp40, an inducible chaperone that protects cells against cell stress, and Rdj2, an abundant cytosolic chaperone were investigated.

1.6.2 Hypothesis and Objectives

The hypothesis of this Master-Thesis is that **"CSPα interacts with and regulates the expression of the BKα subunit of BK channels".** Three specific objectives were used to address this hypothesis.

My first aim was to determine whether CSPα and other J-proteins regulate BK channel expression. To this end, CAD cells were co-transfected with BK channel and J-proteins, and then BK channel expression was determined by Western Blot analyses. The effects of the proteasome inhibitor lactacystin and the agents (quercetin, TEA and dbcAMP) which either influence BK channel or CSPα were evaluated regarding their effect on BK channel expression. I also examined CSPα's effect on BKα-subunit expression in the presence of regulatory β-subunits of the BK channel.

My second objective was to identify regions/amino acids of CSPα which mediate changes in BKα channel protein expression. Therefore N- and C-terminal truncations, J-domain chimeras and HDP-AAA mutations (CSPα and Hsp40) were co-transfected with BK α-subunit and the total and cell surface expression level of BK channel were evaluated by Western Blot analyses. A co-immunoprecipitation strategy was used to determine if CSPα and BK channel interact.

The third aim was to establish a stable BK channel expressing CAD cell line in order to determine the role of J-proteins on endogenously expressed (rather than transient) BK channels. In BK channel stable cell lines, the influence of CSPα and Hsp40 as well as the corresponding HDP-AAA mutations on the cell surface and total BKα protein expression was explored.

1.6.3 Significance

Such J-protein-mediated changes in BK channel expression could lead to critical changes in neural excitability. Furthermore these results could give evidence that BK channel play a role in neurodegeneration and as well play a role in neuroprotection. This line of investigation will eventually lead to an understanding of the full extent of the chaperone machinery and pathways that regulate synaptic transmission.

2 METHODS

2.1 Molecular biology methods

This chapter describes standard procedures used throughout the experiments. All materials/suppliers are listed in the appendix to ensure the reproducibility of the experiments.

2.1.1 Purification of DNA plasmid from bacteria

To isolate plasmid DNA from bacteria, a plasmid-preparation kit (QIAfilter Plasmid Purification, Quiagen) was used. Bacteria from glycerol stocks were streaked out on LB-agar plates (1 % (w/v) Bacto Trypton, 1 % (w/v) NaCl, 0.5 % (w/v) Yeast extract and 1.5 % (w/v) Bacto Agar) with Ampicillin (100 μg/ml) as the selective antibiotic. The plates were incubated over night at 37 °C. Colonies were picked and 2 ml LB medium (1 % (w/v) Bacto Trypton, 1 % (w/v) NaCl, 0.5 % (w/v) Yeast extract) pre-cultures with Ampicillin (100 μg/ml) were prepared and incubated in a shaker incubator (200 rpm, 37 °C) for 2 h. For an overnight culture, 100 ml with Ampicillin was prepared. The pre-culture was added and the culture was incubated in a shaker incubator overnight at 200 rpm and 37 °C. The plasmid purification was performed the following day. Plasmid purification was carried out using the standard protocol described in the QIAfilter plasmid purification handbook.

In more detail, the following protocol was applied: bacteria were harvested by centrifugation at 4 000 rpm and 30 min at 4 °C. The bacterial pellet was resuspended in 10 ml P1 buffer, subsequently 10 ml P2 buffer were added and the solution was mixed thoroughly by inverting the tube and then incubated for 5 min. 10 ml P3 buffer were added and the resulting solution was mixed again. The lysate was poured into the barrel of the QIAfilter cartridge and incubated for 10 min. The lysate was filtered with a QIAfilter cartridge into the previously equilibrated QIAGEN-tip. The clear lysate entered the tip by gravity flow. The tip was washed twice with 30 ml QC buffer. Then the DNA was eluted with 15 ml QF buffer and the DNA was precipitated by adding 10.5 ml isopropanol. The solution was mixed and centrifuged at 14 000 rpm and 30 min at 4 °C. The supernatant was

decanted and the pellet was washed with 5 ml 70 % Ethanol. The solution was centrifuged at 14 000 rpm for 10 min at 4 °C. The resulting supernatant was discarded and the pellet was air dried, then dissolved in TE (10 mM Tris-Cl, pH 8.0 and 1 mM EDTA) buffer and the plasmid DNA concentration was measured.

2.1.2 Glycerol stocks

To store bacteria for extended periods. 800 µl bacteria was mixed with 200 µl Gycerol and stored at -80 °C.

2.1.3 DNA concentration

The DNA concentration was determined with a spectrometer (BioPhotometer, Eppendorf), by measuring the optical densities (OD) at 260 nm and 280 nm. The ratio of OD_{260} nm to OD_{280} nm shows the purity of the DNA. A protein free solution shows a ratio of 1.8-2.0.

2.2 Cell biological methods

To prevent the possibility of contamination, only sterile materials and solutions were used and all manipulations were performed under a sterile hood. The cells were kept in an incubator at 37 °C and 5 % CO_2 in a humidified environment.

2.2.1 Cultivation of CAD cells

The cell line used in these experiments was an adherent CNS catecholaminergic cell line, called CAD (CNS-derived catecholaminergic neuronal cell line). This cell line was established by targeted oncogenesis in transgenic mice and expresses neuronal properties (Qi et al., 1997).

The cells were grown in DMEM/F12 (Gibco® Invitrogen) medium supplemented with 10 % fetal bovine serum (Gibco® Invitrogen) and 1 % Penicillin/Streptomycin. For passaging the cells, the medium was aspirated, the cells were first washed with 1 x PBS

(137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 x 7 H_2O, 1.5 mM KH_2PO_4, pH 7.4) and then incubated with 2 ml Trypsin/EDTA (0.05 % Trypsin in 0.53 mM EDTA, Invitrogen) for 2 minutes. Subsequently, the enzymatic activity of Trypsin/EDTA was stopped by dilution and resuspension in medium. The cells were centrifuged at 2 000 rpm for 2 min. The old medium was discarded and the cells were resuspended in fresh medium, counted with a hemocytometer and seeded at the desired cell amount into either 100 mm cell culture plates or into 6-well plates. The cells were passaged every 3 to 4 days once they had reached ~ 80 % confluence.

2.2.2 Transient transfection of CAD cells

CAD cells were seeded into 6-well plates at a density of 400.000 cells/well or at a density of 2.000.000 cell/dish in a 100 mm culture plate one day before transfection. For one well, 6 µl Lipofectamine and 250 µl Opti-MEM were mixed together and incubated for 5 min at RT (Mix 1). 0.25 to 1 µg cDNA was diluted in 250 µl Opti-MEM and mixed (Mix 2). Mixture 1 and 2 were combined together and incubated for 20 min at RT. The medium on the cells was replaced by Opti-MEM and the mixture was added drop wise to the cells. For a transfection of a 100 mm culture plate the five times higher concentration of cDNA, Lipofectamine and Opti-MEM was used. The cells were incubated at 37 °C and 5 % CO_2 for 6 h, 24 h, and 48 h, respectively, either in the presence or absence of drugs. Afterwards, the cells were harvested and the protein concentration was determined.

2.2.2.1 cDNA Plasmids

Different plasmids were transiently transfected into CAD cells. The tested cDNA plasmids are listed in Table 7. I would like to thanks Robert Winkfein, from the Paul Schnetkamp laboratory for preparing all plasmids in a pCMV plasmid vector. All constructs were generated in a DH5α *E.coli* strain.

Table 7: List of cDNA constructs

Plasmid name	Plasmid	Tag
$CSP\alpha_{1\text{-}198}$ (full length)	pCMV plasmid	myc-tag
Hsp40	pCMV plasmid	myc-tag
Rdj2	pCMV plasmid	myc-tag
$CSP\alpha_{1\text{-}82}$	pCMV plasmid	myc-tag
$CSP\alpha_{1\text{-}90}$	pCMV plasmid	myc-tag
$CSP\alpha_{1\text{-}100}$	pCMV plasmid	myc-tag
$CSP\alpha_{1\text{-}112}$	pCMV plasmid	myc-tag
$CSP\alpha_{\Delta C}$	pCMV plasmid	myc-tag
$CSP\alpha_{83\text{-}198}$	pCMV plasmid	myc-tag
$CSP\alpha_{113\text{-}198}$	pCMV plasmid	myc-tag
$CSP\alpha_{137\text{-}198}$	pCMV plasmid	myc-tag
$CSP\alpha_{1\text{-}198\Lambda}JD_{Hsp40}$	pCMV plasmid	myc-tag
$CSP\alpha_{1\text{-}198\ \Lambda}JD_{Rdj2}$	pCMV plasmid	myc-tag
$CSP\alpha_{1\text{-}198\ \Lambda}JD_{Rme8}$	pCMV plasmid	myc-tag
$CSP\alpha_{HPD\text{-}AAA}$ (full length)	pCMV plasmid	myc-tag
BKα2	pcDNA3.1 plasmid	No tag
BKβ1	pcDNA3.1 plasmid	No tag
BKβ4	pcDNA3.1 plasmid	No tag
Hsc70	pcDNA3.1 plasmid	HA-tag
ATPase of Hsc70	pcDNA3.1 plasmid	HA-tag
Empty pCMV	pCMV plasmid	myc-tag

2.2.3 Establishing a stable BK channel expression CAD cell line

In order to generate a stable cell line that expresses the BK channel proteins, the first step was to test the sensitivity of the CAD cells to the antibiotic zeocin. The BK channel is cDNA is contained within pcDNA3.1 plasmid which consists of a zeocin-

resistant gene. To establish a stable transfected cell line, the minimum concentration required to kill untransfected cells was determined. To achieve this purpose, CAD cells were seeded into 6-well plates at an approximate density of 25 %. Medium with a variety of zeocin (Invitrogen) concentrations (0, 50, 100, 200, 400, 600, 800, and 1000 µg/ml) was added to the cells. The medium was changed every 3-4 days and the amount of surviving cells was observed over 2 weeks.

After determination of the suitable zeocin concentration (800 µg/ml) CAD cells were transiently transfected with the BK channel (as described in 2.2.2) in a 100 mm culture plate. 48 h after transfection, cells were plated and grown in selective medium. Non-transfected cells were used as negative control. The selective medium was switched every 3 to 4 days until cell foci were identified. Cell foci were picked up with cloning cylinders and transferred into 12-well plates before expanding to 6-well plates. The stable expression of the BK channel was determined by Western Blot analysis. Positive clones were plated on 100 mm plates and either used for further experiments or frozen for long-term storage.

2.2.4 Hippocampal neuron cultures preparation (Colicos Laboratory)

Hippocampal neurons were isolated by dissection from new born, postnatal day 0 rats (Sprague Dawley rats) (dissection carried out by Lucas Scott, Colicos Laboratory). After dissection, neurons were digested in a papain containing solution (20 U/ml, 50 µM EDTA, 150 mM $CaCl_2$, 100 mM L-cysteine) for 30 min at 37 °C. The papain solution was prepared in the BME cell culture medium (Basal Medium Eagle, Invitrogen) which contained 0.6 % glucose, 4 % FBS, 0.5 mM L-glutamine, 10 mM HEPES, 2 % B27, 15 mM natrium pyruvate and 100 µg/ml penicillin-streptomycin. On the day of the dissection, hippocampal neurons were transfected by electroporation. Hippocampal neurons were co-transfected with either 0.75 µg CSPα, 0.75 µg $CSP\alpha_{HPD\text{-}AAA}$, 1 µg Hsp40 or 1 µg $Hsp40_{HPD\text{-}AAA}$ cDNA with 0.5 µg cDNA CFP (cyan fluorescent protein). CFP transfected neurons were used as positive transfection control. After electroporation, cells were plated

on chips (Silicon wafers, Quest Silicon) and incubated at 37 °C until live cell imaging or Fluorescence staining was performed.

2.2.5 Live cell imaging in hippocampal neuron culture (Colicos Laboratory)

3, 6 or 12 days after transfection, cell viability was determined by live cell imaging. The chip was transferred from the medium into a small dish containing EBS (135 mM NaCl, 10 mM Glucose, 3 mM $CaCl_2$, 5 mM KCl, 2 mM $MgCl_2$, 5 mM HEPES, pH 7.35, 305 mOsm). Live cell imaging was performed by fluorescent microscopy (Olympus BX61W7 fluorescence slope), using a CFP filter (460-500 nm) and the AstroVid software to capture the corresponding images.

2.3 Protein biochemistry

2.3.1 Preparation of CAD cell lysates

Transfected or treated CAD cells were washed with PBS, treated with 300 µl CAD lysis buffer (40 mM Tris (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 0.1 % SDS, 1 % Triton X-100, 0.5 mM PMSF and protease inhibitor (Sigma)) and incubated briefly for 2-5 min on ice. The lysates were transferred into an Eppendorf tube and incubated on a rotator for 45 min at 4 °C. After this step, the samples were centrifuged at 15 000 rpm for 5 min at 4 °C. The supernatant (soluble fraction) and the pellet (insoluble fraction) were collected and stored at -20 °C.

2.3.2 Protein assay

The protein concentration was determined using the Bradford assay (BioRad). Briefly, a BSA (Bovine Serum Albumin, BioRad) standard curve was prepared to determine the unknown protein concentration of the samples. The BSA absorbance of standards and samples was measured with a spectrometer at a wavelength of 562 nm.

The samples were prepared at four different concentrations. To achieve this, 5 µl, 10 µl, 15 µl and 20 µl aliquots of each sample filled to a final volume of 100 µl with H_2O.

The BioRad detection reagent was diluted 1:4 in H_2O and 5 ml were added to the diluted samples. The samples were filled into cuvettes and were measured with a spectrometer (Ultrospec 3100 pro) and the final concentration was determined by comparing the standard curve to the samples.

2.3.3 SDS-Polyacrylamide-gel electrophoresis (SDS-PAGE) and Western Blotting

30 µg of soluble cell lysate were mixed with 4x Laemmli sample buffer (62.2 mM Tris Base pH 6.8, 7.5 % v/v Glycerol, 2 % w/v SDS, 0.015 mM Bromphenol Blue, 1 % v/v β-Mercaptoethanol and 100 mM DTT) denatured for 1 h at 37 °C. The denatured samples were loaded on a 10 % polyacrylamide resolving gel (30 % acrylamide, 1.5 mM Trip pH 8.8, 10 % SDS, 10 % APS, 1 % Temed, ddH_2O (fill up to desired volume)) and SDS-PAGE was carried out at constant (110 mV) voltage for ~2 h with a running buffer of the following composition: 25 mM Tris, 200 mM Glycine, 0.1 % SDS. A PageRuler prestained protein ladder (Invitrogen) was used as molecular weight marker. Proteins were transferred by the semi-dry method (BioRad) from the SDS-PAGE gel to nitrocellulose (Transfer Buffer: 25 mM Tris, 192 mM Glycine, 20 % Methanol, 0.2 % SDS). Following transfer, SDS-PAGE gels were stained with Coomassie Brilliant Blue. The proteins transferred onto the nitrocellulose membrane were visualized using a Ponceau S solution (Sigma). The nitrocellulose was blocked for non-specific antibody binding with 4 % milk PBS-T solution (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 x 7 H_2O, 1.5 mM KH_2PO_4, pH 6.9 and 0.1 % Tween) for 30 min. After blocking, the membrane was incubated with primary antibody (for the corresponding antibodies see Table 8) either overnight at 4 °C or for 2 h at RT. The membranes were washed 3 to 4 times with blocking solution and then incubated with a horseradish peroxidase-coupled secondary antibody (antibodies listed in Table 9) in 4 % milk in PBS-T solution. After the membranes were washed with PBS-T, protein detection was performed by chemiluminescence using Supersignal West Pico/Dura (Thermo Scientific). Immunoreactive bands were visualized following exposure of membranes to Kodak Film, and the corresponding chemiluminescent signals were

quantified in a BioRad Fluoro Multilayer Max instrument by using the Quantity One 4.2.1. software.

Table 8: List of primary antibodies used for Western Blot analysis

Antibody	Producer	Species	Concentration
Anti- K^+ channel α (monoclonal)	BD Bioscience	Mouse	1:2000
Anti-BKbeta4 potassium channel (monoclonal)	NeuroMab	Mouse	1:2000
Anti-BKbeta1 potassium channel (polyclonal)	Chemicon Millipore	Rabbit	1:2000
Anti-myc-tag (monoclonal)	Clontech	Mouse	1:6000
Anti-HA-tag (12CA5) (monoclonal)	Dr. Dallan Young (University of Calgary)	Mouse	1:2000
Anti-β-actin (monoclonal)	Sigma	Mouse	1:7000
Anti-ubiquitin (FK2)	Calbiochem®	Mouse	1:1000

Thanks to Dr. Dallan Young, Department of Biochemistry and Molecular Biology, University of Calgary who kindly provided me with the 12CA5 anti-HA-tag monoclonal antibody.

Table 9: List with used secondary antibodies for Western Blot analysis

Antibody	Producer	Species	Concentration
Goat anti-mouse IgG (H+L)-HRP	Jackson ImmunoResearch	Mouse	1:2000 to 1:8000
Goat ant-rabbit IgG (H+L)-HRP	Jackson ImmunoResearch	Rabbit	1:2000 to 1:8000

2.3.4 Biotinylation of cell surface protein

CAD cells were transfected with cDNA which encodes a cell surface protein as described in 2.2.2 in 100 mm culture plate. 24 h after transfection, the cells were washed

thrice in PBS (pH 8.0). CAD cells were incubated with EZ-Link Sulfo-NHS-SS Biotin (Thermo Scientific) (1 µg/ml) in PBS for 30 min at 4 °C. As negative control, cells were incubated only with PBS. The reaction was neutralized with 1 % BSA in PBS for 10 min at 4 °C. After neutralization, cells were washed thrice with ice-cold PBS to remove non-reacted biotinylation reagent. Cells were harvested with 1ml 1 % v/v Triton X-100 in PBS and protease inhibitor (complete, EDTA-free, Sigma) by an incubation for 2-5 minutes on ice. The lysates were transferred to an Eppendorf tube and centrifuged at 15 000 rpm for 15 min at 4 °C. The cellular protein concentration was determined as described in 2.3.2.

For the streptavidin pull-down, 1 mg of the total cellular protein lysate was incubated with 100 µl streptavidin agarose resin (50 % slurry) (Thermo Scientific) using a rotator incubation overnight at 4 °C. Beads were rinsed thrice with 1 % Triton X-100 in PBS. Biotinylated proteins were eluted from the beads by adding 2x Laemmli sample buffer (62.2 mM Tris Base (pH 6.8), 7.5 % v/v Glycerol, 2 % w/v SDS, 0.015 mM Bromophenol Blue, 1.2 % v/v β-Mercaptoethanol and 100 mM DTT) for 1 h at 37 °C. After elution of the proteins, samples were centrifuged for 1 min at 3500 rpm and eluted proteins were loaded onto a 10 % SDS-PAGE and were further processed as described in 2.3.3.

2.3.5 Co-immunoprecipitation of proteins

CAD cells were transfected with cDNA as described in 2.2.2 in 100 mm culture plates. 24 h after transfection, cells were lysed with 1 ml 1 % v/v Triton X-100 in PBS and protease inhibitor (complete, EDTA-free, Sigma) by incubation 2-5 minutes on ice. The lysates were transferred to an Eppendorf tube and centrifuged at 15 000 rpm for 15 minutes at 4 °C to remove any insoluble material. The pellet was discarded and the supernatant was transferred into a fresh Eppendorf tube. The total cellular protein concentration was determined (as described in 2.3.2) and 0.7 mg total cellular protein was used for the co-immunoprecipitation experiment. The lysate was pre-cleared by a 1 hour rotation at 4 °C with Protein A/G-coupled agarose beads (50 % w/v slurry) (Pierce). This step was used to remove any material that would otherwise bind non-specifically to beads during later incubation steps. 5 µg primary antibody (antibodies listed in Table 10) was added to the

pre-cleared supernatant and everything was rotated overnight at 4 °C. After this incubation step, 20 µl Protein A/G coupled beads (50 % w/v slurry) were added to the supernatant and the incubation was continued for another 2 h. The Protein A/G-coupled beads were centrifuged at 5 000 rpm for 2 min at 4 °C. The pellet was rinsed thrice with 0.1 % v/v Triton X-100 in PBS. The supernatant from each washing step was discarded. The proteins were eluted from the beads by resuspension in 2x Laemmli samples buffer and an incubation step of 1 h at 50 °C. Proteins were loaded onto a 10 % SDS-PAGE and further processed as described in 2.3.3.

Table 10: List of primary antibodies used for co-immunoprecipitation

Antibody	**Producer**	**Species**
Anti-maxi K (BK_{CA}) channel (polyclonal)	Chemicon Millipore	Rabbit
Anti-myc-tag (monoclonal)	Clontech	Mouse

2.3.6 Fluorescence staining of hippocampal neuron cultures (Colicos Laboratory)

After live cell imaging, hippocampal neurons were fixed with a mixture of 15 % Picric Acid and 4 % PFA in PBS for 20 min at RT. After fixation, the cells were washed thrice with PBS for 5 min and then blocked with blocking solution (5 % donkey serum, 2 % BSA, 0.1 % Triton X-100) in PBS for 1 h at RT. Primary antibody (the employed antibodies are listed in Table 11) was diluted in blocking solution and incubated with the hippocampal neurons under continuous shaking overnight at 4 °C. Afterwards, the cells were washed thrice for 5 minutes with blocking solution and then incubated for 1 h in the dark with the secondary antibody (specific for certain fluorescence wavelengths, see list of antibodies utilized in Table 12) diluted with blocking solution. Another three 5-minute washing steps with PBS were carried out in the dark before acquiring images. Images were captured by AstroVid on a fluorescent microscope with a specific filter for detection (green 550-579 nm for Cy3, red 650-670 nm for Cy5) to monitor the specific binding of the secondary antibody. Images were merged by using ImageJ.

Table 11: List of primary antibodies used for fluorescence staining

Antibody	Producer	Species	Concentration
Anti-maxi K (BK_{CA}) channel (polyclonal)	Chemicon Millipore	Rabbit	1:800
Anti-myc-tag (monoclonal)	Clontech	Mouse	1:1000

Table 12: List of used secondary antibodies used for fluorescence staining

Antibody	Producer	Species	Concentration
Cy3-conjugated (green 550-579 nm)	Jackson ImmunoResearch	Rabbit	1:2000
Cy5-conjugated (red 650-670 nm)	Jackson ImmunoResearch	Mouse	1:1000

2.4 Statistical Analysis

The BK channel expression of the Western Blot analysis was quantified by QualityOne 4.2.1 software. The data were normalized to negative control (pCMV empty vector; 100 %) and presented as mean ± SEM. Statistical calculations were done with Microsoft Excel using t-test (one-way ANOVA). Differences were assumed to be statistical significant if $p < 0.05$ or strong significant if $p < 0.001$.

3 RESULTS

CHAPTER I: Transiently transfected CAD cells

CSPα was first identified in *Drosophila melanogaster* where its deletion resulted in temperature sensitive paralysis and reduced life span (Zinsmaier et al., 1994). In mice, CSPα deficient animals initially function normally but develop a progressive degeneration and mutant mice die around 8 weeks of age (Fernández-Chacón et al., 2004). The rate of degeneration differs among synapses with a marked sensitivity of highly active GABAergic neurons (García-Junco-Clemente et al., 2010). Use-dependent degeneration suggests that the degenerative pathway in CSPα-null mice may well involve a presynaptic channel and/or exocytosis/endocytosis machinery. CSPα is a synaptic vesicle-anchored J-protein that is known to interact with and activate the chaperone Hsc70 (Braun et al., 1996) indicating that CSPα is almost certainly important for presynaptic protein conformational work. A number of proteins have been proposed as client substrates for CSPα (Table 6), however given these large size of the J-protein family (Zhao et al., 2008) it has been challenging to establish if the putative targets are CSPα-specific substrates or J-protein-substrates (i.e. which J-protein targets which client protein). In fact, in mammalian neurons it has not been clearly shown if there is a functional one-to-one correspondence between J-proteins and client proteins. Utilizing a transient co-expression strategy I compared the concentration- and time-dependence influence of CSPα, Hsp40 and Rdj2 on BK channel expression levels. Additionally, CSPα C-terminal truncation, CSPα N-terminal truncation, CSPα chimeras and CSPα HPD-AAA mutants were utilized to identify the regions crucial for modulating steady state BK channel expression.

3.1 J-proteins reduce BK channel expression

At the beginning of the present study it was not known if chaperones altered BK channel expression or function. Initially I set out to determine if J-proteins influence BK channel expression. To determine whether CSPα and other J-proteins (Rdj2 and Hsp40) have an effect on BK channel expression, CAD cells were co-transfected with BKα protein

in the presence and absence of CSPα, Rdj2 or Hsp40 (constructs are listed in Table 7). As negative control, the emtpy pCMV plasmid was co-transfected with BK channel. Cells were harvested 24 h after transfection and protein concentration was measured (described in 2.3.2). 30 µg of the protein samples were separated based on their molecular weight by SDS-PAGE and transferred onto a nitrocellulose membrane (described in 2.3.3). BK channel expression was visualized by using a primary anti- BK channel antibody. A chemiluminescent reaction was used to detect specific bands upon exposure on a Kodak x-ray Film. Quantification was performed on a Bio-Rad Fluor-S MultiImager Max with the Quantity One 4.2.1 software.

3.1.1 CSPα reduces BK channel expression

CAD cells were co-transfected with either 0.25 µg, 0.5 µg or 0.75 µg myc-tagged CSPα cDNA, and with 1 µg BKα channel cDNA. The results of this experiment are illustrated in Figure 4.

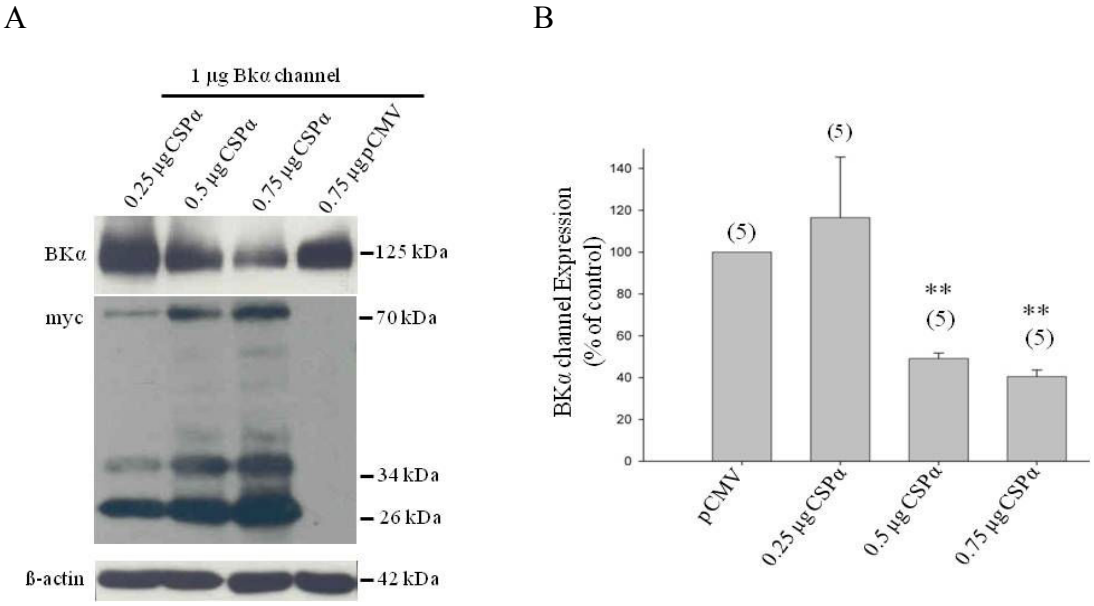

Figure 4: CSPα reduces BK channel expression in a concentration-dependent manner. Panel A. CAD cells were transiently transfected with 1 µg cDNA encoding BK channel and myc-tagged CSPα in different concentrations (0.25 µg, 0.5 µg and 0.75 µg cDNA). As negative control, 0.75 µg empty pCMV plasmid was co-transfected with 1 µg cDNA encoding the BK channel. 24 h after transfection, cells were lysed and protein expression was analyzed by Western blotting with an anti-BK channel antibody to visualize the BK channel. The myc expression is shown as transfection control for CSPα, while ß-actin staining represents the loading control. Panel B. Corresponding mean data compare to pCMV control. * $p < 0.001$

The graph in Figure 4 shows the Western Blot analysis (A) of BK channel co-expressed with CSPα and the corresponding mean data (B). The BK channel α-subunit is a 125 kDa protein. As shown in Figure 4A, with increasing concentration of CSPα cDNA, the BK channel expression decreases. The expression of myc-tagged CSPα is shown in the middle panel, and three distinct protein bands are observed. The mature form of CSPα is 34 kDa palmitoylated protein. The immature CSPα is a 26 kDa protein. CSPα also forms a stable CSPα-CSPα 70 kDa protein dimer in confirmation of previous studies (Braun and Scheller, 1995; Swayne et al., 2003; Xu et al., 2010). The lower panel in Figure 4A shows the β-actin expression, which represents the loading control.

Figure 4B illustrates the corresponding mean data for BK channel expression, normalized to the pCMV control (100 %). The data clearly indicate that, with higher concentrations of CSPα, the BK channel expression is strongly reduced. Transient

expression of 0.25 µg CSPα (116.5 ± 28.9 %) does not change BK channel expression compared to the control (100 %), whereas both the overexpression of 0.5 µg (49.1 ± 2.6 %) and of 0.75 µg (40.5 ± 3.1 %) CSPα show a statistically significant reduction of BK channel compared to the negative control. These results indicate that CSPα has a concentration-dependent reducing effect on BK channel expression.

3.1.2 Rdj2 reduces BK channel expression

Next we examined the possibility that other members of the J-protein family also alter BK channel expression. CAD cells were co-transfected with either 0.25 µg, 0.5 µg, 0.75 µg or 1 µg myc-tagged Rdj2 and 1 µg BK channel. Figure 5 shows the Western Blot analysis (A) and the corresponding mean data (B).

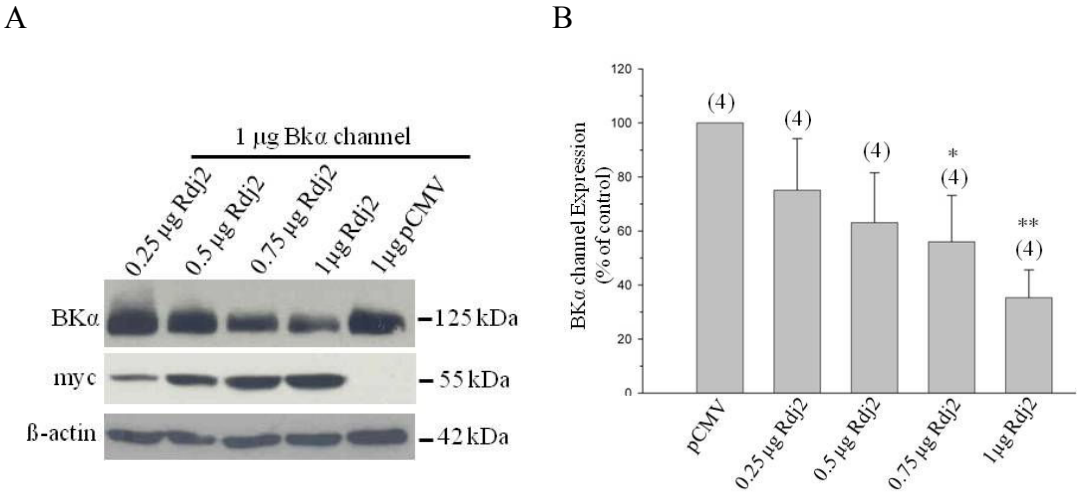

Figure 5: Rdj2 reduces BK channel expression in a concentration-dependent manner. Panel A. CAD cells were transiently transfected with 1 µg cDNA encoding the BK channel and myc-tagged Rdj2 in different concentrations (0.25 µg, 0.5 µg, 0.75 µg and 1 µg). As negative control, 1 µg empty pCMV plasmid was co-transfected with 1 µg cDNA encoding the BK channel. 24 h after transfection, cells were lysed and protein expression was analyzed by Western Blotting with an anti-BK channel antibody to visualize the BK channel. The myc expression is shown as transfection control for Rdj2 while ß-actin represents the loading control. Panel B. Corresponding mean data compare to pCMV control. * $p < 0.05$; ** $p < 0.001$

In the upper panel in Figure 5A, it is seen that the 48 kDa Rdj2 protein reduces BK channel expression and that the reduction is more effective with an increased concentration

of Rdj2 cDNA. The middle panel shows the transfection control of myc-tagged Rdj2 expression. β-actin represents the loading control (lower panel).

Figure 5B displays the corresponding mean data of four separate experiments. The BK channel was normalized to the pCMV control (100 %). Rdj2 cDNA slightly reduces BK channel expression when added in modest amount, i.e. 0.25 µg (75.1 ± 19.1 %) and 0.5 µg (63.1 ± 18.5 %). In contrast, addition of 0.75 µg (56.0 ± 17.2 %) shows a statistically significant effect and the further addition of 1 µg (35.3 ± 10.3 %) Rdj2 cDNA shows a strong statistically significant effect on BK channel expression compared to the pCMV control (100 %).

3.1.3 Hsp40 reduces BK channel expression

The graph in Figure 6 shows the results of the co-expression of BK channel with Hsp40. For this experiment, CAD cells were co-transfected with either 0.25 µg, 0.5 µg or 0.75 µg and 1 µg myc-tagged Hsp40 with 1 µg BK channel. Figure 6 presents the Western Blot results and shows the corresponding mean data of BK channel expression.

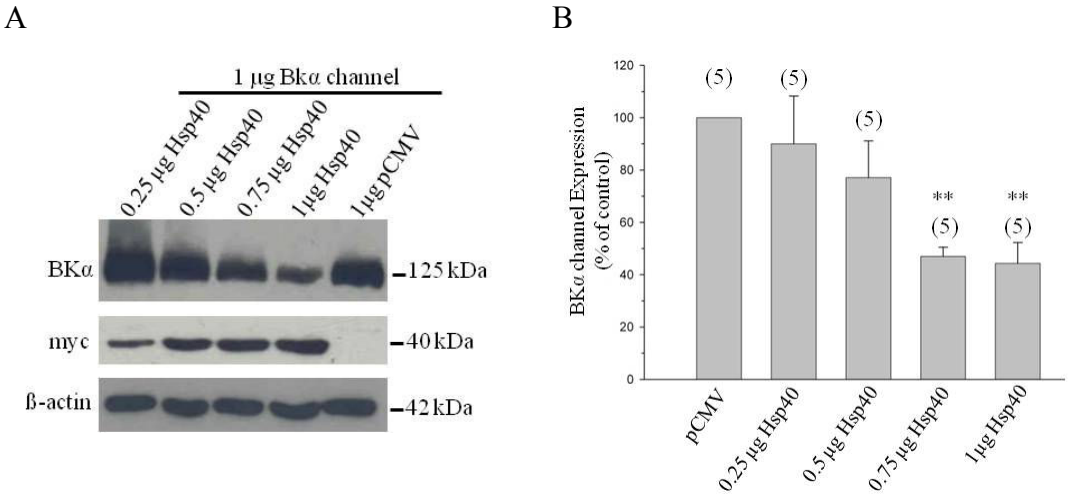

Figure 6: Hsp40 reduces BK channel expression in a concentration-dependent manner. Panel A. CAD cells were transiently transfected with 1 µg cDNA encoding the BK channel and myc-tagged Hsp40 in different concentrations (0.25 µg, 0.5 µg, 0.75 µg and 1 µg). As negative control, 1 µg empty pCMV plasmid was co-transfected with 1 µg cDNA encoding the BK channel. 24 h after transfection, cells were lysed and protein expression was analyzed by Western Blotting with an anti-BK channel antibody to visualize the BK channel. The myc expression is shown as transfection control for Hsp40 while ß-actin represents the loading control. Panel B. Corresponding mean data compared to pCMV control. * $p < 0.001$

Figure 6A (upper panel) shows that BK channel expression is reduced in the presence of Hsp40, and this reduction is increased when a higher amount of Hsp40 cDNA is used. The middle panel illustrates the expression of myc-tagged Hsp40. Hsp40 has a molecular weight of 40 kDa. β-actin was used as loading control (lower panel).

Figure 6B shows the corresponding mean data of five individual experiments, which were normalized to the negative control (100 %). The overexpression of 0.25 µg (90.0 ± 18.3 %) and 0.5 µg (77.1 ± 14.0 %) Hsp40 display non-statistical reductions, while the addition of 0.75 µg (47.0 ± 3.5 %) and 1 µg (44.3 ± 8.0 %) Hsp40 cDNA show a strong statistically significant reduction of the BK channel expression compared to the negative control (100 %).

Overall, these results show that Hsp40 decreases BK channel expression in a concentration-dependent manner.

3.2 J-proteins reduce BK channel expression in a time-dependent manner

Since CSPα, Rdj2 or Hsp40 reduced BK channel expression in a concentration-dependent manner we next determined if these J-proteins have the same effect on BK channel expression over a longer incubation time. First, we evaluated if the BK channel expression is stable over a 48 h incubation time. Figure 7 demonstrates that transient BK channel overexpression is stable over a 48 h incubation period. Therefore, CAD cells were co-transfected with cDNA encoding the BK channel and either CSPα, Rdj2 or Hsp40 cDNA, and incubated for either 24 h or 48 h (as described in 2.2.2). The cells were then harvested and protein concentration was measured. 30 µg of protein samples were run on a SDS-PAGE and transferred onto a nitrocellulose membrane (for the protocol see 2.3.3). The membrane was probed with an anti-BK channel antibody to visualize the BK channel, an anti-myc-tag antibody as control for CSPα, Rdj2 and Hsp40 expression, and β-actin as loading control, respectively. A chemiluminescent reaction was used to detect specific bands upon exposure on a Kodak Film. Quantification was performed on a Bio-Rad Fluor-S MultiImager Max with Quantity One 4.2.1 software.

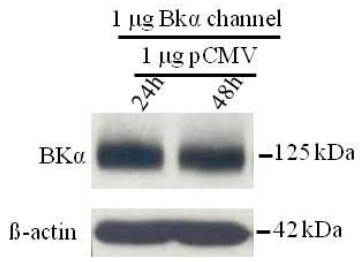

Figure 7: BK channel expression is stable over 48 h incubation time.
CAD cells were transiently transfected with 1 µg cDNA encoding the BK channel with 1 µg empty pCMV plasmid as negative control. 24 h after and 48 h after transfection the cells were lysed and protein expression was analyzed by Western Blotting with an anti- BK channel antibody to visualize BK channel. ß-actin expression represents the loading control.

3.2.1 CSPα reduces BK channel expression in a time-dependent manner

Figure 8 shows the co-expression of 0.25 µg and 0.75 µg myc-tagged CSPα with BK channel over a 24 h- and a 48 h- transfection incubation time. The Western Blot analysis in Figure 8A indicates that a longer incubation time (48 h) decreased BK channel expression at both of the tested concentrations (0.25 µg or 0.75 µg). BK channel expression shows a stronger reduction with a higher concentration of CSPα cDNA. This confirms the concentration-dependent effect of CSPα on BK channel expression described in section 3.1.1. The myc expression (middle bands) shows that the CSPα reduces BK channel expression at 48 h relative to 24 h. The β-actin expression (lower panel) represents the loading control.

Figure 8B shows the corresponding mean data of four separate experiments, which were normalized to the BK channel expression at 24 h (100 %). The results show that 0.25 µg CSPα reduces BK channel expression with a strong statistical significance from 100 % at 24 h to 42.5 ± 9.0 % at 48 h. The co-transfection of 0.75 µg CSPα strongly reduced BK channel expression in a statistically significant manner to 20.4 ± 3.8 % at 48 h compared to 24 h (100 %). Overall, these results indicate that a longer incubation times accentuated CSPα-mediated reduction in BK channel expression.

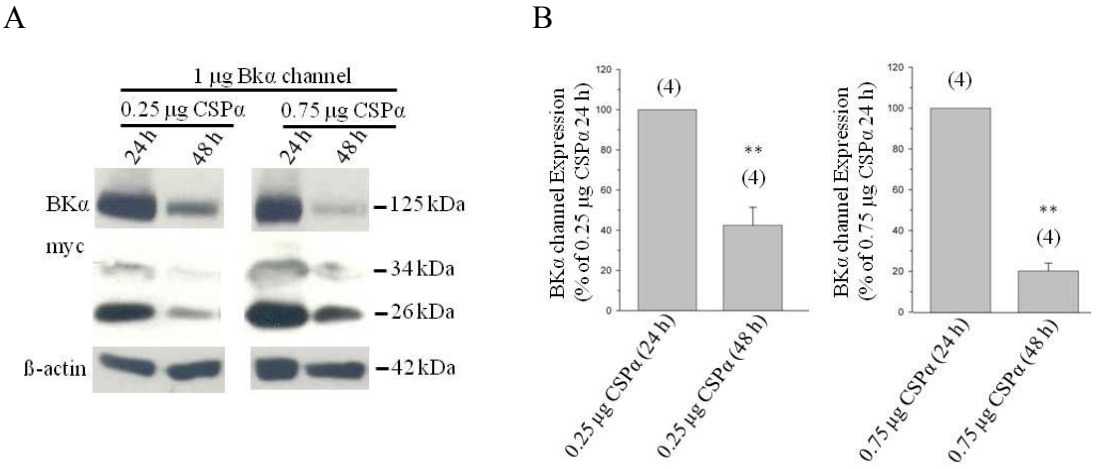

Figure 8: CSPα reduces BK channel expression in a concentration- and time-dependent manner.
Panel A. CAD cells were transiently transfected with 1 µg cDNA encoding the BK channel and myc-tagged CSPα in two different concentrations (0.25 µg and 0.75 µg) and incubated for 24 h and 48 h. The cells were lysed and protein expression was analyzed by Western Blotting with an anti- BK channel antibody to visualize BK channel. Myc expression was used as transfection control for CSPα and ß-actin represents the loading control. Panel B. The corresponding mean data, comparing the 24 h and 48 h effects normalized to the expression level in the presence of 0.25 µg or 0.75 µg CSPα at 24 h
* $p < 0.05$; ** $p < 0.001$

3.2.2 Rdj2 reduces BK channel expression in a time-dependent manner

The time dependency of Rdj2 reduction in BK expression was also evaluated. For these experiments, 0.25 µg and 1 µg Rdj2 cDNA were co-expressed with 1 µg BK channel and transfected cells were incubated 24 h and 48 h. Figure 9 presents the Western Blot analysis and the corresponding mean data. Figure 9A shows that 48 h incubation reduces BK channel more effectively compared to 24 h. This effect is seen with both tested concentrations of Rdj2. The myc expression in Figure 9 presents the transfection control of Rdj2 and the β-actin expression represents the loading control.

The corresponding mean data in Figure 9B were normalized to the expression of the BK channel at 24 h. The co-expression of 0.25 µg Rdj2 reduced BK channel expression in a statistically significant manner from 100 % at 24 h to 66.4 ± 7.7 % at 48 h. 1 µg Rdj2 (55.5 ± 15.5 %) also shows a statistically significant reduction in BK channel expression compared to 24 h (100 %).

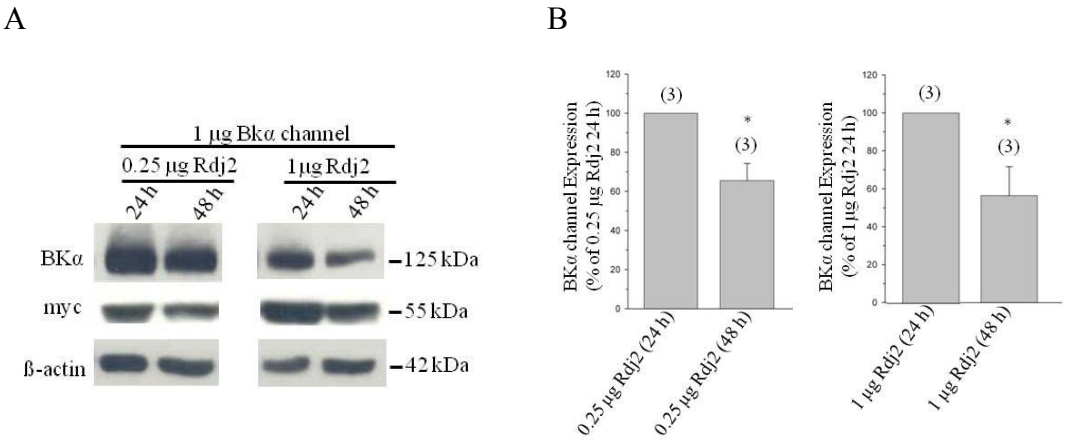

Figure 9: Rdj2 reduces BK channel expression in a concentration- and time-dependent manner.
Panel A. CAD cells were transiently transfected with 1 µg cDNA encoding the BK channel and myc-tagged Rdj2 in two different concentrations (0.25 µg and 1.0 µg) and incubated for 24 h or 48 h. The cells were lysed and protein expression was analyzed by Western Blotting with an anti- BK channel antibody to visualize BK channel. Myc expression was used as transfection control for Rdj2 and ß-actin represents the loading control. Panel B. The corresponding mean data, comparing the 24 h and 48 h effects normalized to the expression level in the presence of 0.25 µg or 0.75 µg Rdj2 at 24 h; * $p < 0.05$

3.2.3 Hsp40 reduces BK channel expression in a time dependent manner

The Western Blot results of the co-expression of the BK channel with Hsp40 at two different time points (24 h to 48 h) and with two different concentrations, 0.25 µg and 1 µg, are shown in Figure 10. The co-transfection of 0.25 µg Hsp40 slightly reduced BK channel expression in the comparison of 48 h to 24 h. A stronger reductive effect on BK channel expression was observed when 1 µg Hsp40 cDNA were used. The myc expression in Figure 10 presents the transfection control of Rdj2 and the β-actin expression represents the loading control.

The mean data in Figure 10B correspond to the Western Blotting results displayed in Figure 10A. A longer incubation time (48 h vs. 24 h) leads to a stronger effect in terms of reducing BK channel expression. 0.25 µg Hsp40 cDNA strongly decreases BK channel expression in a statistically significant manner from 100 % at 24 h down to 57.8 ± 8.4 % after 48 h. 1 µg Hsp40 cDNA decreased BK channel expression to 56.2 ± 11.8 %.

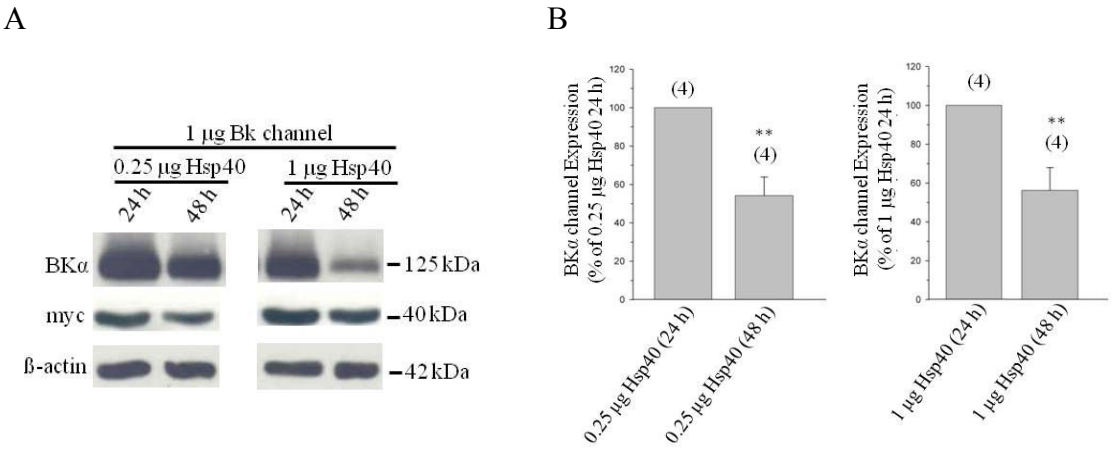

Figure 10: Hsp40 reduces BK channel expression in a concentration- and time-dependent manner.
Panel A. CAD cells were transiently transfected with 1 µg cDNA encoding the BK channel and myc-tagged Hsp40 at two different concentrations (0.25 µg and 1.0 µg) and incubated the cells for 24 h and 48 h. The cells were lysed and protein expression was analyzed by Western Blotting with an anti- BK channel antibody to visualize BK channel. Myc expression was used as transfection control for Hsp40 and ß-actin represents the loading control. Panel B. The corresponding mean data, comparing the 24 h and 48 h effects normalized to the expression level in the presence of 0.25 µg or 0.75 µg Hsp40 at 24 h; * $p< 0.05$; ** $p< 0.001$

Overall, these results indicate that all J-proteins evaluated show a time dependent reduction in BK channel expression.

3.3 Determination of the specifically essential CSPα domain

The next set of experiments focused on identification of the CSPα domain and the individual amino acids which are responsible for the CSPα mediated reduction in BK channel expression. For this purpose, CAD cells were co-transfected with the BK channel and a variety of truncated or mutated myc-tagged CSPα cDNA constructs. Cells were harvested 24 h after transfection and protein concentration was measured. 30 µg protein samples were run on a 10 % SDS-Page and proteins were transferred on to a nitrocellulose membrane and probed with a BK channel antibody to visualize BK channel expression (described in 2.3.3). A chemiluminescent reaction was used to detect specific bands by

recording on Kodak Film. Quantification was performed on a Bio-Rad Fluor-S MultiImager Max with the Quantity One 4.2.1 software. The myc-tag antibody was used to evaluate if the constructs were expressed in the cells and the β-actin antibody was used as loading control.

3.3.1 C-terminal truncation of CSPα

In the first experiments we aimed to identify if the N-terminal region of CSPα plays an important role in reducing the BK channel expression. 1 µg BK channel was co-transfected with 0.75 µg C-terminally truncated CSPα constructs (the structures are illustrated in Figure 11) and incubated for 24 h. As a negative control, 0.75 µg pCMV plasmid was co-transfected with 1 µg BK channel. The tested constructs were $CSP\alpha_{1-198}$ representing the full length CSPα, $CSP\alpha_{1-82}$, $CSP\alpha_{1-90}$, $CSP\alpha_{1-100}$ and $CSP\alpha_{1-112}$. The construct $CSP\alpha_{1-82}$ contains the short N-terminus and the J-domain. In addition to these, the constructs $CSP\alpha_{1-90}$ and $CSP\alpha_{1-100}$ contain parts of the hydrophobic linker region. The $CSP\alpha_{1-112}$ construct lacks the cysteine string and the C-terminal region.

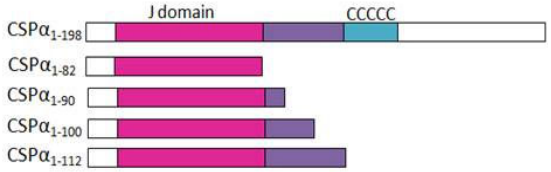

Figure 11: Schematic structure of myc-tagged CSPα C-terminus truncated constructs.
The graph presents a schematic structure of N-terminus myc-tagged full length CSPα and C-terminus truncated myc-tagged CSPα constructs. The top construct illustrates the CSPα full length: The white part N-terminus is followed by the pink J-domain and the violet hydrophobic linker region. The blue part illustrated the cysteine string domain which is followed by the white C-terminal.

The graph in Figure 12A shows the Western Blot analysis of the tested C-terminally truncated CSPα constructs (Figure 11). The upper panel illustrates the BK channel expression. All the five tested constructs show a reduction in BK channel expression. The constructs $CSP\alpha_{1-82}$ and the $CSP\alpha_{1-112}$ produce the strongest effects. The full length $CSP\alpha_{1-198}$, $CSP\alpha_{1-90}$ and $CSP\alpha_{1-100}$ have a slightly weaker reducing effect on BK channel expression compared to $CSP\alpha_{1-82}$ and the $CSP\alpha_{1-112}$. The middle panel shows the expression of myc and represents the transfection control for the tested constructs. The constructs were visualized with a myc-tag antibody separated on a 15 % gel. The lower band shows β-actin expression, representing the loading control.

Figure 12B shows the corresponding mean data of four separate experiments. The BK channel expression was normalized to the negative control (100 %). As shown, all C-terminal CSPα truncations led to a decrease in BK channel expression. $CSP\alpha_{1-198}$ (28.3 ± 16.9 %), $CSP\alpha_{1-90}$ (41.9 ± 10.9 %) and $CSP\alpha_{1-100}$ (48.1 ± 9.5 %) reduce BK channel expression in a statistically significant manner. $CSP\alpha_{1-82}$ (15.1 ± 8.2 %) and $CSP\alpha_{1-112}$ (16.5 ± 8.6 %) show a strong, statistically significant reduction in BK channel expression compared to the negative control.

Taken together, these results indicate that the N-terminal region, which includes the J-domain of CSPα, is functionally important for controlling BK channel expression.

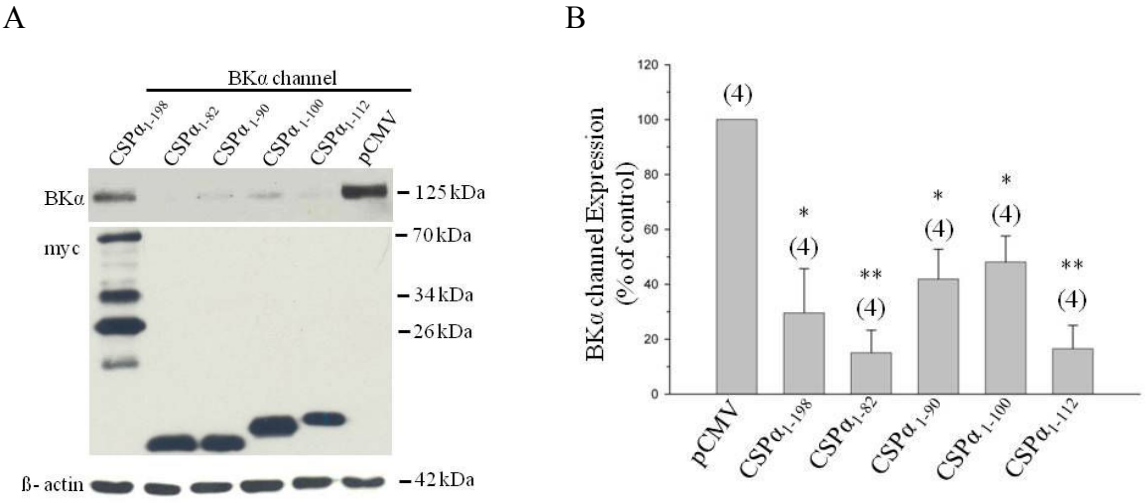

Figure 12: The J-domain of CSPα reduces BK channel expression.
A. Western Blot analysis of BK channel expression in CAD cells which were transiently co-transfected with 1 µg BK channel-encoding cDNA with either 0.75 µg myc-tagged full length CSPα or the constructs which are indicated on top of the corresponding gel lanes and incubated for 24 h. As negative control, 0.75 µg empty pCMV plasmid was co-transfected with 1 µg BK channel-encoding cDNA. 30 µg cell lysate were separated by SDS-PAGE and, probed with an anti-BK channel antibody. The myc expression is shown as transfection control for CSPα and ß-actin presents the loading control. The corresponding mean data is shown in panel B in comparison to the pCMV control; * $p < 0.05$; ** $p < 0.001$

3.3.2 N-terminal truncation of CSPα

We wanted to determine whether additional CSPα regions also modulate BK channel expression. 1 µg BK channel-encoding cDNA was co-transfected with 0.75 µg cDNA encoding various N-terminal CSPα truncations. The tested constructs were full length CSPα, $CSP\alpha_{83\text{-}198}$, $CSP\alpha_{113\text{-}198}$, $CSP\alpha_{137\text{-}198}$ and $CSP\alpha_{\Delta C}$, as displayed in Figure 13. The construct $CSP\alpha_{83\text{-}198}$ lacks the N-terminal J-domain, but contains the hydrophobic linker region, the cysteine string domain and the C-terminal region. The construct $CSP\alpha_{113\text{-}198}$ contains the cysteine string domain and the C-terminal region. The construct $CSP\alpha_{137\text{-}198}$ only contains the C-terminal region. $CSP\alpha_{\Delta C}$ was also tested; this construct has all CSPα domains except the cysteine string domain. As negative control, 0.75 µg empty pCMV plasmid was co-transfected with 1 µg BK channel.

Figure 13: Schematic structure of myc-tagged CSPα N-terminus truncated constructs.
The graph presents a schematic structure of N-terminus myc-tagged full length CSPα and N-terminus truncated myc-tagged CSPα constructs. The top construct illustrates the CSPα full length: The white part of the N-terminus is followed by the pink J-domain and the violet hydrophobic linker region. The blue part illustrates the cysteine string domain which is followed by the white C-terminal.

The results of the Western Blot analysis of the tested constructs are shown in Figure 14. The upper panel in Figure 14A shows the BK channel expression and illustrates that the constructs $CSP\alpha_{1\text{-}198}$, $CSP\alpha_{\Delta C}$ and $CSP\alpha_{83\text{-}198}$ reduced BK channel expression compared to the negative control, whereas constructs $CSP\alpha_{113\text{-}198}$ and $CSP\alpha_{137\text{-}198}$ induce no changes in BK channel expression compared to the pCMV control. The middle panel shows the myc expression and represents the expression control of the tested myc-tagged construct on a 15 % gel. For the construct $CSP\alpha_{83\text{-}198}$, no myc expression was detectable, suggesting that this construct is either secreted (due the N-terminal hydrophobic sequence) or not expressed. The β-actin band (lower panel) represents the loading control.

The corresponding mean data are assembled in Figure 14B. The $CSP\alpha_{1\text{-}198}$ (30.3 ± 15.1 %) and $CSP\alpha_{83\text{-}198}$ (21.8 ± 14.7 %) reduced the BK channel expression in a statistically significant manner. The construct $CSP\alpha_{\Delta C}$ (18.8 ± 7.0 %) shows a strong statistically significant reduction compared to the negative control (100 %). The constructs $CSP\alpha_{113\text{-}198}$ (97.3 ± 30.5 %) and $CSP\alpha_{137\text{-}198}$ (87.3 ± 7.6 %) had no effect on BK channel expression compared to negative control (100 %).

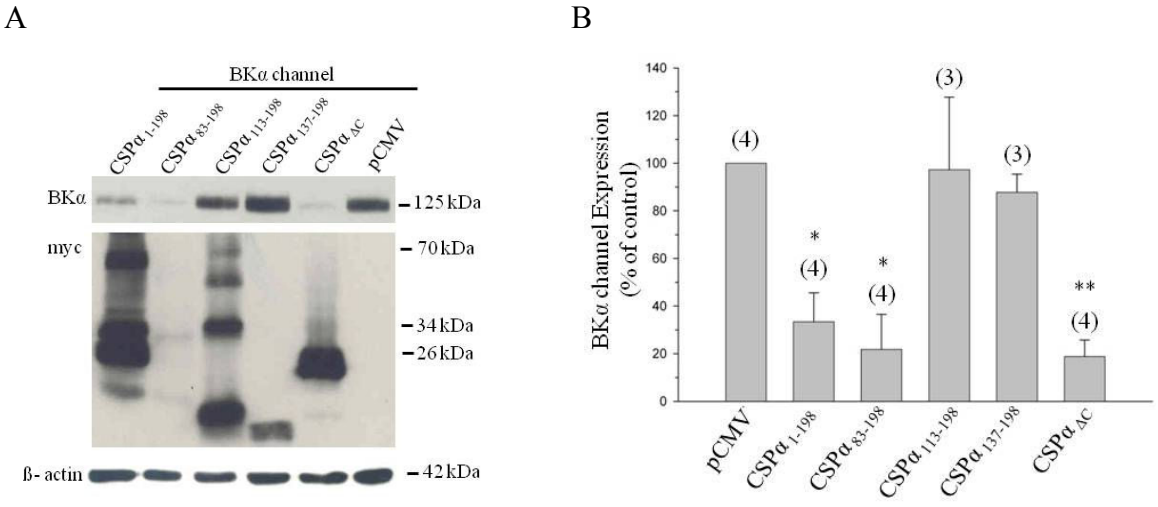

Figure 14: The C-terminus of CSPα does not alter BK channel expression.
Panel A. Western Blot analysis of BK channel expression in CAD cells which were transiently co-transfected with 1 µg BK channel-encoding cDNA with either 0.75 µg myc-tagged full length CSPα or the constructs which are indicated on top of the corresponding gel lanes and incubated for 24 h. As negative control, 0.75 µg empty pCMV plasmid were co-transfected with 1 µg BK channel-encoding cDNA. 30 µg cell lysate were separated by SDS-PAGE, probed with an anti-BK channel antibody. The myc expression is shown as transfection control for CSPα and ß-actin presents the loading control. The corresponding mean data is shown in panel B in comparison to the pCMV control; * $p < 0.05$; ** $p < 0.001$

3.3.3 CSPα chimeras (CSPα with the J-domain of Hsp40, Rdj2 or Rme8)

The previous experiments showed that the N-terminal regions (comprising the N-terminus and the J-domain) reduce BK channel expression. It is of further interest to determine if the J-domains of other J-proteins have an effect on BK channel expression. To evaluate this potential effect, CSPα chimeras were tested. The tested constructs are shown in Figure 15. These constructs have a CSPα background and only their J-domain was replaced with corresponding J-domains from the J-proteins Hsp40, Rdj2 and Rme8.

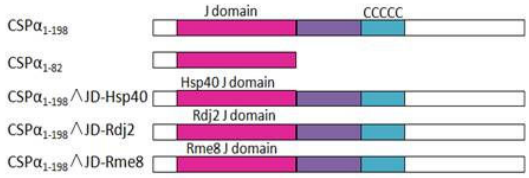

Figure 15: Schematic structure of N-terminus myc-tagged CSPα chimeras.
The graph shows the schematic structures of myc-tagged full length CSPα and CSPα chimera constructs. In the indicated CSPα chimeras' constructs, the J-domain of CSPα was replaced with the J-domain of other J-protein family members (Hsp40, Rdj2 and Rme8). The top construct illustrates the CSPα full length: The white part N-terminus is followed by the pink J-domain and the violet hydrophobic linker region. The blue part illustrated the cysteine string domain which is followed by the white C-terminal.

CAD cells were co-transfected with 1 µg BK channel and 0.75 µg of each construct (Figure 15). As negative control, 0.75 µg pCMV empty plasmid was co-transfected with the BK channel. The results of the Western Blot analysis and the corresponding mean data are shown in Figure 16. The top panel in Figure 16A shows that all CSPα chimeras decrease BK channel expression compared to the negative control. The middle panel illustrates the myc expression and shows that all constructs are equally expressed. The expression of the construct $CSP\alpha_{1\text{-}82}$ is not seen on the Western Blot analyses because the samples were run on a 10 % gel and the small construct run out of the gel (Figure 12 shows the $CSP\alpha_{1\text{-}82}$ construct expression on a 15 % gel). The β-actin expression represents the loading control.

The corresponding mean data in Figure 16B demonstrate that $CSP\alpha_{1\text{-}198}$ (22.6 ± 3.4 %), $CSP\alpha_{1\text{-}82}$ (17.4 ± 8.1 %) and the CSPα chimeras - $CSP\alpha JD_{Hsp40}$ (17.0 ± 3.7 %), $CSP\alpha JD_{Rdj2}$ (23.8 ± 2.0 %) and $CSP\alpha JD_{Rme8}$ (17.5 ± 9.8 %) - show a statistically significant reduction on BK channel expression. The mean data were normalized to the negative control (100 %).

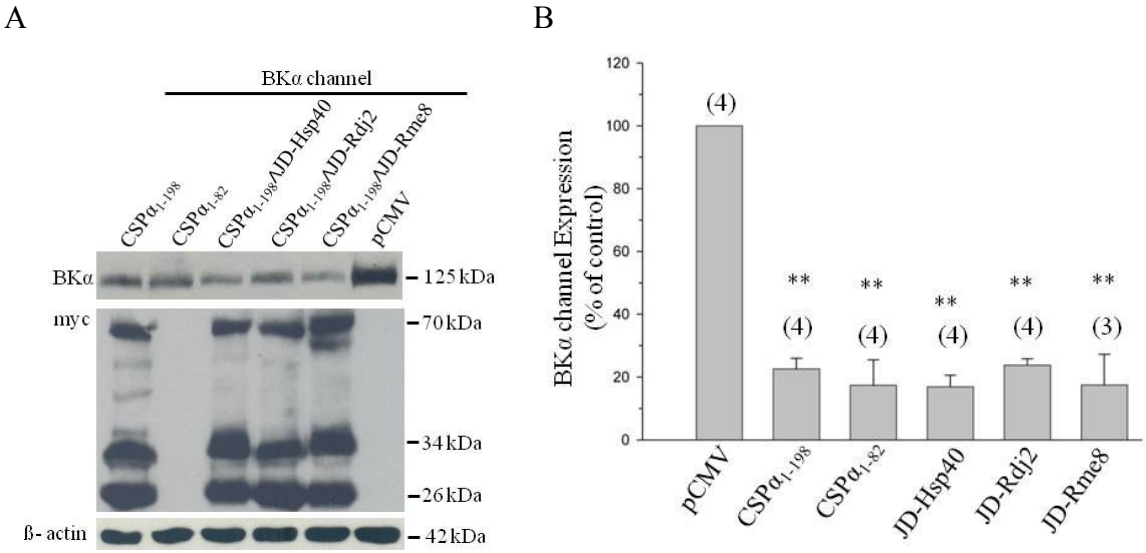

Figure 16: J-domain CSPα chimeras reduce BK channel expression.
Panel A. Western Blot analysis of BK channel expression in CAD cells which were transiently co-transfected with 1 µg BK channel-encoding cDNA with either 0.75 µg myc-tagged full length CSPα or the constructs which are indicated on top of the corresponding gel lanes and incubated for 24 h. As negative control, 0.75 µg empty pCMV plasmid were co-transfected with 1 µg BK channel-encoding cDNA. 30 µg cell lysate were separated by SDS-PAGE, probed with an anti-BK channel antibody. The myc expression is shown as transfection control for CSPα and ß-actin represents the loading control. The corresponding mean data is shown in panel B are in comparison to the pCMV control; ** $p < 0.001$

3.3.4 Mutation of the HPD motif of the J-domain

The results indicated so far that the J-domain is an essential domain for reducing BK channel expression. It is already known that the highly conserved HPD motif within the J-domain is important in activating Hsc70 (Jiang et al., 2007; Kampinga and Craig, 2010). To determine if the HPD motif is essential within the J-domain of CSPα and Hsp40, these three amino acids were mutated to three alanines (AAA). The tested CSPα, the full length Hsp40 and the mutant constructs are shown in Figure 17. 1 µg BK channel cDNA was co-transfected with 0.75 µg CSPα, $CSP\alpha_{HPD\text{-}AAA}$, Hsp40 or $Hsp40_{HPD\text{-}AAA}$ and the empty pCMV plasmid (negative control) in CAD cells.

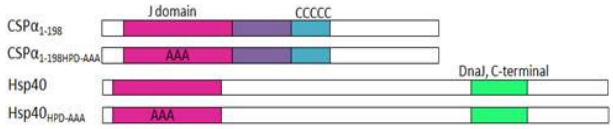

Figure 17: Schematic structure of N-terminus myc-tagged CSPα and Hsp40 constructs. The graph illustrates a schematic structure of the myc-tagged full length CSPα, the $CSP\alpha_{HPD\text{-}AAA}$ mutant, Hsp40 and the $Hsp40_{HPD\text{-}AAA}$ mutant. The CSPα and Hsp40 have a substitution of the highly conserved HPD motif to AAA in the J-domain.

The results of the Western Blot analysis are presented in Figure 18. The upper panel in Figure 18A shows the BK channel expression. As the results demonstrate, full length CSPα reduces BK channel expression, whereas the level of BK channel expression in the presence of $CSP\alpha_{HPD\text{-}AAA}$ is comparable to the negative control. The same effect is seen with the Hsp40 constructs. Hsp40 reduces BK channel expression, but the $Hsp40_{HPD\text{-}AAA}$ mutant appers to have no effect on BK channel expression compared to the negative control. The middle panel presents the expression control (myc expression) of the tested constructs and β-actin represents the loading control.

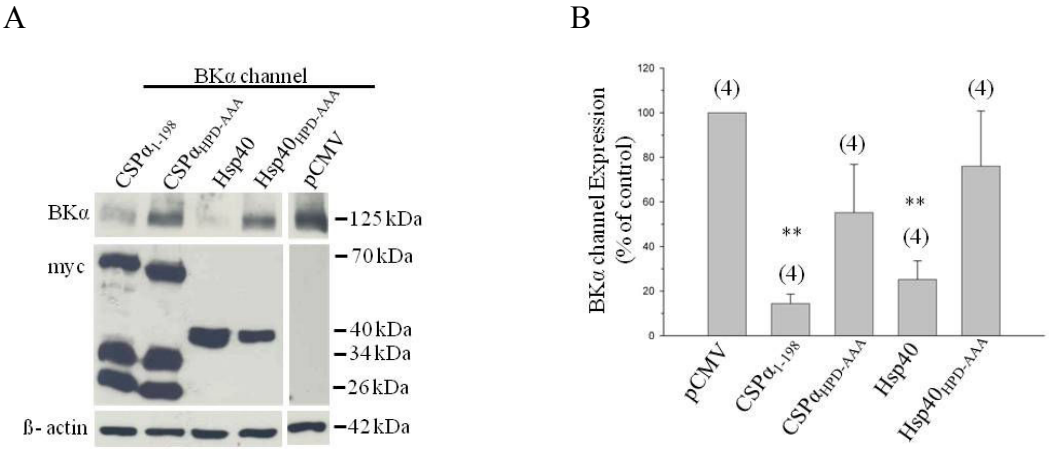

Figure 18: Mutation of J-domain HPD to AAA inhibits the J-domain-mediated reduction in BK channel expression.
Panel A. Western Blot analysis of BK channel expression in CAD cells which were transiently co-transfected with 1 µg BK channel-encoding cDNA with either 0.75 µg myc-tagged full length CSPα or the constructs which are indicated on top of the corresponding gel lanes and incubated for 24 h. As negative control, 0.75 µg empty pCMV plasmid were co-transfected with 1 µg BK channel-encoding cDNA. 30 µg cell lysate were separated by SDS-PAGE, probed with an anti-BK channel antibody. The myc expression is shown as transfection control for CSPα and ß-actin presents the loading control. The corresponding mean data shown in panel B are in comparison to the pCMV control; ** $p < 0.001$

The corresponding mean data in Figure 18B show that $CSP\alpha_{1\text{-}198}$ (14.4 ± 4.7 %) reduces BK channel expression strongly in a statistically significant manner compared to the negative control (100 %), whereas $CSP\alpha_{HPD\text{-}AAA}$ (55.2 ± 21.7 %) shows a modest, but not significant reduction of BK channel expression compared to the negative control (100 %). The same effect is seen when Hsp40 (25.2 ± 8.4 %) and $Hsp40_{HPD\text{-}AAA}$ (76.0 ± 24.7 %) are co-expressed with the BK channel. Hsp40 shows a statistically significant reduction, while $Hsp40_{HPD\text{-}AAA}$ co-transfected produces no change in BK channel expression compared to the negative control. Overall, both HPD mutants eliminated the J-protein mediated reduction in BK channel expression. These results indicate that the HPD motif is critical within the J-domain with respect to reducing BK channel expression.

3.4 Determination of the influence of Hsc70 overexpression on BK channel expression

The heart of the cellular chaperone machinery is the chaperone Hsc70 which is essential for numerous cellular processes and requires the activation of J-proteins (Kampinga and Craig, 2010). Within the J-protein, the HPD motif is the most important sequence in binding and activating the ATPase of Hsc70 (Braun et al., 1996). The results in 3.3.4 indicated that a mutation in the HPD motif inhibits the J-protein-mediated reducing effect on BK channel expression. Therefore, it is suggested that Hsc70 plays an important role in this effect. In this experiment we evaluated the possible effect of Hsc70 itself on BK channel expression. Hsc70 has a low ATPase activity which can be increased via J-protein binding (Braun et al., 1996).

CAD cells were transiently co-transfected with 1µg BK channel cDNA, 1 µg HA-tagged Hsc70 cDNA or 1 µg HA-tagged ATPase domain of Hsc70 cDNA, and 1 µg pCMV plasmid (negative control). The results of the Western Blot analysis are presented in Figure 19. The upper panel shows the BK channel expression and indicates that BK channel expression is slightly increased when Hsc70 is co-expressed although this change is not statistically significant. The co-expression of the ATPase domain of Hsc70 shows no change in BK channel expression compared to the negative control. The HA-tag expression represents the protein expression of the tested Hsc70 and ATPase domain constructs. The HA-tag antibody shows background bands at 70 kDa and 50 kDa, but, notably, the 70 kDa band is more intense when Hsc70 is co-expressed, which indicates that full-length Hsc70 is expressed in CAD cells.

The corresponding mean data in Figure 19B show no significant changes in BK channel expression when either Hsc70 (132.9 ± 30.3 %) or the ATPase domain of Hsc70 (69.7 ± 18.9 %) is co-expressed with the BK channel, compared to the negative control (100 %). Overall, the results indicate that Hsc70 itself has no effect on the BK channel expression and that the activation through J-proteins is essential.

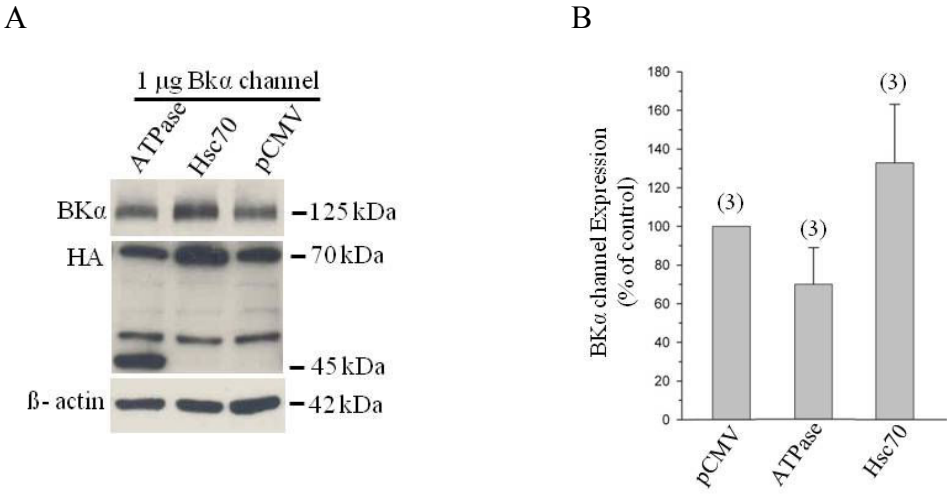

Figure 19: Overexpression of Hsc70 has no effect of BK channel expression.
Panel A. CAD cells were transiently co-transfected with 1 µg cDNA encoding the BK channel, 1 µg HA-tagged Hsc70 and Hsc70 ATPase. As negative control, 1 µg empty pCMV plasmid was co-transfected with 1 µg cDNA encoding the BK channel. 24 h post-transfection, the cells were lysed and protein expression was analyzed by Western Blotting with an anti-BK channel antibody to visualize the BK channel. The HA-tag specific antibody is shown as expression control and ß-actin is shown as loading control. The corresponding mean data shown in panel B are in comparison to the negative control.

3.5 Determination of the influence of CSPα on BK channel expression at the cell surface

The results obtained so far demonstrate that CSPα and other J-proteins reduce BK channel expression in the total cellular protein lysate. We next evaluated if this effect of CSPα influences BK channel expression at the cell surface. For this purpose, a cell surface labeling assay was carried out (described in 2.3.4). BK channels at the cell surface were labeled with biotin and a streptavidin pull-down assay was used to extract the biotinylated cell surface proteins from the total cellular protein pool. Western Blot analysis was used to detect the biotinylated BK channel and establish if the BK channel expression at the cell surface is changed.

CAD cells were transiently co-transfected with 1 µg BK channel cDNA and either 0.75 µg CSPα cDNA or 0.75 µg $CSP\alpha_{HPD\text{-}AAA}$ cDNA. As negative control, empty pCMV plasmid was co-transfected with 1 µg BK channel. 24 h after transfection, CAD cells were

labeled with biotin, harvested and protein concentration was measured. 1 mg of the total cellular protein was used for the streptavidin pull-down assay. Western Blot analysis was carried out to identify the BK channel in the biotinylated and cellular protein samples.

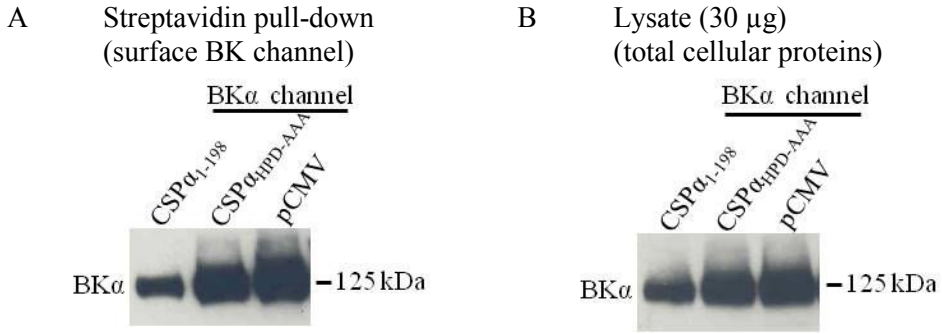

Figure 20: CSPα reduces BK channel expression at the cell surface.
CAD cells were transiently co-transfected with 1 µg cDNA encoding BK channel and 0.75 µg CSPα, 0,75 µg $CSP\alpha_{HPD\text{-}AAA}$ or 0,75 µg pCMV (negative control). 24 h post-transfection the cells were labeled for 30 min with Biotin at 4°C. The cells were harvested and protein concentration was measured; 1 mg of total protein was used for the overnight pull-down. The pull-down proteins (A) and 30 µg of total cellular proteins (B) were electrophoresed and the protein expression was analyzed by Western Blot with anti-BK channel antibody to visualize BK channel at the surface (A) or in the total protein pool (B). Data are representative of 4 separate experiments.

Figure 20A shows that less BK channel was biotinylated in the presence of CSPα (lane 1), in contrast to $CSP\alpha_{HPD\text{-}AAA}$ (lane 2) mutant or negative control (lane 3). The overexpression of $CSP\alpha_{HPD\text{-}AAA}$ (lane 2) mutant shows a slightly weaker BK channel expression than the negative control (lane 3). Figure 20B shows the Western Blot analysis of total cellular protein and confirms the results of Figure 20A. CSPα (lane 1) reduces BK channel expression and $CSP\alpha_{HPD\text{-}AAA}$ (lane 2) leads to a similar BK channel expression as the negative control (lane 3). Overall, these results indicate that CSPα decreases the total amount of BK channel in the cell and also its expression at the cell surface.

3.6 BK channel and CSPα are co-immunoprecipitated

Next we evaluated whether CSPα and BK channel physically interact with each other. It is known that CSPα and the BK channel are expressed at the same site in neurons, i.e. at the pre-synaptic terminal (Zhao et al., 2008). CSPα is additionally expressed in the endoplasmic reticulum (ER) (Schmidt et al., 2009; Zhang et al., 2002; Zhang et al., 2006), where numerous proteins are synthesized.

To address the question if CSPα and BK channel interact with each other, co-immunoprecipitation experiments were carried out (for protocol see 2.3.5). CAD cells were co-transfected with 1 µg cDNA encoding the BK channel and 0.75 µg myc-tagged CSPα, $CSP\alpha_{HPD\text{-}AAA}$ and pCMV (negative control). 24 h after transfection, cells were harvested and protein concentration was measured. 0.7 mg total cellular protein lysate were used for co-immunoprecipitation. The primary antibodies against the BK channel (polyclonal) or myc-tag (monoclonal) were used for the pull-down. To evaluate if the BK channel and CSPα are co-immunoprecipitated, Western Blot analysis was carried out. The nitrocellulose membrane was probed for BK channel or myc-tagged CSPα.

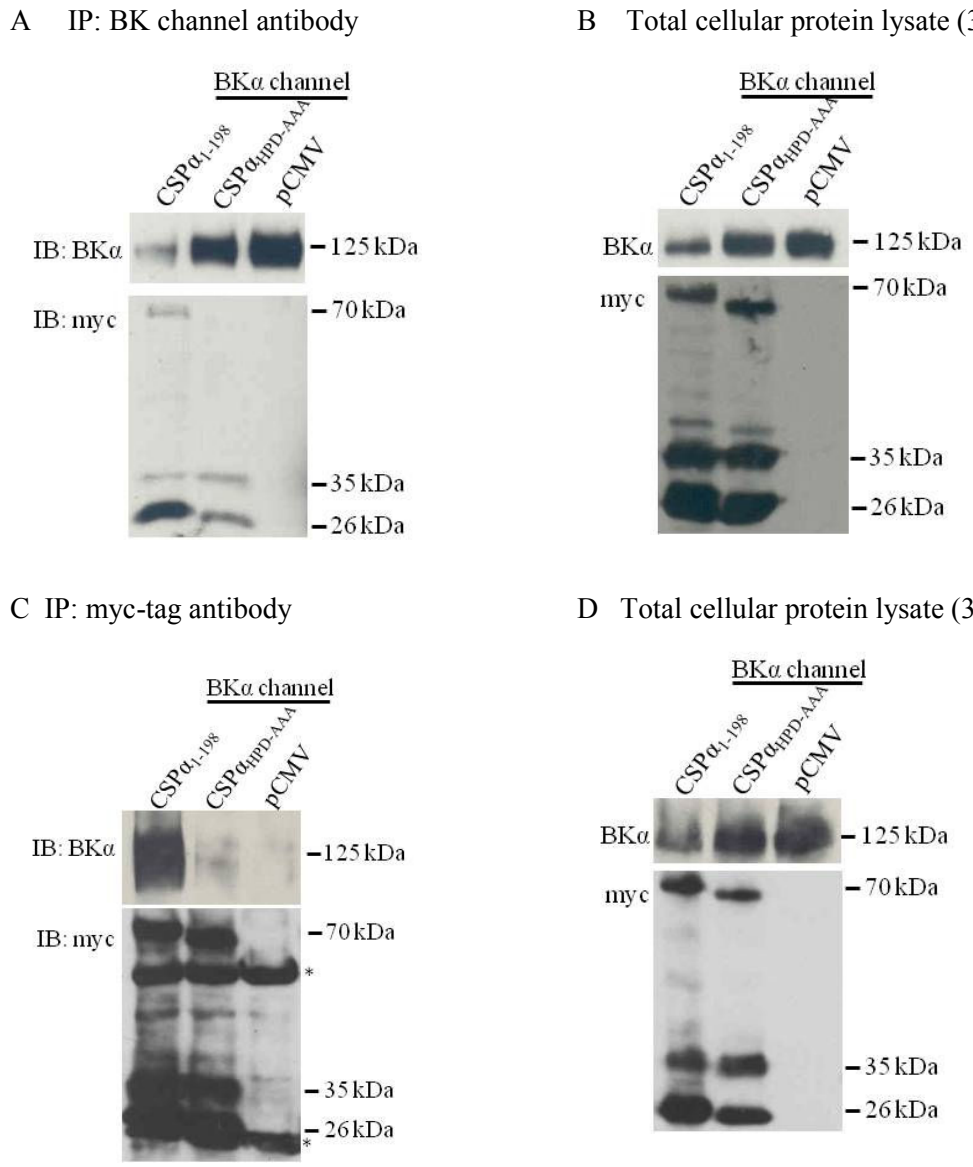

Figure 21: CSPα co-immunoprecipitates with the BKα channel
CAD cells were transiently co-transfected with 1 µg BK channel and 0.75 µg myc-tagged CSPα, 0.75 µg myc-tagged $CSP\alpha_{HPD\text{-}AAA}$ and 0.75 µg pCMV (negative control). 24 h after transfection, the cells were harvested and protein concentration was measured. 0.7 mg soluble protein were pre-cleared with Protein A/G-coupled agarose beads and co-immunoprecipitation was carried out with either BK channel antibody (A) or myc-tag antibody (C) (5 µg) overnight. The samples were separated by SDS-PAGE and the co-immunoprecipitation (A and C) and total protein expression (B and D) were analyzed by Western Blotting with an anti-BK channel and an anti-myc-tag antibody to visualize BK channel and myc-tagged CSPα. B and D show the total cellular protein (30 µg). * Heavy- and light-chain of the myc-tag antibody. Data are representative of three separate experiments.

The results in Figure 21 show that CSPα and BK channel co-immunoprecipitate. The top panel in Figure 21A shows that CSPα reduces BK channel expression and that $CSP\alpha_{HPD\text{-}AAA}$ shows a comparable expression to the negative control. The lower panel in Figure 21A shows that BK channel co-immunoprecipitates with myc-tagged CSPα. Notably, the immature CSPα (26 kDa) band is stronger compared to the mature form of CSPα (34 kDa) or the CSPα-CSPα dimer (70 kDa). Figure 21B present the Western Blot analysis of the total cellular protein and indicates that a similar amount of CSPα and $CSP\alpha_{HPD\text{-}AAA}$ protein was expressed in transient CAD cells.

The top panel in Figure 21C demonstrates that myc-tagged CSPα co-immunoprecipitates with the BK channel. The upper panel shows the BK channel expression and demonstrates that a high amount of CSPα co-immunoprecipitates with the BK channel, whereas only a small amount of the BK channel co-immunoprecipitates with $CSP\alpha_{HPD\text{-}AAA}$ mutant. The negative control shows that BK channel protein is not detectable in the immunoprecipitate when CSPα is not co-transfected. The bands of 55 kDa and 26 kDa represent the heavy- and light- chain of the monoclonal myc-tag antibody. The total cellular protein lysate of this experiment is presented in Figure 21D.

Overall, these results suggest that CSPα and BK channel interact with each other. As illustrated in Figure 21, a high amount of immature CSPα interacts with the BK channel.

3.7 Determination of the influence of the BK β-subunit on the CSPα-mediated effect

In most BK channel-expressing cells, BK channels are not expressed alone at the cell surface. They usually have an auxiliary β-subunit which gives the BK channel more functional diversity (Behrens et al., 2000; Brenner et al., 2000b). We next addressed the questions: Does CSPα and the BK β-subunit compete for the same binding sites at the BK α-subunit? Does CSPα-mediated reduction of BK channel expression occur in the presence of the BK β-subunit and/or does CSPα also reduce the expression of the BK

β-subunit? The neuronally expressed BKβ4 and the smooth muscle expressed BKβ1 were tested, regarding the effect of CSPα on BK channel expression.

3.7.1 Determination of the influence of BKβ1 on the CSPα-mediated effect

CAD cells were transiently transfected with 1 µg BK channel, 1 µg BKβ1 and either 0.75 µg CSPα or pCMV plasmid (negative control). The corresponding Western Blots are illustrated in Figure 22.

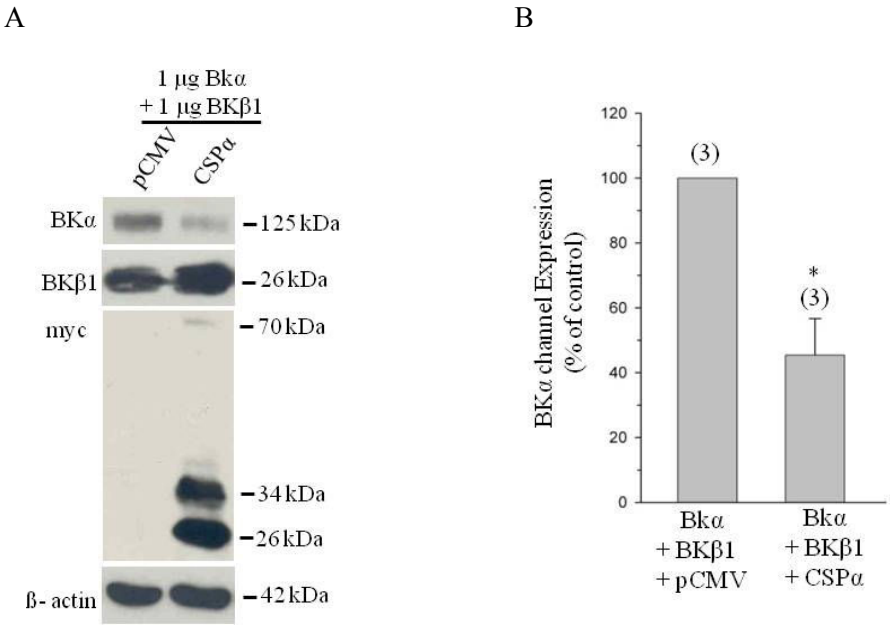

Figure 22: BKβ1-subunit does not influence the CSPα-mediated reduction of BK channel expression.
Panel A: CAD cells were transiently transfected with 1 µg BK channel, 1 µg BKβ1-subunit and with either 0.75 µg CSPα or pCMV plasmid (negative control). 24 h after transfection, cells were lysed and protein expression was analyzed by Western Blotting with an anti-BK channel and anti-BKβ1-subunit antibody to visualize the co-expression of the BK channel with BKβ1. Myc expression is shown as transfection control for CSPα and β-actin represents the loading control. The graphs shown in panel B represent the corresponding mean data of BK channel expression when BKβ1 is co-expressed with CSPα or pCMV plasmid. * $p < 0.05$

The results in Figure 22A indicate that CSPα markedly reduces BK channel expression (upper panel) but does not change BKβ1 expression (second panel). The third panel from the top shows the expression control for CSPα (myc expression) and the lowest panel shows the expression of β-actin, representing the loading control. The corresponding mean data on BK channel expression are presented in Figure 22B. The mean data were normalized to the negative control. The co-expression of BKβ1 does not influence CSPα-mediated reduction in BK channel expression. The overexpression of CSPα reduces BK channel expression in a statistically significant manner to 45.4 ±11.3 % compared to the negative control (100 %).

3.7.2 Determination of the influence of BKβ4 on the CSPα-mediated effect

CAD cells were co-transfected with 1 µg BK channel, 1 µg BKβ4 and 0.75 µg CSPα or pCMV plasmid (negative control). 24 h after transfection, cells were lysed, protein concentration was measured and Western Blot analysis was carried out. Western Blot analysis results are shown in Figure 23.

A B

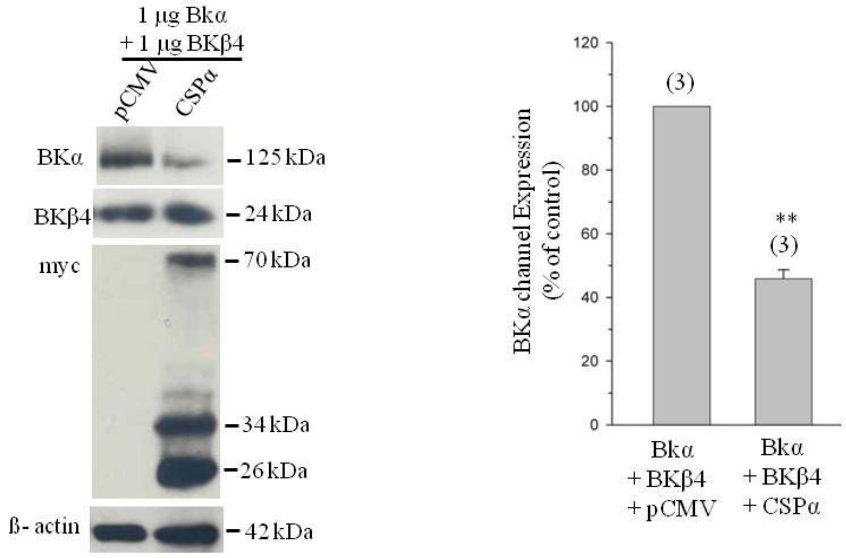

Figure 23: BKβ4-subunit does not influence the CSPα-mediated reduction of BK channel expression.
Panel A: CAD cells were transiently transfected with 1 µg BK channel, 1 µg BKβ4-subunit and with either 0.75 µg CSPα or pCMV plasmid (negative control). 24 h after transfection, cells were lysed and protein expression was analyzed by Western Blotting with an anti-BK channel and anti-BKβ4-subunit antibody to visualize the co-expression of the BK channel with BKβ4. Myc expression is shown as transfection control for CSPα and β-actin represents the loading control. The graphs shown in panel B represent the corresponding mean data of BK channel expression when BKβ4 is co-expressed with CSPα or pCMV plasmid. * $p < 0.001$

The Western Blot analysis in Figure 23A shows that CSPα reduces BK channel (upper panel) expression, but had no effect on the BKβ4 expression (second panel). The expression of CSPα is shown in the middle panel and the β-actin expression (lower panel) represents the loading control. The corresponding mean data on BK channel expression from Figure 23B shows that BK channel expression (45.9 ± 2.8 %) is strongly reduced in a statistically significant manner when CSPα is co-transfected, compared to the negative control (100 %). These results indicate that BKβ4 does not influence the CSPα-mediated effect on BK channel expression and that CSPα only affects BK channel function, not BKβ4 expression. Taken together, these results show that CSPα reduces BK channel expression regardless of the presence of BKβ1 or BKβ4.

3.8 Biotinylation of BK channel, BKβ1 and BKβ4

Since CSPα reduces BK channel expression but not the expression of BKβ1 or BKβ4, we wanted to investigate if the auxiliary β-subunits are expressed at the cell surface. Therefore, CAD cells were co-transfected with BK channel and either BKβ1 or BKβ4. 24 h after transfection, CAD cells were labeled with biotin, harvested and protein concentration was measured. 1 mg of the total cellular protein was used for the streptavidin pull-down assay. Western Blot analysis was carried out to confirm BKβ1 or BKβ4 expression at the cell surface.

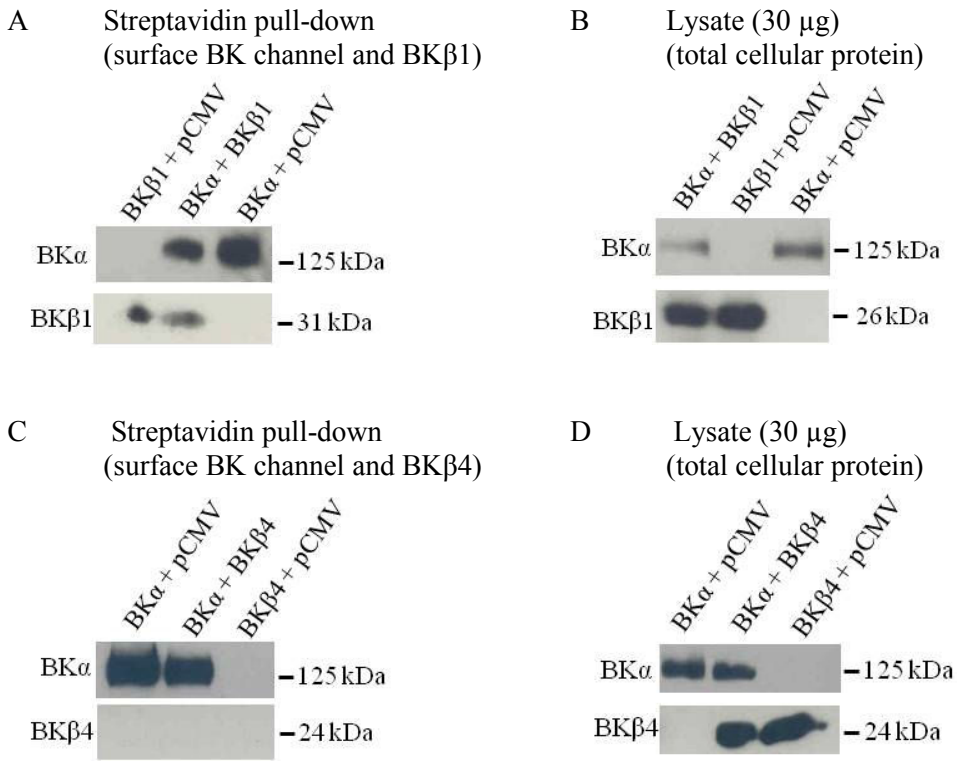

Figure 24: Biotinylation of BK channel, BKβ1- and BKβ4-subunits at the cell surface. CAD cells were transiently co-transfected with 1 µg BK channel, 1 µg BKβ1-subunit (A) or 1 µg BKβ4-subunit (C) with 1 µg pCMV plasmid (negative control). 24 h post-transfection the cells were labeled for 30 min with Biotin at 4°C. The cells were harvested and protein concentration was measured; 1 mg of total protein was used for the overnight pull-down. The pull-down proteins (A and C) and 30 µg of total cellular proteins (B and D) were electrophoresed and the protein expression was analyzed by Western Blot with anti-BK channel antibody to visualize BK channel at the surface (A and C) or in the total protein (B and D) and with anti-BKβ1 subunit or anti-BKβ1-subunit antibody to visualize BK channel, BKβ1 (A and B) and BKβ4 (C and D). Data are representative of three separate experiments.

The results of the cell surface-labeled BK channel with BKβ-subunits are presented in Figure 24. The results in Figure 24A demonstrated that the glycosylated BKβ1 (31 kDa) is biotinylated at the cell surface, when it is either alone (with pCMV plasmid) or co-expressed with BK channel. Figure 24B shows that Western Blot analysis of the total cellular protein lysate and indicates that both proteins are expressed. However, the results of the cell surface biotinylation of BK channel and BKβ4 in Figure 24C illustrate that BKβ4 is not biotinylated and thus does not appear to be expressed at the cell surface. But

interestingly, the Western Blotting results of the total protein lysate demonstrate that BKβ4 is expressed in the cells. This result could be due to the fact that the cDNA used was from different species and BKβ4 was not able to traffic to the cell surface.

3.9 Drug treatment

Finally, we wanted to determine if drugs influence the CSPα-mediated effect on BK channel expression. The following drugs were considered for testing: lactacystin, a proteasome inhibitor, quercetin, which shows a neurodegenerative effect on neurons via an unknown mechanism; TEA, a BK channel blocker, and dbcAMP (dibutyryl cAMP) an analogue of cAMP (cyclic adenosine monophosphate).

3.9.1 Lactacystin treatment

First we wanted to evaluate if a proteasome inhibitor could inhibit the degradation of BK channel. Almost all proteins get degraded through a proteasome dependent mechanism. For this experiment CAD cells were co-transfected with CSPα in two different concentrations (0.25 µg and 0.75 µg) and 1 µg BK channel. As negative control 0.25 µg or 0.75 µg pCMV plasmid was co-transfected with 1 µg BK channel. 6 h post-transfection the cells were treated for 18 h with lactacystin in five different concentrations (0 µM, 2.5 µM, 5 µM 10 µM and 25 µM). The effect of the lactacystin treatment was analyzed by Western Blot analyses. The results are shown in Figure 20.

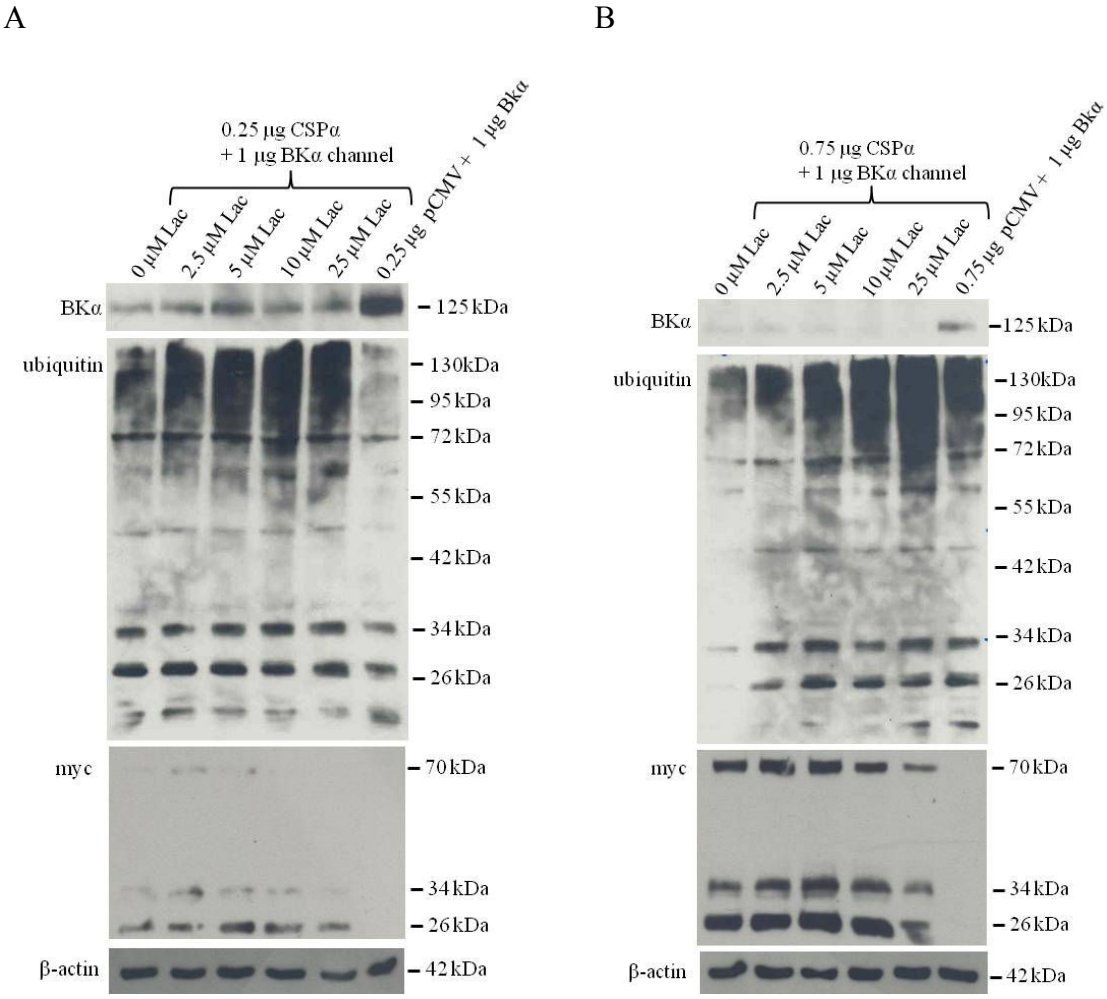

Figure 25: Lactacystin increases ubiquitinated proteins.
CAD cells were transiently co-transfected with 1 µg cDNA encoding BK channel and myc-tagged CSPα in two different concentrations (A. 0.25 µg and B. 0.75 µg). As negative control, empty pCMV plasmid (A. 0.25 µg and B. 0.75 µg) was co-transfected with 1 µg cDNA encoding BK channel. 6 h after transfection, the cells were treated for 18 h with Lactacystin in five different concentrations (0 µM, 2.5 µM, 5 µM, 10 µM and 25 µM). Cells were lysed and protein expression was analyzed by Western Blotting with an anti-ubiquitin antibody to visualize ubiquitinated proteins and an anti-BK channel antibody to visualize the BK channel. Myc expression represents the transfection control of CSPα and β-actin represents the loading control. Data are representative of three separate experiments.

Figure 25A illustrates the results of the co-transfection of 0.25 µg CSPα with the BK channel in the presence of different concentrations of lactacystin. The upper panel shows that the BK channel expression is not changed between untreated cells (lane 1) and

lactacystin treated-cells (lanes 2-5). The expression of ubiquitinated proteins (second panel) shows that the amount of ubiquitinated protein increases with an increase of lactacystin concentration. The third panel shows myc-tagged CSPα expression and the β-actin expression illustrated in the lowest panel represents the loading control. Figure 25B shows a similar outcome of the experiment in which cells were co-transfected with a higher amount of CSPα (0.75 µg) cDNA with BK channel. As already shown in Figure 25A, the Lactacystin treatment (lane 2-5) shows no changes in BK channel expression compared to the untreated control (lane 1). As seen in Figure 25A, lactacystin treatment increases the amount of detected ubiquitinated proteins (second panel). Overall, the lactacystin treatment increased the amount of ubiquitinated protein in the cellular lysate but did not influence the CSPα mediated effect on BK channel expression.

3.9.2 Quercetin, TEA and dbcAMP treatment

In this set of experiments we wanted to evaluate if treating cells with quercetin, TEA and dbcAMP influences the CSPα-mediated effect on BK channel expression.

Quercetin is a flavonoid used as a nutritional supplement, memory enhancer, and in the treatment of altitude sickness and cancer. Its mechanism of action is still unknown, but in a recent publication (Xu et al., 2010) it has been demonstrated that quercetin promotes CSPα dimerization and inhibits synaptic transmission. Thus, it was of interest to determine if quercetin has an effect on CSPα and thereby on the CSPα-mediated effect on BK channel expression.

TEA specifically blocks K^+ channels and inhibits potassium channel function by binding within the conduction pathway (Lenaeus et al., 2005). Therefore we intended to determine if TEA alters the CSPα-mediated effect on BK channel expression. The reason to test a K^+ channels blocker was tested if it may changes the BK channel conformation a thus inhibit the CSPα-mediated effects.

The last drug we aimed to test was dbcAMP, an analog to cAMP and a second messenger which is essential for many biological processes. Increase in intracellular cAMP concentration activates PKA. CSPα as well BK channels are phosphorylated by PKA which

lead to changes of their function and activity (Evans et al., 2001; Tian et al., 2004). Membrane permeable dbcAMP was used because cAMP does not get through the cell membrane into the cell.

The CAD cells were co-transfected with 1 µg BK channel and 0.75 µg CSPα. As negative control, 0.75 µg pCMV plasmid was co-transfected with 1 µg BK channel. 6 h after transfection, the cells were treated for 18 h with 50 µM quercetin (in DMSO), 10 mM TEA, or 1 mM dbcAMP. Because quercetin was the only drug which was diluted in DMSO, DMSO treatment was used as control. Western Blot analysis was carried out to evaluate the effect of the tested drugs on BK channel expression. In Figure 26 are the Western Blot results of the drug treatment.

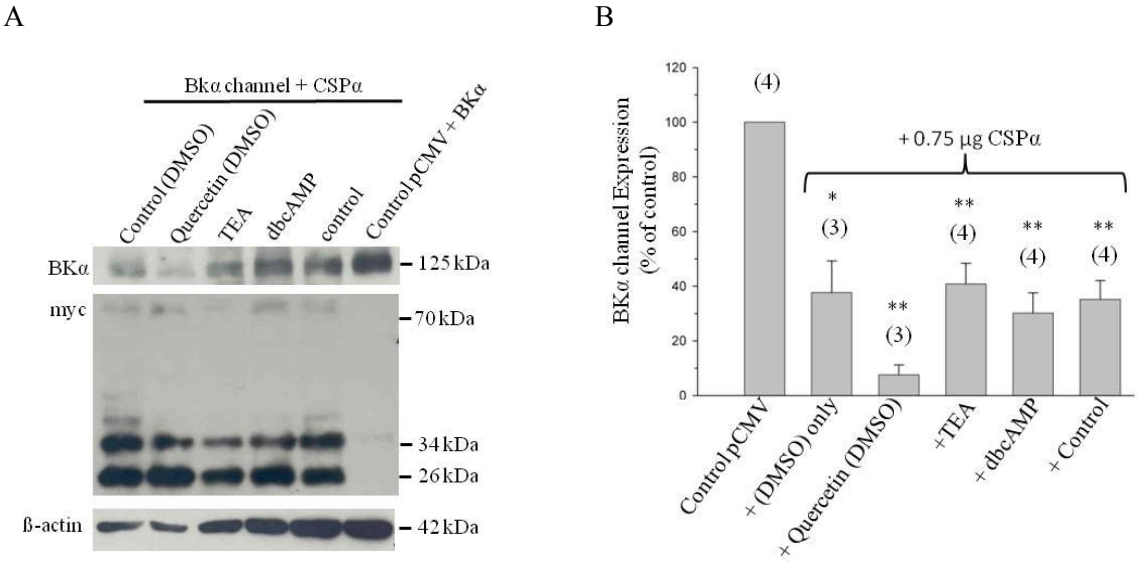

Figure 26: Quercetin, TEA and dbcAMP treatment of CAD cells.
Panel A. CAD cells were transiently co-transfected with 1 µg cDNA encoding the BK channel and 0.75 µg myc-tagged CSPα or empty pCMV plasmid (negative control). 6 h after transfection, the cells were treated for 18 h with 50 µM quercetin (in DMSO), 10 mM TEA, 1 mM dbcAMP or DMSO (control). Cells were lysed and protein expression was analyzed by Western Blotting with an anti-BK channel antibody to visualize the BK channel. Myc expression is shown as transfection control for CSPα constructs and ß-actin represents the loading control. Panel B. Corresponding mean data, compared to the pCMV negative control. * $p < 0.05$; ** $p < 0.001$

The top panel in Figure 26A shows the Western Blot analysis of BK channel expression upon drug treatment. Only the treatment with 50 μM quercetin (lane 2) reduces BK channel expression more strongly than CSPα does (DMSO control; lane 1). The other tested drugs, TEA (lane 3) and dbcAMP (lane 4), appeared to be ineffective and BK channel was expression comparable to the untreated CSPα control (lane 5). The middle panel represents the transfection control (myc expression) and the lowest panel depicts the loading control (β-actin expression).

The corresponding mean data in Figure 26B show the BK channel expression normalized to the negative control (100 %). The quercetin treated cells (lane 3) show the strongest reduction in BK channel expression compared to the DMSO control (lane 2). In the presents of CSPα, BK channel expression was reduced to (37.7 ± 11.6 %) with DMSO only, whereas quercetin (7.6 ± 3.6 %) had a statistically significant effect on BK channel expression compared to the control (lane 1; 100 %). The difference in BK channel expression observed in untreated cells (lane 2) compared to quercetin treated cells (lane 3) is not statistically significant. The TEA treatment (lane 4; 30.2 ± 7.4 %) and the dbcAMP treatment (lane 5; 35.2 ± 6.9 %) show a significant reduction compared to the negative control (100 %), but no change compared to the CSPα control (lane 6; 40.9 ± 7.5 %).

3.9.3 Quercetin treatment

Given the effect of quercetin treatment on BK channel expression, we wanted to investigate if quercetin changes BK channel expression when CSPα is not co-expressed. For this purpose, CAD cells were transfected only with the BK channel and treated with quercetin (in DMSO) or DMSO alone (negative control). As in the previous experiment (chapter 3.9.2), CAD cells were treated for 18 h with 50 μM quercetin or DMSO. A Western Blot analyses was carried out to evaluate BK channel expression. The results are presented in Figure 27.

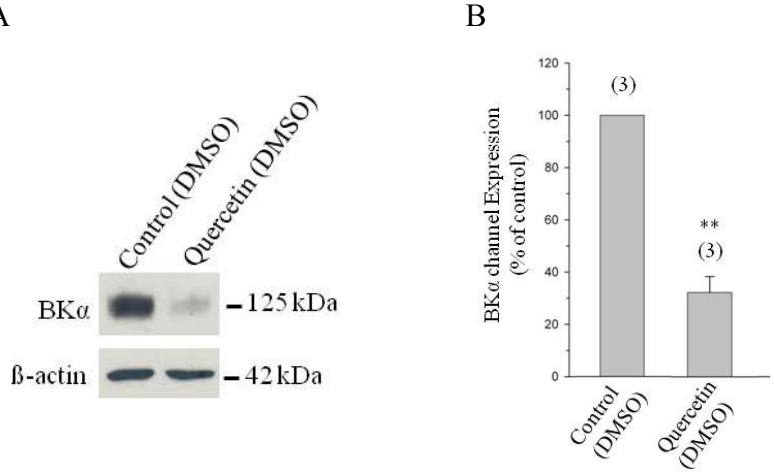

Figure 27: Quercetin reduces BK channel expression.
Panel A. CAD cells were transiently transfected with 1 µg cDNA encoding the BK channel. 6 h after transfection, the cells were treated for 18 h with 50 µM quercetin (in DMSO) and DMSO (negative control). Cells were lysed and protein expression was analyzed by Western Blot with anti-BK channel antibody to visualize BK channel. The ß-actin represents the loading control. Panel B. Corresponding mean data, compared to the pCMV negative control. ** $p < 0.001$

Figure 27A demonstrates that quercetin strongly reduces BK channel (upper panel) expression compared to the DMSO control. The lower panel represents the loading control (β-actin).

The corresponding mean data in Figure 27B show that quercetin (32.6 ± 6.1 %) reduces BK channel expression compared to the DMSO control (100 %). This result indicates that quercetin targets the BK channel and the elevated expression of CSPα is not necessary for the effect of quercetin.

4 DISCUSSION

CHAPTER I: Transiently transfected CAD cells

In the experiments described in this chapter I found that CSPα and other J-proteins reduce BK channel expression in a concentration- and time-dependent manner in CAD cells and that the HPD motif within the J-domain is crucial for the CSPα-mediated reduction in steady-state BK channel expression. The results also indicate that CSPα reduces BK channel expression at the cell surface in transiently transfected CAD cells. Furthermore, I demonstrated that CSPα and BK channel associate with each one another.

4.1 Reduction of BK channel expression by J-proteins

In this study I demonstrated that J-proteins such as CSPα, Rdj2 and Hsp40 reduce BK channel expression; these observations are demonstrated in section 3.1. CSPα, Hsp40 and Rdj2 were chosen because of their distribution in neurons (Zhao et al., 2008). They are expressed either in the same cell compartment as the BK channel or in close proximity to the former. The unique J-protein CSPα is a presynaptic anchored J-protein and is important for synaptic vesicles function (Mastrogiacomo et al., 1994; Zinsmaier et al., 1994). It shows a unique structure compared to other J-proteins. In addition to the highly conserved J-domain, it contains a cysteine string domain and a hydrophobic linker region. Hsp40 is a ubiquitously expressed class II cytosolic J-protein that is up-regulated in response to stress in most cells. The transient up-regulation of Hsp40 (and other stress-induced chaperones) is neuroprotective. Structural analyses show that the J-domain is followed by a glycine/phenylalanine linker region and a chaperone DnaJ C-terminal region. It was shown that Hsp40 can interact with CSPα to form a neuroprotective protein complex (Gibbs et al., 2009). Rdj2 is a 48 kDa, class I, J-protein found in both cytosolic and membrane fractions. Rdj2 is involved in the isoprotenerol-mediated G-protein signaling pathways (Rosales-Hernandez et al., 2009). All three J-proteins have a highly conserved J-domain (~ 70 amino acid sequence consists of four helices, helices II and III that contain a highly conserved HPD domain) that is important in interacting with Hsc70 and enhances

the ATPase activity (Kampinga and Craig, 2010). Outside of the J-domain, the proteins show diverse structures that give different cells their distinct functional properties. Hsp40 and Rdj2 share a 53 % sequence similarity with CSPα.

All J-proteins showed similar effects on BK channel expression. The results demonstrated that the tested J-proteins significantly reduce BK channel expression in a concentration-dependent manner section (3.1). J-proteins had the greatest effect on the BK channel when using a high cDNA concentration. The strongest effect is seen by the co-expression of CSPα with the BK channel. Even a small amount of CSPα cDNA (Figure 4), i.e. 0.5 µg, reduced BK channel expression in a statistically significant manner compared to Hsp40 and Rdj2. The Rdj2 (Figure 5) showed the least reduction, 0.75 µg cDNA of Rdj2 was needed to decrease BK channel significantly. Hsp40 (Figure 6) showed a significant BK channel reduction when using 0.75 µg cDNA. The highest amount of CSPα cDNA used was 0.75 µg. A higher concentration of CSPα decreased cell viability and thus experimental reproducibility. The J-proteins Hsp40 and Rdj2 did not change the cell viability and thus the amount of 1 µg cDNA Hsp40 and Rdj2 was used in co-transfection experiments with the BK channel. The effect of J-proteins on BK channel expression was also determined in the insoluble cell fraction (data not shown). J-protein-mediated reduction in BK channel expression was also seen in the insoluble fraction and was comparable to the total cellular protein, as presented in 3.1. The corresponding Western Blot results are not presented in this master thesis because the results showed comparable effects, and gave no further insight into the regulation of BK channel by J-proteins. However it does show that the loss of BK channel was not due to its translocation to detergent-insoluble membrane domain.

A recent publication by the group of Young and Shrier showed a similar effects of J-proteins with regards to another member of the K^+ channel family (Walker et al., 2009). They demonstrated that J-proteins; DJA1 (Hsp40), DJA2 (Rdj2) and DJA4 reduced hERG potassium channel expression. The hERG gene encodes a potassium channel which is responsible for the rapidly activating delayed rectifier K^+ current (I_{Kr}) in the heart. In their publication (Walker et al., 2009) they demonstrated that overexpression of J-proteins in a transient and a stably transfected system reduced the level of the mature

(fully-glycosylated) form of hERG exported from the ER. The group of Frizzell found comparable results for CFTR (Zhang et al., 2002; Zhang et al., 2006). They demonstrated that overexpression of CSPα decreased CFTR expression in a concentration-dependent manner. The effects of other J-proteins on CFTR expression has not been investigated yet.

These findings altogether, including our results suggest that J-proteins play a critical role e.g. as quality control machinery for hERG, CFTR and BK channel biogenesis and/or degradation. Overall, the results indicate that J-proteins have the ability to reduce hERG, CFTR or BK channel expression. These findings raise many questions regarding the molecular details underlying J-protein mediated channel reduction. So far the results have not established whether the response is J-protein specific. As demonstrated in this thesis and in recent publications (Schmidt et al., 2009; Walker et al., 2009; Zhang et al., 2002; Zhang et al., 2006) the reductive effect does not appear to be specific to one J-protein or to one client protein. Hsp40 reduced BK channel and also hERG expression (Walker et al., 2009). CSPα reduced both BK channel expression and CFTR (Schmidt et al., 2009; Zhang et al., 2002; Zhang et al., 2006). It is important to note that the experiments were not done in the same cell lines. Also, the results indicated that some of the tested J-proteins demonstrate greater reductions for certain channels. In this thesis, CSPα triggered a greater reduction in BK channel expression compared to Hsp40 and Rdj2. Walker et al., (2009) demonstrated that DJA2 had slightly stronger effects on hERG expression than to DJA1 and DJA4. One possibility is that these co-chaperones form multimeric complexes, it remains to be determined whether some J-proteins specifically interact with one client protein, or if numerous J-proteins are involved in regulating each client protein. Also the endogenous co-chaperones may well influence the J-protein mediated effects on hERG, CFTR or BK channel expression.

To further investigate the effect of J-proteins on BK channel expression, the incubation period was extended from 24 h to 48 h. The results in section 3.2 indicate that the tested J-proteins showed a greater effect on BK channel expression when incubation time was increased to 48 h. The longer incubation time showed also that, independently from the tested concentration of cDNA of the tested J-proteins, BK channel expression was significantly reduced at 48 h compared to 24 h. As already pointed out, the co-transfection

of CSPα (Figure 8) with the BK channel had a greater effect on BK channel expression than either Rdj2 (Figure 9) or Hsp40 (Figure 10). Preliminary experiments in Figure 7 showed that BK channel was stably expressed over 48 h, indicating that the BK channel expression in transiently transfected cells is constant over 48 h and the observed effect is due to the function of J-proteins and thus not due to a decreased BK channel expression. The results also demonstrated that expression of myc-tagged CSPα, Hsp40 and Rdj2 was decreased at 48 h compared to 24 h. The strongest reduction of myc expression was seen in the co-transfection of CSPα with the BK channel (Figure 8). Rdj2 (Figure 9) and Hsp40 (Figure 10) showed only a weak reduction in myc expression at 48 h compared to 24 h. These observations suggest that the BK channel and J-proteins may bind to each other and get degraded as a complex. Pulse chase experiments in the study of Walker et al. (2009) demonstrated that the J-proteins had a greater effect on hERG over a longer incubation time. They determined the reduction of hERG channel compared to a negative control at 0 h, 1 h, 2 h, 4 h, 10 h and 24 h, and noted a reduction of J-proteins over a longer incubation time, which raised the possibility that J-proteins and hERG were degraded as complex.

Altogether, these results demonstrate that, in the transiently transfected system tested here, the J-proteins CSPα, Rdj2 and Hsp40 all reduced BK channel expression; CSPα produced the strongest effect.

4.2 The J-domain within the CSPα is functionally important

The aim of this part of the study was to identify the CSPα domains and individual amino acids within these domains that are important in regulating BK channel expression. CSPα was chosen because it showed throughout the first set of experiments the greatest effect on BK channel expression. This study showed that the functionally essential domain is the J-domain and within the J-domain the amino acids histidine, proline and aspartic acid. This sequence is also known as the HPD motif and is crucial for CSPα interaction with Hsc70 to facilitate and/or maintain Ca^{2+} triggered neurotransmission. Four different experiments were carried out to identify the essential domain and motifs.

First, C-terminal CSPα truncations were tested (Figure 12). These experiments study showed that all C-terminal truncations still reduced BK channel expression. The construct $CSP\alpha_{1-82}$ and $CSP\alpha_{1-112}$ triggered a similar reduction of BK channel expression comparable to that reduced by $CSP\alpha_{1-198}$ (full length), $CSP\alpha_{1-90}$ and $CSP\alpha_{1-100}$. This effect may be due to the structure folding capacity of the truncations. These results provided the first evidence that the N-terminal region of CSPα with the highly conserved J-domain plays an essential role in reducing BK channel expression. Secondly, N-terminal CSPα truncations and a CSPα construct lacking the cysteine string domain were tested (Figure 14), to determine if regions of CSPα outside the J-domain also play a role in BK channel expression reduction. The Western Blot results demonstrated that the construct consisting of a J-domain ($CSP\alpha_{1-198}$ and $CSP\alpha_{\Delta C}$) reduced BK channel expression, whereas $CSP\alpha_{113-198}$ and $CSP\alpha_{137-198}$ did not able to reduce BK channel expression (seen in Figure 14). Interestingly, the construct $CSP\alpha_{83-198}$ which does not include the J-domain also reduced BK channel expression, but the expression of the myc-tagged protein was not detectable by Western Blotting. It is possible that this construct was post-transitionally modified or secreted because of the hydrophobic N-terminus and thus it may have been able to reduce BK channel expression without being detectable in the Western Blot analysis. The reason why this construct reduced BK channel was not further investigated here, but it would be an important theme for future experiments. In the third set of experiments, the role of the J-domain was further investigated by testing CSPα chimeras (Figure 16). The J-domain of CSPα was replaced with J-domains of Hsp40, Rdj2 and Rme8 and the resulting chimeras were tested for their effects on BK channel expression. All three tested CSPα chimeras caused a strongly significant reduction of BK channel expression (Figure 16). As already indicated by the first study, Hsp40 and Rdj2 were able to reduce BK channel expression. In this step, it was proven that the crucial J-domain of CSPα is interchangeable. Overall, this study indicates that the J-domains in a subset of J-proteins are functionally conserved. The J-domain is highly conserved among the J-protein family, and includes the HPD motif which is crucial for the interaction and activation of the ATPase of Hsc70 (Kampinga and Craig, 2010). The CSPα J-domain (amino acids from 15 to 82) shows a high sequence identity with the J-domain of Hsp40 (95 %), Rdj2 (97 %) and Rme8

(76 %). The fourth and last experiment was to identify the specific amino acids essential for reducing BK channel expression (Figure 18). It has already been demonstrated that the HPD motif in the J-domain is important with respect to the Hsc70 ATPase (Kampinga and Craig, 2010). Mutation of this motif showed that the J-protein function prevents the activation of Hsc70 (Zhao et al., 2008). Therefore, the HPD motif of CSPα and Hsp40 was mutated to three alanines and the effect of the AAA mutant on BK channel expressions was tested. The results (Figure 18) showed that the constructs $CSP\alpha_{HPD\text{-}AAA}$ and $Hsp40_{HPD\text{-}AAA}$ were not able to reduce BK channel expression. This particular study suggests that, within the J-domain, the HPD motif is essential for the interaction with Hsc70 and therefore decreases BK channel expression. Consistent with our findings, the group of Frizzell demonstrated that a CSPα HPD mutant (CSPαH43Q) failed to reduce CFTR expression and also had a reduced interaction with Hsc70 compare to WT CSP (Schmidt et al., 2009; Zhang et al., 2006). As well Walker at al. (2009) showed that the DJA1 and DJA2 mutants lacking the J-domain had no effect on hERG channel expression.

Overall, my study demonstrated that the J-domain is the most important domain within CSPα to reduce BK channel expression. Since, the HPD motif is essential to interact with and activate Hsc70 ATPase this suggests that in turn triggers the J-protein-mediated effect on BK channel expression (Braun et al., 1996). HPD mutations prevented the activation of Hsc70 and thereby reversed the J-protein-mediated reduction of BK channel expression.

4.3 Hsc70 co-expression does not change BK channel expression

J-proteins are necessary to bind and activate the ATPase of Hsc70 and thus mediate folding processes (Braun et al., 1996; Kampinga and Craig, 2010). J-proteins are not only necessary to activate the ATPase of Hsc70, they are also important to hold a client protein in a unfolded state after protein synthesis and target it to Hsc70, where the final folding processes occur (Kampinga and Craig, 2010). Since the HPD motif of the J-domain is critical for reducing BK channel expression we next evaluated if Hsc70 by itself has an effect on BK channel expression independently of J-proteins. To this end, CAD cells were co-transfected with BK channel and either Hsc70 or ATPase domain of Hsc70 (without a

substrate binding domain). The results in Figure 19 indicated that neither Hsc70 nor the ATPase domain were able to change BK channel expression which suggests that activation of Hsc70 through J-protein binding and the targeting of BK channel to Hsc70 by J-proteins is necessary to reduce BK channel expression. According to Braun at al. (1996) and Zhang et al. (1999), Hsc70 activity has a weak activity which can increase up to ~12 to 14 times upon J-protein binding. Schmidt et al. (2009) mutated CSPα (CSPαH43Q) did not observe a change CFTR expression. Furthermore, co-immunoprecipitation experiments demonstrated that co-expression of CSPα increased the association of Hsc70 with CFTR. In contrast, CSPα mutant failed to bind and activate Hsc70 showing a loss of its ability to induce Hsc70-CFTR association (Schmidt et al., 2009). This finding points out that CSPα is necessary to target the client protein for Hsc70 folding or degradation. Also, in line with our observations Walker et al. (2009) showed that overexpression, of Hsc70 did not change hERG expression.

4.4 Reduced BK channel expression at the cell surface induced by CSPα

The physiological role of the BK channel is to contribute to cell surface repolarization with an efflux of K^+ ions and thus to bring the cell surface back to its resting potential (Salkoff et al., 2006). The BK channel is functional at the cell surface and thus it is important to determine if CSPα influences the cell surface expression of BK channels. Usually BK channels get synthesized in the ER and then traffic to the cell surface where they exert their function. So far, our study has shown that CSPα reduces BK channel expression in the total cellular protein pool. To determine if CSPα changes BK channel expression at the cell surface, a biotin cell surface labeling assay was carried out. The results in Figure 20 showed that CSPα reduced BK channel expression at the cell surface. This indicates that, overall, CSPα reduces the amount of BK channel both inside the cell and at the cell surface. The Western Blot analysis in Figure 20 showed a similar effect for both the biotinylated proteins and the total cellular proteins; CSPα reduces BK channel expression, whereas $CSP\alpha_{HPD\text{-}AAA}$ has no effect on BK channel expression compared to the negative control. It is important to note that these findings cannot be directly compared

with each other, because different amounts of cellular proteins were used and analyzed by SDS-PAGE. 1 mg of the total cellular protein pool was used to extract the biotinylated proteins which were then separated by SDS-PAGE. In contrast only 30 µg total cellular protein lysate was loaded on a SDS-PAGE. The results represent four separate experiments with identical outcomes; CSPα reduces BK channel expression in the biotinylated protein pool as well as in the total cellular protein. CSPα reduces the overall expression of the BK channel and thus also affects BK channel expression at the cell surface.

It has already been described that the drug MESNA (sodium 2-mercaptoethanesulfonate) can strip biotin from cell surface proteins (Gee et al., 2010). With this treatment, it is possible to determine if biotin penetrates into the cell or only binds to cell surface proteins. Preliminary results (data not shown) demonstrated that the MESNA completely stripped the biotin from cell surface proteins, and thus it was not possible to detect biotinylated proteins by Western Blot analysis. These findings indicate that CSPα changes BK channel expression at the cell surface. A peroxidase-conjugated streptavidin secondary antibody was used as positive control for biotinylated proteins on Western Blots (data not shown).

It is important to mention that this study is not directly comparable with the first study (section 3.1) due to a different cell lysis protocol. For the first study cells were lysed as described in 2.3.1 with a lysis buffer containing 0.1 % SDS and 1 % Triton X-100, incubated 45 min at 4 °C and a centrifugation step at 15 000 rpm for 5 min at 4 °C. The cell lysis procedure for the biotinylated proteins (as described in 2.3.4) was done with only 1 % Triton X-100 and centrifugation was carried out at 15 000 rpm at 4 °C for 15 min. Triton X-100 is used to extract native proteins from insoluble cell fractions, whereas SDS is used to denature proteins. Also the acceleration and time of centrifugation are important factors for extracting proteins from the insoluble cell fraction. Therefore, it is not possible to compare directly CSPα-mediated reduction on BK channel expression of the first study with the total cellular protein of this study. Nevertheless, both studies show that CSPα reduces BK channel expression.

In contrast, our findings are not comparable which the J-protein-mediated reduction of hERG and CFTR (Schmidt et al., 2009; Walker et al., 2009; Zhang et al., 2002; Zhang et

al., 2006). hERG and CFTR are channels which undergo glycolysation. These channels get core-glycosylated in the ER and full-glycosylated after export from the ER. It is suggested that the presents of fully-glycosylated hERG or CTFR represents a marker of channel expression at the cell surface. The overexpression of the J-proteins (DJA1, DJA2 and DJA4) reduced the expression of the full-glycosylated CFTR and changed the ratio between synthesized hERG channel and fully processed hERG. The results indicate that J-proteins (DJA1, DJA2 and DJA4) inhibit the export of these channels from the ER. The same results were found when CSPα was overexpressed with CFTR. CSPα reduced the mature form of CFTR and increased the expression of immature CFTR (Schmidt et al., 2009; Zhang et al., 2002; Zhang et al., 2006). BK channel does not undergo glycosylation and thus it is difficult to identify where in the cell CSPα or J-proteins act to reduce BK channel expression. The cell surface labeling experiments indicate that BK channels are delivered to the cell surface but that their expression is reduced to the same extent as in the total cellular lysate. This reduction could be due to reduced export from the ER or an increased endosomal recycling (degradation) from the cell surface. Further investigations are needed to determine what aspects of the secretory endocytic pathway CSPα influences.

4.5 BK channel and CSPα interact with each other

Since CSPα reduces BK channel expression we next investigated whether CSPα and BK channels interact. As mentioned earlier, the BK channel and CSPα are distributed at the presysnaptic terminal. Recent publications (Schmidt et al., 2009; Zhang et al., 2002; Zhang et al., 2006) demonstrate that CSPα is also expressed in the ER in epithelial cells. It has not yet been determined whether neurons also express CSPα in the ER or the Golgi. The question is, do CSPα and the BK channel interact in the ER at an early stage of BK channel maturation, in the Golgi complex during trafficking, or at the pre-synaptic terminal? The results in Figure 21 showed that CSPα and the BK channel co-immunoprecipitate, and thus, that they bind to each other. CSPα and $CSP\alpha_{HPD\text{-}AAA}$ were used to investigate the nature of this interaction. Western Blot analysis showed that a large amount of immature CSPα (26 kDa) was co-immunoprecipitated with the BK channel

whereas only a weak interaction was seen with mature CSPα (34 kDa). This finding indicates that the BK channel and CSPα interact before CSPα is post-translationally modified by palmitoylation. These results give the first evidence that CSPα and BK channel may interact at an early stage of BK channel maturation, either in the ER or the Golgi. The Western Blot analysis of the total cellular protein lysate demonstrated that CSPα and $CSP\alpha_{HPD\text{-}AAA}$ were equally expressed and so it can be ruled out that the effect seen is due to the amount of CSPα used for transfection with the BK channel. The results in Figure 21 also showed that the BK channel primarily co-immunoprecipitates with myc-tagged CSPα. The myc-tagged $CSP\alpha_{HPD\text{-}AAA}$ co-immunoprecipitates only to a small extent with the BK channel.

Overall, these results indicate that CSPα interacts with the BK channel; furthermore, the strong interaction of immature CSPα with the BK channel suggests that the interaction might occur at an early stage in BK channel maturations or during trafficking.

Co-immunoprecipitation experiments with hERG and either DJA1 or DJA2 demonstrated that J-proteins primarily interact with an immature forms of hERG (Walker et al., 2009), consistent with our findings that a high amount of immature CSPα interacts with BK channel. Furthermore, the group of Shrier and Young (Walker et al., 2009) showed that the overexpression of J-proteins changed the interaction of hERG and Hsc70. This could be due to an Hsc70-mediated degradation process of hERG channel. Whether CSPα and BK channel also associate with Hsc70 and thus form a channel-chaperone complex has not been investigated yet. It is however suggested, based on results described in sections 3.3.4 and 3.4 that Hsc70 plays a key role in the CSPα-mediated reduction on BK channel expression and may also target the BK channel for degradation.

4.6 Co-expression of the BK β-subunit does not influence the CSPα-mediated effect on BK channel expression

In many tissues, auxiliary β-subunits are co-localized with the BK channel, thus providing more functional diversity. The human genome encodes four BK β-subunits; all four show a distinct cell distribution and thus various functional properties in different

tissues (Kim et al., 2007b; Salkoff et al., 2006; Tanaka et al., 2004). In this study, we evaluated whether BKβ-subunits influences the effect of CSPα on BK channel expression. One possible scenario is that BKβ-subunits and CSPα compete for a binding site on the BK channel. Another possibility is that CSPα also changes the expression of the BKβ-subunits. The results indicated that CSPα only influences BK channel expression but does not change the BK β-subunit expression. Two BK β-subunits were tested; BKβ1-subunit and BKβ4-subunit. The BKβ1-subunit was identified as a glycosylated 31 kDa protein in smooth muscles, hair cells and certain neurons and it is known to affect gating and Ca^{2+} sensitivity in the BK α-subunit (Tanaka et al., 2004). Interestingly, it was demonstrated earlier that BKβ1-subunit may increases or decreases the BK channel trafficking in different splice variants of BK channel (Kim et al., 2007b; Toro et al., 2006). Nevertheless, our results indicated that the co-expression of BKβ1-subunit did not change BK channel expression. BKβ4-subunit, which is highly expressed in the brain (Orio et al., 2002), also did not change BK channel expression. The results of this study indicated that the BK β-subunits do not inhibit the CSPα-mediated effect, and that CSPα does not change the expression of BKβ-subunits. It can be concluded that the CSPα-mediated effect influences primarily the expression if the BKα-subunit.

So far, the cellular locations where BK channels and the BKβ-subunits associate to form a complex remains controversial; it is possible that they assemble into a complex either before getting delivered to the cell surface or directly at the cell surface. The biotinylation of the BK channel and the BKβ1-subunit indicates that both proteins get synthesized and delivered to the cell surface (results shown in Figure 24). Preliminary co-immunoprecipitation experiments (data not shown) gave evidence that the BK channel and the BKβ1-subunit form a complex. It was not possible to detect BKβ4-subunit at the cell surface or immunoprecipitated with the BK channel. One explanation for our observation could be that the tested protein were from different species and were not able to interact with each other. The BK channel which we used is a mouse protein, while the tested BKβ1-subunit is bovine and the BKβ4-subunit is a human protein. Perhaps not all species-specific protein constructs can interact with each other. The bovine BKβ1-subunit and the human BKβ4-subunit share a 90 % amino acid sequence homology. Whereas the BKβ4-subunit

sequence homology between human, mice and bovine is 100 %, which may indicate that there are other factors play a role in protein assembly.

Overall, these experiments indicate that the BKβ-subunit expression did not change the CSPα-mediated effect on BK channel expression, suggesting that CSPα acts exclusively on the BK α-subunit.

4.7 Influence of drug treatment on the CSPα-mediated effect on BK channel expression

It has recently been shown (Xu et al., 2010) that quercetin treatment affects CSPα dimerization however, its influence on CSPα function remains unknown. To obtain further insight, we evaluated the effect of the drug treatment on the CSPα-mediated reduction of BK channel expression. Therefore we investigated the effects of the proteasome inhibitor lactacystin, the flavonoid quercetin, the BK channel blocker TEA, and dbcAMP - a membrane permeable second messenger analogue.

4.7.1 Lactacystin increases the amount of ubiquitinylated proteins

Lactacystin is a specific proteasome inhibitor used to evaluate proteasomal activity of cells *in vitro*. Lactacystin is one of the most commonly used proteasome inhibitors and it is naturally produced by *Streptomyces bacteria*. The main function of the proteasome is to degrade non-essential proteins by proteolysis. Proteins are tagged with ubiquitin as a marker for degradation. This process is essential for the cell, to remove unfolded or damaged proteins. Our study indicated that the treatment of CAD cells with lactacystin did not influence the CSPα-mediated effect on BK channel expression (Figure 25). BK channel expression was not altered in either untreated or lactacystin-treated cells. All CSPα transfected cells showed the already described reduction in BK channel expression compared to the negative control. As expected, treatment with Lactacystin increased the amount of ubiquitylated proteins. Due to the inhibition of the proteaosome, the ubiquitylated proteins could not be degraded. Overall, these results indicated that the specific proteasome inhibitor lactacystin did not alter CSPα's reduction in BK channel

expression. In contrast, the group of Young and Shrier demonstrated that lactacystin treatment of hERG co-transfected with J-proteins restored the mature hERG expression (Walker et al., 2009). J-proteins had no effect in the presence of lactacystin on the core- or full-glycosylated hERG and thus had no effect on the trafficking efficiency of hERG channel suggesting that hERG gets degraded through a proteasome-dependent manner in contrast to BK channel expression. Treatment with MG132, another proteasome inhibitor was ineffective to restore CFTR when co-transfected with CSPα (Schmidt et al., 2009). The inhibition led to an accumulation of ubiquitylated proteins, comparable to our findings. The fact that inhibiting the proteasome failed to prevent the CSPα mediated reduction in expression of either BK channel expression or CFTR suggests that the proteasome is not involved.

4.7.2 Quercetin enhances the CSPα mediated effect on BK channel expression

In the following experiments the influence of quercetin, TEA and dbcAMP, on CSPα's reduction in BK channel expression was evaluated. The results demonstrated that TEA and dbcAMP had no effect on BK channel expression compared to the CSPα control. Only the quercetin treatment showed an enhancement of the CSPα-mediated reduction on BK channel expression. Quercetin is a flavonoid and it is widely used as a memory enhancer and for reducing altitude sickness and cancer, by mechanisms that still remain largely unknown. It has been recently shown (Xu et al., 2010) that quercetin stabilizes the CSPα-CSPα dimer and inhibits synaptic transmission. Our results suggested that quercetin stabilizes CSPα and thus enhances the CSPα-mediated effect in BK channel expression.

TEA is a selective drug used to block potassium channels. The aim of testing TEA was to determine if it would alter the CSPα reductive effect on BK channel expression. However, our results indicated that TEA treatment did not alter the CSPα-mediated effect.

cAMP is an important second messenger. It activates protein kinase A (PKA) and thus it is important for the adenyl-cyclase-signal transduction pathway. In this study the effect of dbcAMP, a cAMP analogue, was evaluated. PKA activation triggers in turn the phosphorylation of numerous proteins, thus generating a signaling cascade within the cell. Since cAMP-dependent PKA has been was reported to phosphorylate CSPα (Evans et al.,

2001) and BK channels are reported to show altered activity through phosphorylation by PKA (Tian et al., 2004; Zhou et al., 2001) we tested if the effect of dbcAMP on the CSPα-mediated reduction of BK channel expression. Our data indicate that a PKA signaling cascade is not involved in CSPα-mediated reduction of BK channel expression (results in Figure 26).

Taken together, these findings indicate that of the agents tested only quercetin was able to enhance the CSPα-reductive effect on BK channel expression.

4.7.3 Quercetin enhances reduces BK channel expression

Next we determined if quercetin reduces BK channel expression via CSPα and or directly on BK channel expression. Xu et al. (2010) first described that quercetin inhibits neurotransmission, but did not explain how. Our study pointed out that quercetin drastically reduced BK channel expression even when CSPα was not co-transfected with the BK channel (results in Figure 27). These findings indicated that quercetin alters BK channel expression through an independent mechanism and which is independent of overexpressed CSPα. However it cannot be ruled that the effect of quercetin is independent of CSPα because CAD cells express endogenous CSPα. This study suggested that quercetin might inhibit neurotransmission by down-regulating the BK channel, which would lead to reduce in hyperpolarization, changes in the intra- and extra-cellular Ca^{2+} homeostasis and alterations in neurotransmitter release. Further investigations need be done to determine whether quercetin targets the BK channel *in vivo* and thus inhibits neurotransmission.

5 RESULTS

CHAPTER II: Stable transfected BK channel expressing CAD cells

The experiments in Chapter I evaluated BK channel steady state levels in CAD cells transiently co-expressing BK channel and various chaperones and CSPα constructs. Since BK channel is initially synthesized in the ER, transported through the secretory pathway to its functional site at the plasma membrane where it is eventually removed from the plasma membrane and degraded by endocytic and proteolysis pathways, J-protein-mediated reduction in BK channels levels could be due to either reduced movement through the secretory pathway or increased degradation through the proteolysis pathways. To further investigate the CSPα/BK channel relationship, a stable BK channel expression cell line was generated and the effect of CSPα and Hsp40 on the steady-state expression of BK channel was evaluated.

A stable BK channel expression CAD cell line was generated, according to the method described in section 2.2.3. Western Blot analysis was used to identify positive clones for their stably expressed BK channel. Figure 28 shows one positive clone which stably expressed BK channel protein. In total, 20 clones were screened for stable BK channel expression and three positive clones were found.

Figure 28: Generation of a CAD cell line that stably expresses the BK channel protein. 20 CAD cell clones were screened for the stable expression of the BK channel protein

5.1 Examination of the influence of J-proteins (CSPα and Hsp40) on BK channel expression in CAD cell lines stably expressing the BK channel

To test the effect of J-proteins on BK channel expression in cell lines that stably express BK channels, cells were transfected with either 0.75 µg myc-tagged CSPα, $CSP\alpha_{HPD\text{-}AAA}$, Hsp40, $Hsp40_{HPD\text{-}AAA}$ or pCMV plasmid (negative control). 48 h after

transfection, cells were harvested, protein concentration was measured and Western Blot analysis was carried out. The membrane was probed with a BK channel antibody to detect the stable BK channel expression and CSPα or Hsp40 expression was visualized with a myc-tag antibody. The results are presented in Figure 29.

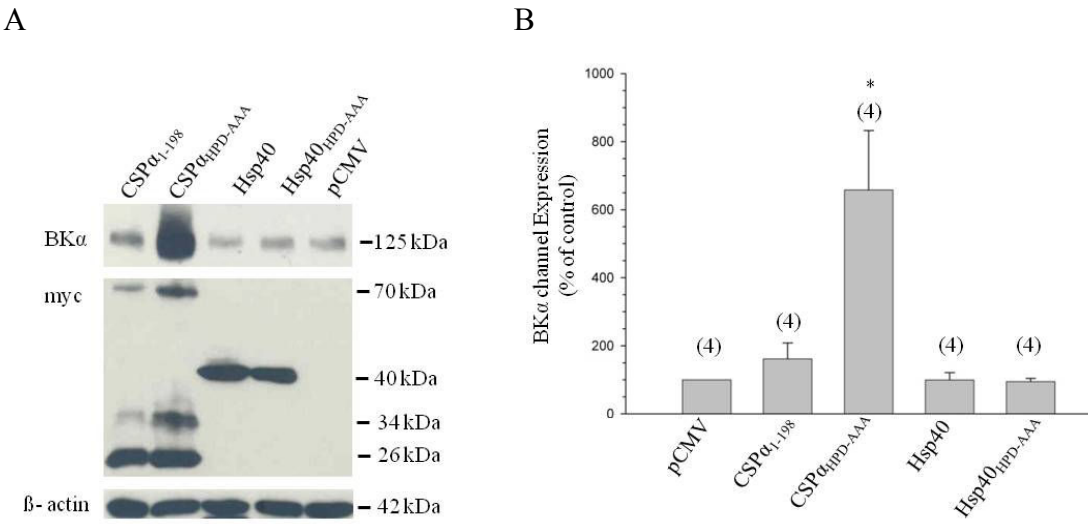

Figure 29: $CSP\alpha_{HPD\text{-}AAA}$ increases BK channel expression in stably transfected, BK channel expressing CAD cells.
Panel A. Western Blot analysis of BK channel expression in stably BK channel expressing CAD cells which were transiently transfected with 0.75 µg myc-tagged full length CSPα, CSPα, $CSP\alpha_{HPD\text{-}AAA}$, 1 µg Hsp40 or $Hsp40_{HPD\text{-}AAA}$ and incubated for 48 h. As negative control, 0.75 µg empty pCMV plasmid was transfected. 30 µg cell lysate were separated by SDS-PAGE, probed with an anti-BK channel antibody. The myc expression is shown as transfection control for CSPα and ß-actin presents the loading control. The corresponding mean data are shown in panel B in comparison to the pCMV control; * $p< 0.05$

The results in Figure 29 show the Western Blot analysis and the corresponding mean data. The results in Figure 29A indicate that CSPα, Hsp40 and $Hsp40_{HPD\text{-}AAA}$ have no effect on BK channel expression compared to the negative control. The co-expression of $CSP\alpha_{HPD\text{-}AAA}$ shows a drastic increase in BK channel expression. The corresponding mean data (Figure 29B) indicate that while CSPα increased BK protein expression slightly (161.2 ± 46.8 %), $CSP\alpha_{HPD\text{-}AAA}$ elicited a statistically significant increase in BK channel expression (657.5 ± 175.4 %) compared to the negative control (100 %). Hsp40

(99.3 ± 21.7 %) and $Hsp40_{HPD-AAA}$ (94.7 ± 9.5 %) were not found to alter BK channel expression compared to the negative control. Overall, these results indicate that in CAD cells stably expressing BK channel, CSPα/$CSP\alpha_{HPD-AAA}$ specifically alters BK channel expression while Hsp40/$Hsp40_{HPD-AAA}$ does not change BK channel expression in a steady-state expressing CAD cell line.

5.2 Examination of the influence of CSPα on BK channel expression at the cell surface in a stable BK channel expressing cell line

Next, we aimed to determine if the overexpression of $CSP\alpha_{HPD-AAA}$ also has an effect on BK channel expression at the cell surface. Stable BK channel expressing CAD cells were transfected with either 0.75 µg myc-tagged CSPα, $CSP\alpha_{HPD-AAA}$ or empty pCMV as negative control. The cells were biotinylated and processed as described in 2.3.4. 1 mg of total protein was used for a streptavidin pull-down assay and Western Blot analysis was carried out. The membrane was probed with a BK channel antibody to detect biotinylated BK channel at the cell surface.

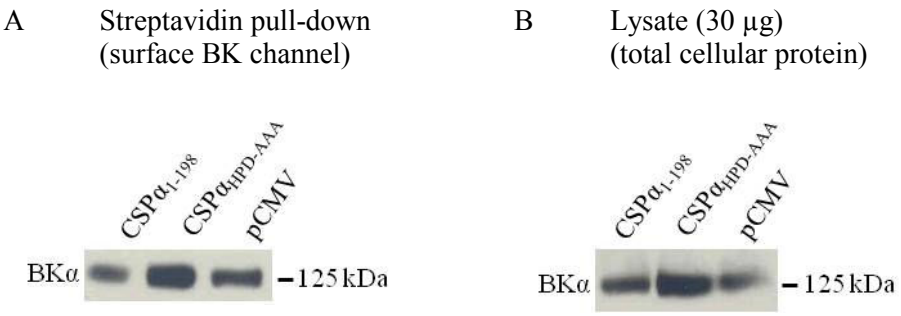

Figure 30: $CSP\alpha_{HPD-AAA}$ increases BK channel expression at the cell surface in stably transfected CAD cells expressing the BK channel.
Stably expressing BK channel CAD cells were transiently transfected with 0.75 µg myc-tagged CSPα, 0.75 µg $CSP\alpha_{HPD-AAA}$ and 0.75 µg pCMV (negative control). 48 h after transfection the cells were labeled for 30 min with Biotin at 4°C. The cells were harvested and protein concentration was measured; 1 mg of total protein was used for the overnight pull-down. The pull-down proteins (A) and 30 µg of total cellular proteins (B) were electrophoresed and the protein expression was analyzed by Western Blot with anti-BK channel antibody to visualize BK channels at the surface (A) or in the total protein (B). Data are representative of three separate experiments.

The Western Blot analysis of the cell surface biotinylation of the stable BK channel-expression cell line is presented in Figure 30A. The results demonstrate that CSPα (lane 1) has no effect on BK channel expression at the cell surface compared to the negative control (lane 3). However, $CSP\alpha_{HPD\text{-}AAA}$ (lane 2) increased the cell surface expression of BK channel. Figure 30B shows that the total cellular protein lysate is comparable to that found in Figure 29A. $CSP\alpha_{HPD\text{-}AAA}$ (lane 2) increased BK channel protein expression compared to CSPα (lane 1) and to the negative control (lane 3).

Overall these results indicated that in CAD cells that stably express BK channels, $CSP\alpha_{HPD\text{-}AAA}$ increased the BK channel expression in both the total cellular lysate and the cell surface.

6 DISCUSSION

CHAPTER II: Stable transfected BK channel expressing CAD cells

In chapter II I demonstrated that a dominant negative form of CSPα, namely $CSP\alpha_{HPD\text{-}AAA}$ increased endogenous BK channel expression in a stably BK channel expressing CAD cell line.

6.1 $CSP\alpha_{HPD\text{-}AAA}$ increases BK channel expression in a stably transfected BK channel expressing cell line

Surprisingly, a CAD cell line stably expressing the BK channel $CSP\alpha_{HPD\text{-}AAA}$ showed a drastic, statistically significant increase in BK channel expression (Figure 29). Whereas CSPα shows no changes in BK channel expression. Since $CSP\alpha_{HPD\text{-}AAA}$ is not expected to activate Hsc70, it is predicted to act as a dominant negative regulator for endogenous BK channel. These results suggest that interference with intrinsic cellular CSPα chaperone activity increases BK channel protein expression. The reason why $CSP\alpha_{HPD\text{-}AAA}$ overexpression triggered a drastic increase in BK channel expression in CAD cells stably expressing BK channels while CSPα reduced BK channel expression in CAD cells transiently expressing BK channels remains to be confirmed, but a number of possibilities exist. For example, the ratio of BK channel:CSPα expressed in the transient expression system may well be different than that in the stably expressing BK channel cell lines. Another possibility would be that CSPα may also influence the cDNA plasmid prior to BK channel entry into the secretory pathway. Despite these differences, a CSPα dominant negative in CAD cells that steady-state express BK channel increases cellular BK channel while an increase in CSPα levels in a transient co-transfection system reduces BK channel. Thus the conclusion is the same; CSPα reduces BK channel protein expression. In contrast, the overexpression of DJA1 and DJA2 in a stably expression hERG cell line showed the same results as in the transient transfected system. (Walker et al., 2009).

Furthermore, J-protein selectivity was higher in CAD cells stably expressing BK channels as evidenced by the fact that Hsp40 and $Hsp40_{HPD\text{-}AAA}$ had no effect on BK

channel expression compared to the negative control. These results indicate that the effects of CSPα/CSPα$_{HPD-AAA}$ are more specific than that of Hsp40. Hsp40 reduced BK channel expression in the transiently transfected system (Chapter I), but did not alter the expression in the cells stably expressing the BK channel. Additionally, in the transiently transfected system, CSPα$_{HPD-AAA}$ had no effect on BK channel expression, whereas CSPα drastically decreased BK channel expression.

The incubation time of 24 h which was used for almost all studies in Chapter I, was increased to 48 h for this study; this was done because preliminary results showed the 24 h incubation time showed only weak changes in BK channel expression. The study in Chapter I indicated that an increase in incubation time increased the inhibitory effect of tested J-proteins on the BK channel. The results presented in this chapter indicate that, with an increase in incubation time, both the CSPα and CSPα$_{HPD-AAA}$ mediated effect on BK channel expression was enhanced. An explanation for this effect could be that the steady-state BK channel expression is more stable than the transient transfected BK channel expression and thus the CSPα-mediated reduction needs a longer time to change the BK channel expression. Another possibility is that the endogenous CSPα and the overexpressed myc-tagged CSPα or CSPα$_{HPD-AAA}$ are competing, and thus the effect is only seen after a longer incubation time.

6.2 CSPα$_{HPD-AAA}$ changes BK channel expression at the cell surface in a cell line stably expressing the BK channel

The results in 5.1 (Figure 29) raised the question if CSPα$_{HPD-AAA}$ also influences BK channel expression at the cell surface. The results (Figure 30) showed that CSPα$_{HPD-AAA}$ increased BK channel expression at the cell surface while CSPα had no effect, compared to the negative control. This finding is consistent with the previous study. The results obtained for the biotinylated proteins and the total cellular protein lysate are not directly comparable, given that 1 mg of total cellular protein was used to extract the biotinylated proteins from the total protein pool that ran on a SDS-PAGE, whereas only 30 µg of the total cellular protein were used for separation by SDS-PAGE. As already discussed in chapter I, the cell

lysis and centrifugation procedures used differed from one another in these two cases. CSPα overexpression did not alter BK channel expression but the overexpression of the $CSP\alpha_{HPD\text{-}AAA}$ mutant resulted in a strong increase in BK channel expression. This result is seen in the total cellular lysate as well as the surface expression of the biotinylated cell surface protein.

Taken together these results identify CSPα as a mayor presynaptic regulator of BK channel expression.

7 OVERALL DISCUSSION AND FUTURE DIRECTIONS

This study provides the first evidence that J-proteins modulate the cellular levels of BK channels. The study in Chapter I demonstrated that J-proteins have the ability to reduce BK channel expression in a transient transfection system. Among the tested J-proteins (CSPα, Hsp40 and Rdj2), CSPα showed the strongest action to reduce BK channel expression. The HPD motif in the J-domain is critical for this function and essential to activate Hsc70. Although CSPα works through the interaction with Hsc70, overexpression of Hsc70 alone does not influence cellular levels of BK channel protein indicating the requirement of J-proteins in this process. CSPα was found to reduce BK channel expression at the cell surface as well as total cellular BK channels. Co-immunoprecipitation experiments indicate that the association of CSPα and BK channels is more robust than the association of $CSP\alpha_{HPD\text{-}AAA}$ and BK channels. Interestingly, a large amount of immature (i.e. not palmitoylated) CSPα was found to associate with BK channels. The study in Chapter II further supports a role for CSPα in the regulation of BK channel expression. Experiments in stably BK channel-expressing cells showed that CSPα did not change BK channel expression, whereas the $CSP\alpha_{HPD\text{-}AAA}$ mutant led to an increase in BK channel expression in the total cellular protein lysate and at the cell surface. It is suggested that the mutation in the HPD motif, CSPα function to activate Hsc70 is inhibit and thus is as acting as a dominant negative for BK channel expression. The influence of the CSPα/$CSP\alpha_{HPD\text{-}AAA}$ system on stable BK channel-expressing cell lines was selective given that the J-proteins Hsp40/$Hsp40_{HPD\text{-}AAA}$ did not change BK channel expression. The finding that Hsp40 is ineffective to change BK channel expression in stably BK channel expressing cell cold be explained by the fast that Hsp40 is a cytosolic co-chaperone, whereas recent results indicate that CSPα is also expressed in the ER, where BK channel protein get processes before it get delivered to the cell surface by the secretory pathway.

These experiments raise the following questions: How does CSPα negatively regulate expression of cellular BK channels? And is CSPα important for the homeostatic expression of BK channels in neurons? A working model is illustrated in Figure 31. In this model, CSPα is a negative regulator of BK channel expression that plays a role at an early

stage of BK channel biogenesis, maturation or trafficking. CSPα together with Hsc70 acts as the molecular control machinery by yet-to-be-established mechanisms making BK channel triage decisions. Our model proposes that CSPα controls the homeostatic expression of the BK channel. However, the $CSP\alpha_{HPD\text{-}AAA}$ mutant has lost its function to activate Hsc70 and thus cannot regulate BK channel expression. Evidence for this model is as follows: first, CSPα does not change BK channel expression in the stably BK channel-expressing cells, whereas the $CSP\alpha_{HPD\text{-}AAA}$ mutant increased BK channel expression in the intracellular and cell surface pool; secondly, CSPα and BK channel interact with each other. The strong interaction of the BK channel with the immature CSPα gives the first suggestion that these two proteins interact with each other at an early stage of channel maturation. The co-immunoprecipitation experiments also indicate that the BK channel interacts with CSPα and not with $CSP\alpha_{HPD\text{-}AAA}$. This suggests that $CSP\alpha_{HPD\text{-}AAA}$ has lost its ability to interact with Hsc70 and also its ability to regulate BK channel expression, by eliminating the trafficking of the BK channel to the cell surface and/or target the BK channel for ER-associated degradation.

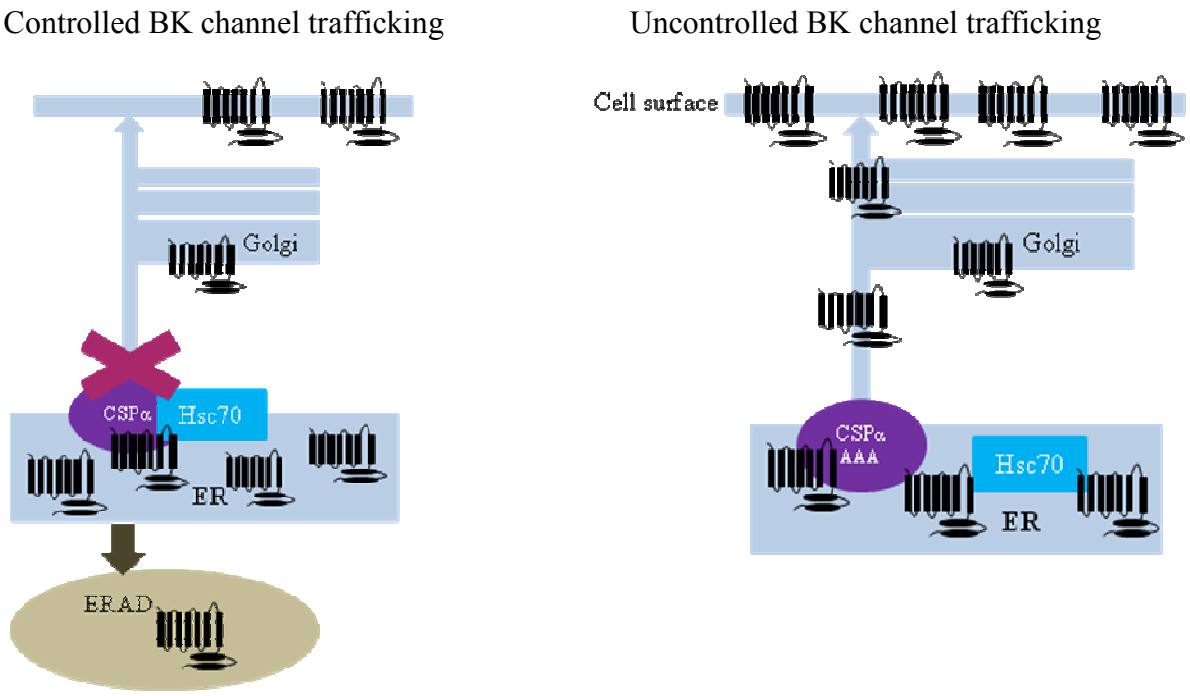

Figure 31: Working model: CSPα is a negative regulator of BK channel expression. CSPα regulates the exit of nascent BK channel of the ER. When CSPα has a mutation in the highly conserved HPD motif, it is no longer able to bind and activate Hsc70; this inhibits Hsc70 interaction with the BK channel. (ERAD: ER-associated degradation)

Despite the working model, changes in BK channel expression alter action potential, high-frequency firing and evoked neurotransmitter release. Possible changes in pre-synaptic action potential amplitude, as well high-frequency firing neuron and neurotransmitter release in comparison of WT CSPα (left panel) and $CSP\alpha_{HPD\text{-}AAA}$ (right panel) overexpression are demonstrated in Figure 32. Figure 32A illustrate that overexpression of $CSP\alpha_{HPD\text{-}AAA}$ reduces and shorten action potential and as well increased hyper-polarization which is due to elevated BK channel expression (as indicated in Figure 31). Figure 32B demonstrate that $CSP\alpha_{HPD\text{-}AAA}$ overexpression reduces and prolong firing rate and thus has negative effects of high-frequency firing neurons. Additional increase in BK channel expression also reduces evoked neurotransmitter release, which is illustrated in Figure 32. The reduction of evoked neurotransmitter release in CSPα-null mice was already reported (Umbach et al., 1994; Zinsmaier et al., 1994).

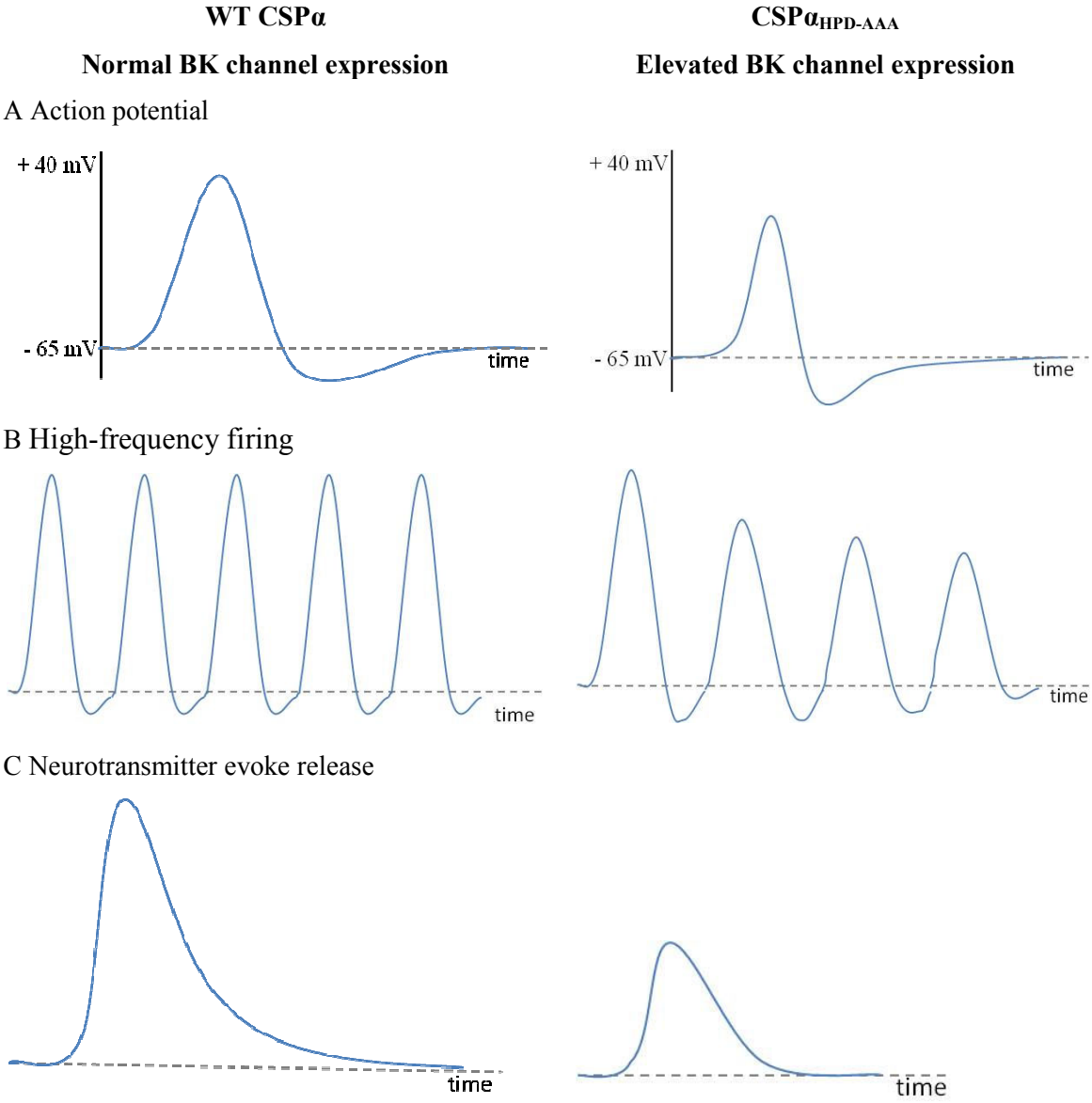

Figure 32: Possible effects of CSPα$_{HPD-AAA}$ on action potential, high-frequency firing and neurotransmitter release.
Panel A illustrates changes in action potential amplitude; Panel B shows changes in high-frequency firing neuron and Panel C demonstrates changes in evoked neurotransmitter release in comparison of WT CSPα (left panel) and CSPα$_{HPD-AAA}$ (left panel) overexpression.

CSPα is abundant in neuronal tissue and important for synaptic vesicle function, but the precise function of CSPα remains still unclear. In this Master's thesis we identified a

new client protein for CSPα; the BK channel. BK channels at the presynaptic level provide a homeostatic mechanism for regulating synaptic transmission by limiting the influx of extracellular Ca^{2+} through voltage-gated Ca^{2+} channels (Salkoff et al., 2006). Various studies have now shown that changes in BK channel function expression can alter neurotransmitter release (Martire et al., 2010; Robitaille et al., 1993; Sausbier et al., 2004). Genetic studies in BK channel-null mice showed an increase in neurotransmitter release at the neuronal junction (Meredith et al., 2004; Sausbier et al., 2004). Up-regulation of BK channel by growth hormones or BK channel activation leads to an increase in hyper-polarization, suppresses neuronal firing and increases the threshold for action potential firing and the amplitude of hyper-polarization, respectively (Holm et al., 1997; Kim et al., 2007a; Song et al., 1999). Because it is known that BK channels are co-localized with voltage-gated Ca^{2+} channels in many neurons; changes in BK channel function can change Ca^{2+} homeostasis and thus affect a large number of neuronal signaling events and processes. Also knockout studies of CSPα in mice and flies have been a progressive neurodegeneration. It has been reported that CSPα knockout changes neuronal Ca^{2+} homeostasis leading to changes in neurotransmitter release; however this has been controversial. Neuronal Ca^{2+} homeostasis and Ca^{2+} signaling regulate multiple neuronal functions, including synaptic transmission, plasticity and cell survival and thus are essential for neuronal function. Dysregulation in Ca^{2+} homeostasis significantly changes neuronal function and leads to neuronal degeneration (Wojda et al., 2008). Interestingly, both CSPα deletions/mutation, as well as changes in BK channel expression, have been reported to change Ca^{2+} homeostasis and neurotransmitter release, leading us to speculate that CSPα controls BK channel homeostasis/expression and thus is neuroprotective. If this is true, it may explain why larval flies with CSPα deletions show an minimal neurotransmitter release at the NMJ (Bronk et al., 2005). Uncontrolled traffic of BK channel to the presynaptic terminal in CSPα null mutants would increase the threshold for action potential firing and the amplitude of hyper-polarization, reducing uptake of Ca^{2+} into the cell and suppressing neurotransmitter release.

Further experimental studies should be carried out to test our model and the hypothesis that CSPα is neuroprotective due to its function as a negative regulator of BK

channel expression. The model in Figure 31 suggested that CSPα is expressed in the inner membrane of the ER and thus controls the exit of BK channel from the ER. First, it has to be proven that CSPα is indeed expressed in the ER. The Frizzell group (Schmidt et al., 2009; Zhang et al., 2002; Zhang et al., 2006) have shown that CSPα is expressed in the ER in epithelial cells, where it regulates the maturation of CFTR. To determine if CSPα is expressed in the ER of neurons and other cell types, two potential experimental approaches can be used. In the first approach, cells could be transfected with CSPα and intact ER could be extracted from the cell. The expression of CSPα in the ER can then be evaluated by Western Blotting. However, if CSPα is shown to be expressed in the ER, the next steps should be the transfection of CSPα in stable BK channel-expressing CAD cells and the determination of the CSPα-mediated effect on BK channel expression in the ER. Changes in BK channel expression in the ER induced by CSPα would indicate that the interaction of CSPα and BK channel occur in the ER, which would subsequently lead to changes in BK channel expression at the cell surface. The second approach would be immunocytochemistry, with confocal microscopy evaluation to determine the co-localization of CSPα with an ER marker, for example Calnexin in neurons or CAD cells. Co-localization would also indicate that CSPα is expressed in the ER. After confirming CSPα expression in the ER, the next step would be to test for co-localization of the BK channel with CSPα in the ER. If CSPα and BK channel are not co-localized in the ER, immunocytochemistry evaluated by confocal microscopy would provide another option to identify their cellular localization.

Changes in BK channel expression at the cell surface would be followed by changes in the excitability of the cell. Electrophysiology experiments, such as whole patch clamping, would allow for the functional analysis of CSPα - or $CSP\alpha_{HPD\text{-}AAA}$ - mediated changes in BK channel expression. The experiments could possibly establish that the overexpression of $CSP\alpha_{HPD\text{-}AAA}$ in the stable BK channel expressing cells increases functional BK channel expression at the cell surface by measuring an increase in the overall cell current. Changes in cell excitability would be followed by possibly important physiological alterations in Ca^{2+} homeostasis or neurotransmitter release.

It is known that BK channels are co-localized with voltage-gated Ca^{2+} channels in many cells and thus it would interesting to determine if changes in BK channel expression would change the de- and re-polarization phases of neurons. Ca^{2+}-live cell imaging in neuronal cultures would be an interesting tool to investigate these effects. Such an experimental approach would be highly interesting to determine the CSPα-mediated effect on BK channel expression in neurons with respect to changes in Ca^{2+} homeostasis or neurotransmitter release.

The co-immunoprecipitation experiments showed that CSPα interacts with the BK channel. The next step would be to determine whether Hsc70 is also a component of this complex.

Further co-immunoprecipitation experiments are required to identify specific interaction domains within CSPα and the BK channel. Firstly, it would be interesting to determine the interaction domain of CSPα which interacts with the BK channel. For such a study, CSPα truncations that were used to identify the functionally-specific domain of CSPα could be used to determine the domain of CSPα responsible for interacting with the BK channel. Furthermore, it is of interest to determine the BK channel domain which interacts with CSPα. Therefore, co-immunoprecipitation of CSPα with BK channel 'core' and 'tail' domains would identify regions of the BK channel that interacts with CSPα. It is suggested that the C-terminal region of CSPα interacts with the intracellular 'tail' domain, which represents two thirds of the whole BK channel protein.

The preliminary results in the Appendix section shows the evaluation of fluorescence staining of BK channel expression with CSPα and Hsp40 in rat hippocampal neurons. The quantification of fluorescence staining is challenging and therefore an easier approach would be to investigate the effects of CSPα and Hsp40 on BK channel expression in rat hippocampal neurons by quantitative Western Blot analysis. This would indicate whether CSPα or Hsp40 change BK channel expression in differentiated neurons. Another approach would be to evaluate specific localizations of both proteins and possibly determine their co-localization by confocal microscopy. This would allow one to investigate if CSPα is a negative regulator of BK channel expression.

Furthermore, the roles of other proteins, such as CHIP (C-terminus Hsc70-interacting protein) or Hsp90, could be investigated. The Frizzell group (Schmidt et al., 2009; Zhang et al., 2002; Zhang et al., 2006) have proposed a novel role for CSPα at the ER in epithelial cells; according to their model, CSPα negatively regulates CFTR progression at post-ER compartments. Their model was based on the following findings: overexpression of CSPα blocked the maturation of CFTR and this blockage required Hsc70/Hsp70, because mutation in the J-domain showed no effects on CFTR expression (Zhang et al., 2002; Zhang et al., 2006). A later study (Schmidt et al., 2009) provided evidence that CSPα is involved in proteasomal-mediated CFTR degradation. The results demonstrated that the overexpression of CSPα increased the association of CFTR with Hsc70/Hsp70 and with the E3 ubiquitin ligase CHIP. Additionally, it has been proven that CSPα and CHIP interact directly and CSPα increases the CFTR ubiquitinylation. Interactions between CSPα, Hsp70/Hsc70 and CHIP suggest a model in which CSPα coordinates the formation of a complex that facilitates the degradation of CFTR as it blocks CFTR exit from ER. It would be of interest to test for a CHIP interaction with CSPα, which activates the BK channel for proteasomal-mediated degradation.

Hsp90 is one of the most abundant proteins in the cell. The Hsp90 chaperone network gives assistance in protein folding, cell signaling, and tumor repression. Additionally, it was also shown to be involved in the biogenesis/maturation efficiency of several membrane proteins. Especially, Hsp90 is required for the folding of a subset of client proteins that are believed to have difficulties in reaching a native conformation (Young et al., 2001). The Hsc70/Hsp90 network has been characterized for the CFTR and glucocorticoid receptors (Grad and Picard, 2007; Okiyoneda et al., 2010). Recent publications provide evidence that the Hsp90 chaperone complex participates in the biogenesis of K_{ATP} channels (Yan et al., 2010). The Shyng group demonstrated that K_{ATP} is associated with Hsp90, Hsp40 and Hsc70. Additionally, inhibition of Hsp90 function markedly reduces surface expression of K_{ATP} channel and an overexpression of Hsp90 significantly increases surface expression (Yan et al., 2010). These findings indicate that Hsp90 facilitates K_{ATP} channel biogenesis by increasing the folding/processing efficiency.

It would be interesting to test if Hsp90 is also involved in BK channel maturation/trafficking.

In this Master's thesis I proved the hypotheses that: **"CSPα interacts with and regulates the expression of the BKα subunit of BK channels"** and provided the first experimental evidence that CSPα regulates the BK channel expression. Further investigations have to be performed to evaluate the precise role of CSPα in BK channel maturation in the ER and/or its trafficking to the cell surface.

8 RESULTS

APPENDIX: Hippocampal neurons

BK channels are abundant in neurons and important for membrane repolarization after stimulation (Salkoff et al., 2006). Thus, it is important to determine if CSPα and Hsp40 influences BK channel expression in neurons. Therefore, hippocampal neurons were isolated by dissection from neonatal rats at postnatal day 0. On the day of dissection the neurons were co-transfected by electroporation with 0.75 µg cDNA of either myc-tagged CSPα or $CSP\alpha_{HPD\text{-}AAA}$, or with 1 µg of either Hsp40 or $Hsp40_{HPD\text{-}AAA}$ with 0.5 µg CFP (cyan fluorescent protein) as positive transfection control and plated on chips. 3, 6 and 12 days after transfection, live cell imaging (described in 2.2.5) and fluorescence staining (described in 2.3.6) were carried out.

8.1 Live cell imaging of rat hippocampal neurons transfected with CSPα and $CSP\alpha_{HPD\text{-}AAA}$

Figure 33 shows the live cell imaging of rat hippocampal neurons transfected with CSPα or $CSP\alpha_{HPD\text{-}AAA}$. The neurons were co-transfected with CFP as positive control and CFP positive cells were observed under a fluorescence microscope with a CFP filter (460-500 nm). Cell viability, transfection efficiency and formation of synapses were determined by live-cell imaging of neurons. Figure 33A shows that transfection efficiency was high and no changes in viability were observed among CSPα (first panel), $CSP\alpha_{HPD\text{-}AAA}$ (middle panel) and the CFP control (last panel) 3 days following transfection. Figure 33B shows neurons 6 days after transfection. Compared to Figure 33A, it is seen that the axons and dendrites are longer and that there are more synapses (these are seen as white dots in-between axons). No differences in cell viability were observed among CSPα (first panel), $CSP\alpha_{HPD\text{-}AAA}$ (middle panel) and CFP control (last panel). 12 days after transfection (Figure 33C) viability is reduced in CSPα- and $CSP\alpha_{HPD\text{-}AAA}$- and CFP-transfected neurons. Primary neurons are difficult to culture over a long period of time, especially after electroporation with cDNA plasmids, which may be a reason for the

reduced cell viability observed after 12 days (Figure 33C). Overall, these results indicated that, at all stages, transfections of CSPα and CSPα$_{HPD-AAA}$ had no negative effect on neurons viability compared to the CFP control.

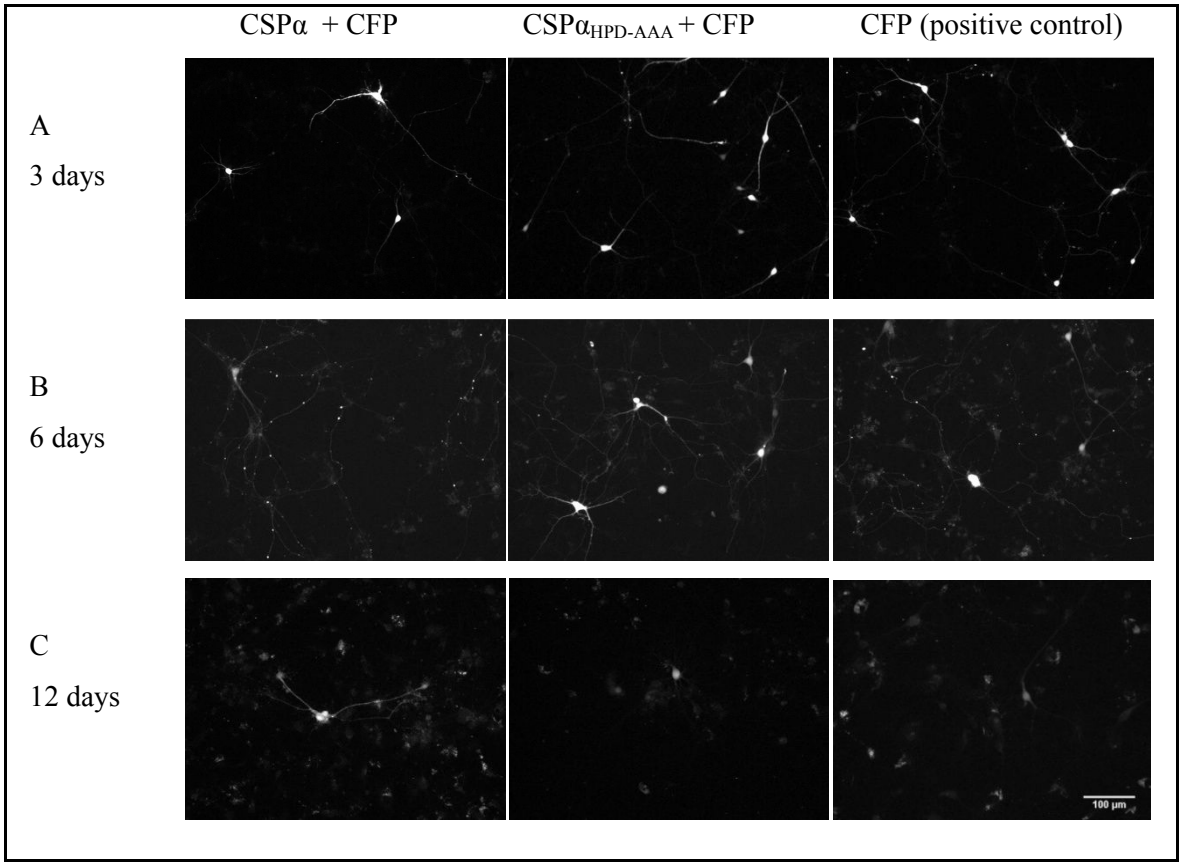

Figure 33: Live cell imaging of rat hippocampal neurons transfected with CSPα (left) or CSPα$_{HPD-AAA}$ (middle).
Hippocampal neurons were isolated by dissection from P0 rats and co-transfected by electroporation with 0.75 µg myc-tagged CSPα or CSPα$_{HPD-AAA}$ cDNA and 0.5 µg CFP as positive transfection control. Cell viability was determined 3 (A), 6 (B) and 12 (C) days after transfection. Live cell imaging was performed by fluorescence microscopy (10 x magnification), using a CFP filter and the AstroVid software to capture the corresponding images.

8.2 Immunofluorescence staining of CSPα and the BK channel in rat hippocampal neurons

After live cell imaging, neurons were fixed and fluorescence staining (for the protocol, see 2.3.6) was carried out. The neurons were stained with a specific BK channel polyclonal antibody to detect the endogenous BK channel expression and a monoclonal myc-tag antibody to detect the overexpression of myc-tagged CSPα and CSPα$_{HPD-AAA}$. Immunofluorescence experiments focused on 3 and 6 days following transfection when viability was good.

The images obtained 3 days after transfection (Figure 34A) show that BK channel expression (first panel, green) is localized at the soma and axon level. Myc-expression (middle panel, red) shows that CSPα expression also occurs at the soma and axon level. The merged images in the last panel indicate that these two proteins are partially co-localized. The same was observed for CSPα$_{HPD-AAA}$ overexpression. There were no obvious changes in BK channel distribution expression when CFP (positive transfection control), CSPα or CSPα$_{HPD-AAA}$ were overexpressed in neurons. Figure 34B shows the fluorescence staining images 6 days after transfection when either CSPα or CSPα$_{HPD-AAA}$ was overexpressed in neurons. The images in Figure 34B show that the BK channel is expressed throughout the cell soma and at the axon level. It seems that there is a slight BK channel accumulation in neurons when CSPα is overexpressed. The overexpression of CSPα$_{HPD-AAA}$ shows a concomitant BK channel expression throughout the neuron. The CSPα or CSPα$_{HPD-AAA}$ expression (middle panel) is indicated by the myc-tag antibody. These two proteins are expressed everywhere in the cell, but show reduced expression in the nucleus. The merged image in the last panel of Figure 34B suggests co-localization of the BK channel and of CSPα or CSPα$_{HPD-AAA}$.

Further investigations are required to demonstrate if there is indeed co-localization of BK channel with CSPα or CSPα$_{HPD-AAA}$ and where exactly it occurs within the cell. Additionally, it remains to be established if the overexpression of CSPα is followed by an accumulation of BK channel in the cell soma.

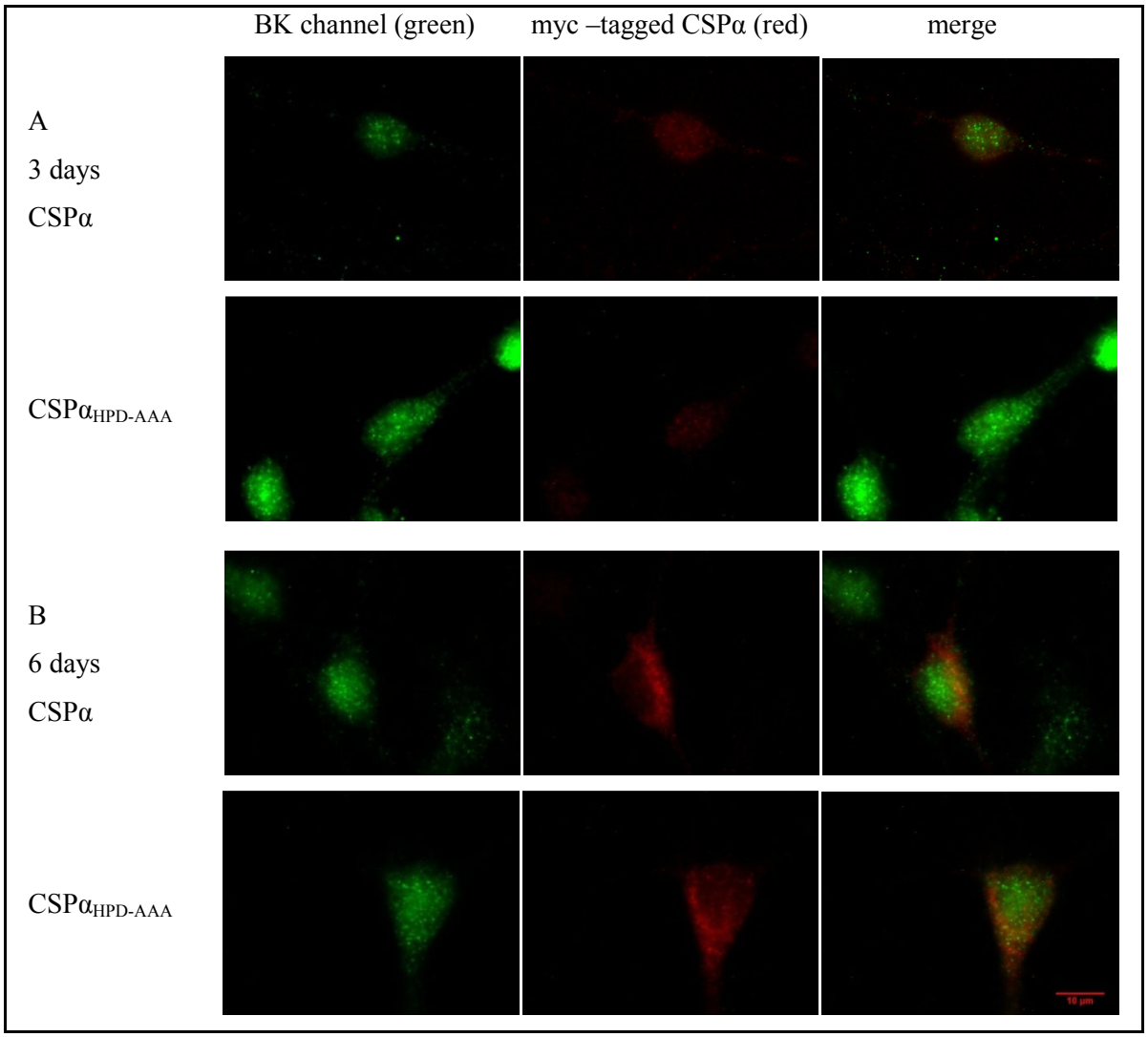

Figure 34: Immunofluorescence staining of CSPα and the BK channel in rat hippocampal neuron.

Hippocampal neurons were isolated by dissection from P0 rat and co-transfected by electroporation with 0.75 µg myc-tagged CSPα or CSPα$_{HPD-AAA}$ cDNA and 0.5 µg CFP as positive transfection control. 3 (A) and 6 (B) days after transfection, immunefluorescence staining was carried out with polyclonal anti-BK channel antibody to visualize endogenous BK channel expression (green) and monoclonal anti-myc-tag antibody to visualize CSPα expression (red). Cell imaging was performed by fluorescent microscopy.

8.3 Live cell imaging of rat hippocampal neurons transfected with Hsp40 and $Hsp40_{HPD-AAA}$

It is known that Hsp40 is important in stress response in neurons (Zhao et al., 2008). In this experiment we evaluated the role of Hsp40 and $Hsp40_{HPD-AAA}$ on BK channel expression in neurons. Hippocampal neurons were electroporesed with either 1 µg cDNA Hsp40 or $Hsp40_{HPD-AAA}$ and 0.5 µg CFP as positive control. 6 days after transfection, images of neurons were captured by live cell imaging and cell viability was evaluated. The corresponding results are shown in Figure 35 and they demonstrate that the overexpression of Hsp40 or $Hsp40_{HPD-AAA}$ led to no changes in cell viability compared to the positive control. The investigated neurons formed long axons and built a network with other neurons.

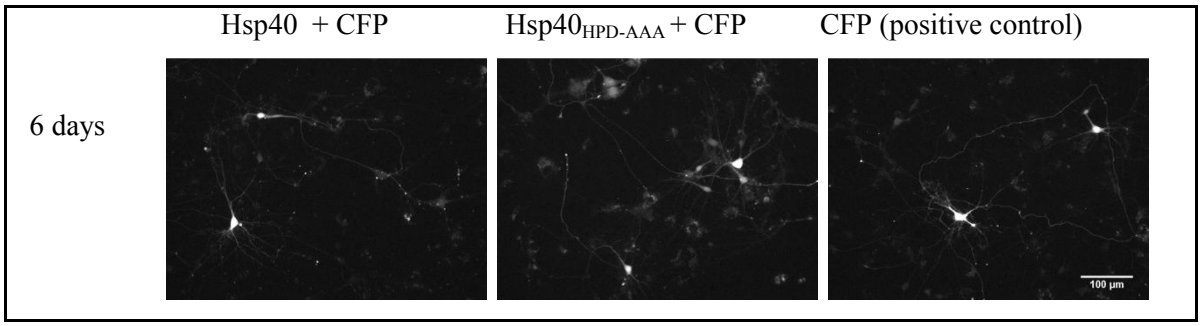

Figure 35: Live cell imaging of rat hippocampal neurons transfected with either Hsp40 or $Hsp40_{HPD-AAA}$.
Hippocampal neurons were isolated by dissection from P0 rats and co-transfected by electroporation with 1µg myc-tagged Hsp40 cDNA and 0.5 µg CFP as positive transfection control. Cell viability was determined 6 days after transfection by live cell imagining, using a fluorescent CFP filter and the AstroVid software to capture the corresponding images.

8.4 Immunofluorescence staining of Hsp40 and the BK channel in rat hippocampal neurons

After live cell imaging, cells were stained for endogenous BK channel expression and myc-tagged Hsp40 and fluorescence microscopy was carried out. Neurons were stained with a BK channel antibody to visualize the endogenous BK channel and a myc-tag antibody to detect overexpressed myc-tagged Hsp40. The BK channel was detected with a secondary Cy3-conjugated antibody at 550-579 nm and myc expression with a secondary

Cy5-conjugated antibody at 650-670 nm. Corresponding fluorescence images are presented in Figure 36, with BK channel expression in green, myc-tagged CSPα expression in red and the merged channel. The images indicate that the BK channel is present in the cell soma and axons. Hsp40 is seen through the whole neuron, including nucleus, cell soma and axons. The merged image suggests possible co-localization. Further methods should be used in future studies to determine co-localization.

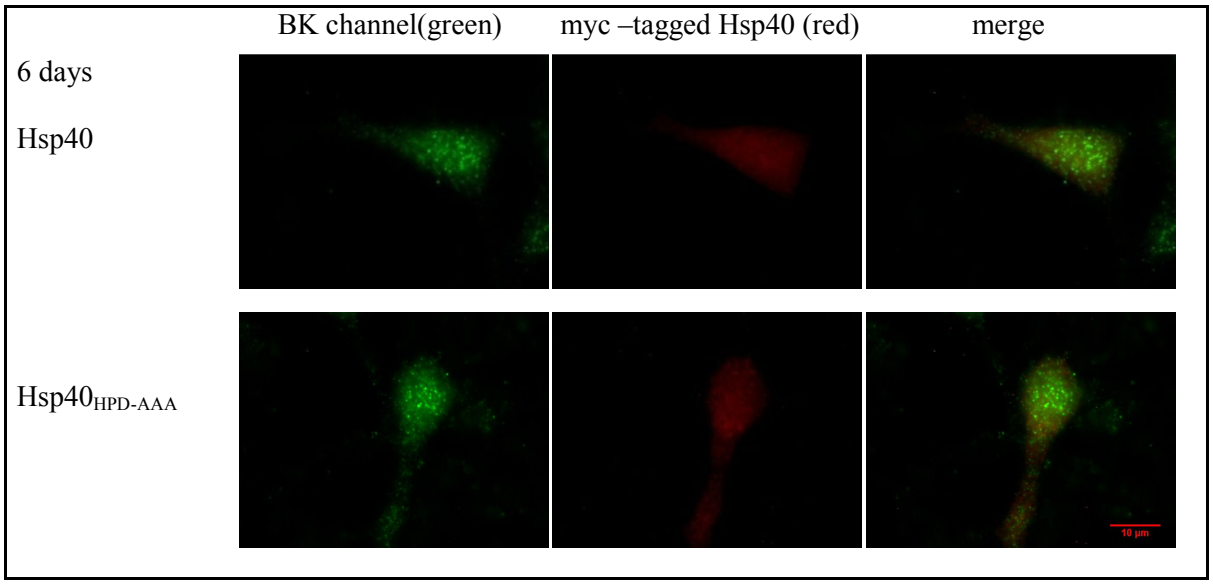

Figure 36: Immunofluorescence staining of Hsp40 and the BK channel in a rat hippocampal neuron.
Hippocampal neurons were isolated by dissection from P0 rat and co-transfected by electroporation with 0.75 µg myc-tagged Hsp40 or $Hsp40_{HPD\text{-}AAA}$ cDNA and 0.5 µg CFP as positive transfection control. 6 days after transfection, immunefluorescence staining was carried out with polyclonal anti-BK channel antibody to visualize endogenous BK channel expression (green) and monoclonal anti-myc-tag antibody to visualize Hsp40 expression (red). Cell imaging was performed by fluorescent microscopy.

9 DISCUSSION

APPENDIX: Hippocampal neurons

The preliminary results in the fluorescence staining experiments indicate that experiments in hippocampal cultures are feasible and that CSPα or Hsp40 partially co-localizes with the BK channel. Further experiments are needed to determine where in the neurons CSPα or Hsp40 are co-localized with BK channel and also if these two J-proteins have indeed an effect on BK channel expression in the neurons.

The images seen in Appendix are the results of a single set of experiments. Rat hippocampal neurons (acknowledgments to Lucas Scott, Colicos Lab for carrying out the dissection and helping me with fluorescence staining and microscopy) were transfected and plated on several chips and thus it was possible to evaluate cell viability and fluorescence staining (qualitative and not quantitative) over more than one time point. To evaluate the effect of CSPα or Hsp40 on the BK channel, this experiment needs to be repeated for at least another three times to make clear statistically relevant suggestions. In this first set of experiments, it was possible to determine the specificity and working concentrations of the primary and secondary antibodies for fluorescence staining. The polyclonal BK channel showed a better binding capacity than the monoclonal BK channel antibody (data not shown). Polyclonal CSPα and Hsp40 antibodies were also tested. Staining showed a stronger expression of CSPα and Hsp40 because the antibodies also detected endogenously expressed CSPα and Hsp40 in the neurons; the detected staining pattern was comparable to the monoclonal myc-tag antibody. For the secondary antibodies we also investigated specific binding and detection at the different wavelengths used or when myc-tagged CSPα or Hsp40 were not transfected. Therefore it was possible to demonstrate that the detected signal was specific.

The electroporation of neurons as a method was also evaluated. It is widely accepted that neurons are difficult to transfect with cDNA. They are large cells and thus more efficient methods are often required. Neurons have a slow recovery time from permablization, which reduces cell viability after transfection compared to other cells. They express transfected genes less efficiently and slower because neurons are post-mitotic cells.

The plasmid can reach the nucleus in mitotic cells during the cell cycle, whereas in post-mitotic cells this can only occur through nuclear pores (Mertz et al., 2002).

Overall, electroporation showed a good transfection efficacy and thus it was possible to evaluate antibody binding for future experiments. One disadvantage of the method was that neurons show reduced cell viability. To determine if CSPα or Hsp40 are co-localized in specific cell compartments, further investigations will be required.

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