The multiple functions of the KChIP proteins

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by

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Contents

Abstract

Intracellular Ca^{2+} signals carry out crucial roles in neurons. In order to interpret these Ca^{2+} signals into different changes in neuronal function, a number of Ca^{2+} sensor proteins are required to respond selectively to defined signals and modulate neuronal activity appropriately. These Ca^{2+} sensing proteins include the neuronal calcium sensor (NCS) proteins. The NCS proteins contain EF-hand Ca^{2+} binding domains and provide selectivity by their differing Ca^{2+} affinities, localisation, kinetics and expression patterns. This thesis focuses on one particular sub-family of the NCS proteins, the K^+ channel interacting proteins (KChIPs). Previous studies have demonstrated the ability of the KChIPs to regulate the function and traffic of Kv4 potassium channels, which play important roles in the control of neuronal excitability. However, one of the KChIPs, KChIP3, has been demonstrated to have two other well-characterised functions and as a consequence has been given two other names. Firstly it was shown to interact with the presenilins and was subsequently named calsenilin. Furthermore, it was demonstrated to play a role in the regulation of transcription through binding to the DRE DNA motif, hence it has also been named DREAM.

Here I have explored the extent to which the KChIPs share similar functional properties by carrying out a comparative study of selected isoforms of KChIPs 1-4 and some of their known functions, utilising GFP-variant fusion proteins expressed in COS-7 and PC12 cells. Co-expression of all four KChIPs with the Kv4.2 channel resulted in increased traffic of the channel to the plasma membrane, indicating that all four KChIPs share this particular functional property. By contrast, only KChIP2 was demonstrated to have an effect upon the traffic of the Kv1.4 channel to the plasma membrane, with traffic of the channel inhibited by co-expression with KChIP2. Similarly, only KChIP3 co-localised with presenilin-1, indicating that whilst the KChIPs do possess the ability to perform multiple functions in common with each other, they also maintain distinct individual properties.

Another NCS protein, NCS-1, has also been reported to be able to carry out some of the functional roles of the KChIPs, such as the stimulation of traffic of Kv4 channels. Whilst unable to confirm these interactions in this study, I have investigated the potential for the KChIPs to share one of the functional properties of NCS-1, a role in the stimulation of exocytosis by utilising a well established assay. Of the KChIPs examined, KChIP3 specifically was found to significantly increase secretion from PC12 cells in response to purinergic receptor stimulation. This increase in secretion appeared to be as a result of a modification of the Ca^{2+} signal seen in response to ATP stimulation, with KChIP3 expression resulting in a prolonged increase in $[Ca^{2+}]_i$. These data provide evidence for a novel and specific role of KChIP3 and suggest that the KChIPs may have distinct roles in regulation of neuronal function.

Abbreviations

AA-NAT	arylalkylamine N-acetyltransferase
Ab	antibody
Aβ	β-amyloid
ADP	adenosine diphosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
APP	amyloid precursor protein
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAPS	Ca^{2+}-dependent activator protein for secretion
cGMP	cyclic guanosine monophosphate
COS cells	cell line derived from African green monkey kidney
CNS	central nervous system
C-terminal	carboxy-terminal
DAG	diacylglycerol
DNA	deoxyribonucleic acid
DRE	downstream-regulatory-element
DREAM	downstream-regulatory-element-antagonist modulator
ECFP	enhanced cyan fluorescent protein
ECL	enhanced chemiluminescence
EGFP	enhanced green fluorescent protein
EGTA	ethylene glycol-bis (2-aminoethylether) N,N,N',N'-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EPSP	excitatory postsynaptic potential
ER	endoplasmic reticulum
EYFP	enhanced yellow fluorescent protein
FAD	familial Alzheimer's disease
FCS	foetal calf serum
fra-2	Fos-related antigen-2
GCAP	guanylyl cyclase-activating protein
GDNF	glial cell line-derived neurotrophic factor
GH	human growth hormone
HEK293 cells	human embryonic kidney 293 cells
HeLa cells	cell line derived from human cervical carcinoma
HEPES	N-(2-hydroxyethyl) piperazine-N'-(2ethanesulfonic acid)
HRP	horseradish peroxidase
HS	horse serum
ICER	inducible cAMP early repressor
IL1RAPL protein	interleukin-1 receptor accessory protein-like protein
$InsP_3$	inositol 1,4,5-trisphosphate
IP	immunoprecipitation
I_{to}	transient outward current

KChIP	potassium channel interacting protein
Kv channel	voltage-gated potassium channel
LTD	long-term depression
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
mRNA	messenger ribose nucleic acid
NAIP	neuronal apoptosis inhibitory protein
NCS	neuronal calcium sensor
NCX	Na^{+}/Ca^{2+} exchanger
NFT	neurofibrillary tangles
NMDA	N-methyl-D-aspartate
N-terminal	amino-terminal
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBT	PBS containing 1% Triton-X-100 and 0.3% BSA
PC12 cells	cell line derived from rat adrenal pheochromocytoma
PCR	polymerase chain reaction
PI(4)K	phosphatidylinositol 4-OH kinase
PIPES	piperazine-N, N'-bis[2-ethanesulfonic acid]
PLD	phospholipase D
PMCA	plasma membrane Ca^{2+}-ATPase
PS	presenilin
PtDIns[4]P	phosphatidylinositol-4-phosphate
PtDIns[4,5]P2	phosphatidylinositol 4,5-bisphosphate
RNA	ribose nucleic acid
ROC	receptor-operated channel
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SERCA	sarco (endo) plasmic reticulum Ca^{2+}-ATPase
TGN	*trans*-Golgi network
Tris	tris (hydroxymethyl) aminomethane
Triton-X-100	t-octylphenoxypolyethoxethanol
VILIP	visinin-like protein
VOC	voltage-operated channel
VSVG	vesicular stomatitis virus glycoprotein

CHAPTER 1:

Introduction

1.1 Ca^{2+} signalling in neurons

Ca^{2+} is an important intracellular messenger, with intracellular Ca^{2+} signals carrying out crucial roles in neurons, such as controlling neurotransmitter release, regulating gene transcription and cell death, modulating channel function and inducing changes in synaptic plasticity (Berridge *et al.*, 2000; Carafoli, 2002). Neurons generally maintain a resting intracellular concentration of Ca^{2+} within the range of 40-100nM (Maravall *et al.*, 2000; Sabatini *et al.*, 2002), but the receipt of a neurotransmitter signal from another neuron, or some other form of stimulation, can lead to a change in intracellular Ca^{2+} concentration that can vary both temporally and spatially. These changes can range from being highly localised and transient Ca^{2+} elevations to longer lasting and global changes throughout the neuron (Augustine *et al.*, 2003). The changes in intracellular Ca^{2+} concentration can be facilitated by a number of extracellular and intracellular sources of Ca^{2+}. For example, Ca^{2+} can enter the cell through plasma membrane channels such as voltage-gated Ca^{2+} channels or NMDA (N-methyl-D-aspartate) receptors, or be released from intracellular stores such as the ER in response to messengers such as inositol 1,4,5-trisphosphate (Berridge, 1998).

As the Ca^{2+} concentration is over 1000 fold higher in the extracellular space than in the cytosol, the opening of Ca^{2+} channels in the plasma membrane results in an influx of calcium. Voltage gated channels open during membrane depolarisation and are involved in the generation of Ca^{2+} signals in presynaptic terminals that modulate the exocytotic machinery and trigger the rapid release of neurotransmitters

(Sabatini & Regehr, 1996; Stanley, 1997; Turner *et al.*, 1999; Yoshihara *et al.*, 2003). Receptor operated channels are controlled by neurotransmitters and are predominantly located at postsynaptic sites. NMDA receptors for example are activated by the binding of their ligand, glutamate, and they open and allow Ca^{2+} to flow into the cell.

Other cell surface receptors are not ionotrophic as NMDA receptors are, and as such are not permeable to Ca^{2+} ions. Instead, these metabotrophic receptors act to stimulate polyphosphoinositide metabolism resulting in the release of Ca^{2+} from intracellular stores (Frenguelli *et al.*, 1993). Phosphatidylinositol 4,5-bisphosphate (PtDIns[4,5]P2) is hydrolysed to form the second messengers diacylglcerol (DAG) and inositol 1,4,5-trisphosphate ($InsP_3$), and it is $InsP_3$ that triggers Ca^{2+} release from intracellular stores such as the ER (Streb *et al.*, 1983; Pin & Duvoisin, 1995).

The ER is perhaps the most important intracellular Ca^{2+} store. In neurons it extends as a continuous network throughout the whole cell, including the dendrites and dendritic spine (Broadwell & Cataldo, 1983, 1984; Spacek & Harris, 1997). The Golgi can also act as an intracellular Ca^{2+} store (Pinton *et al.*, 1998), whilst mitochondria can also take up large amounts of Ca^{2+} and thus are also involved in regulating cytosolic Ca^{2+} levels (Rizzuto, 2001), but the ER would appear to be the main Ca^{2+} store. It can release Ca^{2+} through the activation of $InsP_3$ receptors or ryanodine receptors either by their individual ligands ($InsP_3$ and cyclic ADP ribose respectively) (Henzi & MacDermott, 1992) or by Ca^{2+} itself through a process

known as calcium induced calcium release, where Ca^{2+} signals can be amplified, propagating Ca^{2+} waves that can in turn develop into global events (Verkhratsky & Shmigol, 1996). Ca^{2+} signals can also be highly localised, where only small groups of $InsP_3$ or ryanodine receptors open ('elementary events') (Bootman & Berridge, 1995; Bootman *et al.*, 1995). In addition, the morphological characteristics of neurons can also affect Ca^{2+} signals. Ca^{2+} concentration can be increased in dendritic spines without changes in the adjacent dendrite due to the limited diffusion of Ca^{2+} through the narrow necks of the spines – the head of the dendritic spine (volume ~0.01-1μm^3) is connected to the parent dendrite by a thin neck (diameter ~0.1μm) which leads to a geometric constriction and thus prevents diffusion of the Ca^{2+} signal (Sabatini *et al.*, 2001; Sabatini *et al.*, 2002).

Termination of Ca^{2+} signals and the return of the intracellular Ca^{2+} concentration to its resting level can occur via a variety of mechanisms. Ca^{2+} can be sequestered back into the ER by the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) pumps (Lipscombe *et al.*, 1988) or it can be pumped out of the cell. In the latter case, Ca^{2+} can be extruded from the cell either by plasma membrane Ca^{2+}-ATPase (PMCA) activity or via the Na^+/Ca^{2+} exchanger, where three Na^+ ions are exchanged for the removal of one Ca^{2+} ion from the cell (Fierro *et al.*, 1998). The SERCA and PMCA pumps utilise the energy provided by ATP hydrolysis to transport Ca^{2+} against its concentration gradient from the cytosol to the ER lumen or indeed out of the cell. Both of these pumps have high Ca^{2+} affinities and play a vital role in maintaining resting Ca^{2+} concentrations within the cytosol (Berridge *et al.*, 2003).

The distinct types of Ca^{2+} signal that occur, differing temporally, spatially and in magnitude, contribute partially to the diversity of neuronal functions controlled by Ca^{2+} (Berridge *et al.*, 2000). However, in order for these different signals to be interpreted into different changes in neuronal function, a number of Ca^{2+} sensor proteins are required that are able to respond selectively to defined signals and modulate cellular activity appropriately. The effects of these Ca^{2+} sensing proteins can depend upon a variety of factors, such as their localisation in relation to the Ca^{2+} signal, the affinity of the protein for Ca^{2+} and their interaction with other proteins (Ikura & Ames, 2006).

One of the most widely studied Ca^{2+} sensing proteins is the ubiquitously expressed protein calmodulin (Chin & Means, 2000) and a variety of its cellular functions, such as its role in synaptic plasticity, have been defined in detail (Lisman *et al.*, 2002). Another Ca^{2+} sensing protein, synaptotagmin, is now well established as the Ca^{2+} sensor for fast neurotransmission (Fernandez-Chacon *et al.*, 2001). A number of other Ca^{2+}-binding proteins related to calmodulin are enriched or expressed solely in the nervous system where they carry out distinct roles in the regulation of neuronal function. These include the neuronal calcium sensor (NCS) protein family, which provide selectivity by their differing Ca^{2+} affinities, localisation, kinetics and expression patterns (Burgoyne & Weiss, 2001; O'Callaghan & Burgoyne, 2003) and have been implicated in a wide range of Ca^{2+} signalling events in neurons and photoreceptors (Burgoyne & Weiss, 2001; Burgoyne *et al.*, 2004). A basic model of calcium signalling is shown in figure 1.

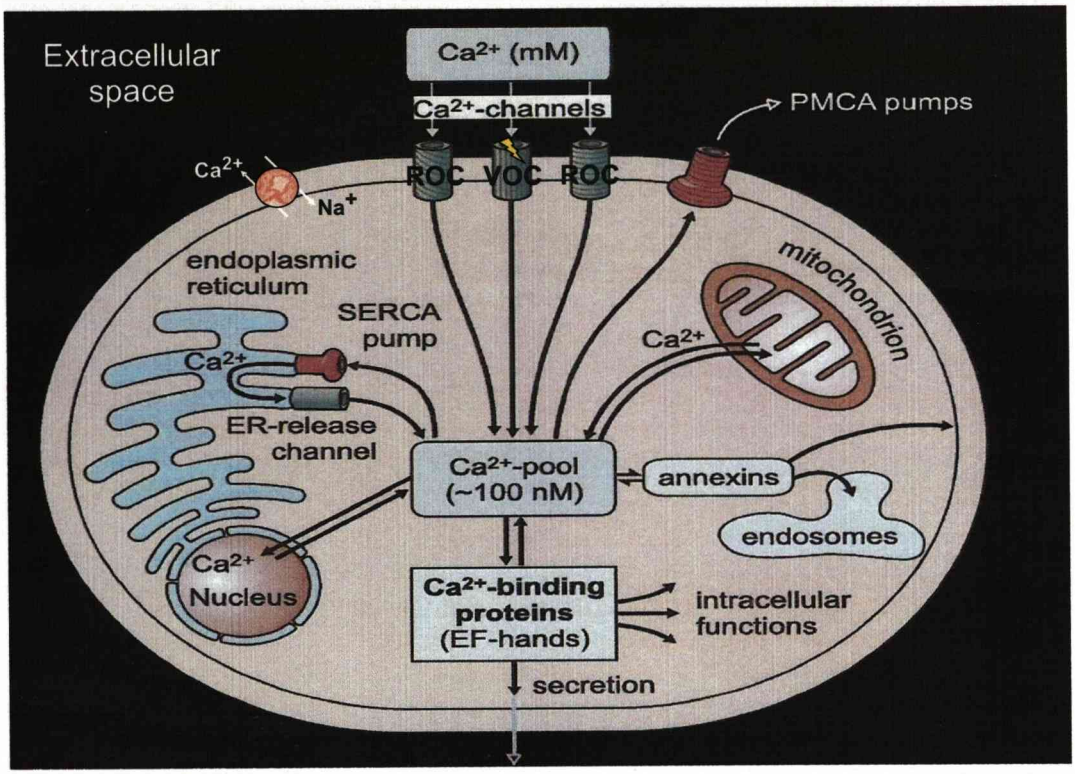

Figure 1. Basic model of intracellular calcium signals.
Influx of external Ca^{2+} is mediated by voltage-operated (VOC) or receptor operated channels (ROC) controlled by ionotropic neurotransmitters. Metabotrophic receptors can also be activated to stimulate polyphosphoinositide metabolism resulting in the release of calcium from intracellular stores through the activation of $InsP_3$ or ryanodine receptors by the appropriate second messengers. The Ca^{2+} signal can be terminated by a number of mechanisms that include removing Ca^{2+} from the cell by the plasma-membrane Ca^{2+}-ATPase (PMCA) or the Na^+/Ca^{2+} exchanger, or sequestering Ca^{2+} back into the ER by the action of the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA).
(derived and modified from the Proceedings of the 9th meeting of the European Calcium Society, 2006).

1.2 The Neuronal Calcium Sensor (NCS) proteins

1.2.1 General Overview

The NCS proteins have been identified in multiple organisms ranging from yeast to man, with the NCS protein family being encoded by 14 genes in the human genome. It is likely that the 14 members of the family are conserved throughout all mammalian species, and in some cases this diversity is increased by the existence of splice variants. They are small EF-hand containing proteins (generally 22-25kDa) that can bind Ca^{2+} with micromolar or submicromolar affinities (0.1-1μM), meaning that they can bind Ca^{2+} at levels not far above resting intracellular levels. Upon Ca^{2+} binding, the NCS proteins undergo conformational changes enabling their interaction with and subsequent regulation of other proteins, leading to changes in physiological function. They are primarily expressed in neurons and neuroendocrine cells but have also been shown to be expressed in tissues other than the brain, including the heart and testes (Braunewell & Gundelfinger, 1999; An *et al.*, 2000; Burgoyne & Weiss, 2001).

The NCS proteins include NCS-1 (neuronal calcium sensor-1), recoverin, the VILIPS/neurocalcins (visinin-like proteins), GCAPs (guanylyl cyclase-activating proteins) and KChIPs (potassium channel interacting proteins) (Braunewell *et al.*, 1997; Braunewell & Gundelfinger, 1999; Burgoyne & Weiss, 2001) and have a vast array of functions that include effects on neurotransmitter release (McFerran *et al.*,

1998), learning and memory (Gomez *et al.*, 2001), receptor and channel trafficking (Kabbani *et al.*, 2002; O'Callaghan *et al.*, 2003a), and regulation of channel function (An *et al.*, 2000; Burgoyne & Weiss, 2001) to name but a few. During evolution the NCS family has become progressively more complex, such that there are now five classes of NCS proteins (A-E) that have been defined based upon their amino acid sequences. This information is summarised in figure 2. Three NCS proteins are expressed in *C.elegans* whilst *D.melanogaster* has four NCS proteins. Zebrafish have two NCS-1 orthologues (Blasiole *et al.*, 2005), up to eight class B proteins, a recoverin, at least eight GCAPs (Palczewski *et al.*, 2004) and five KChIPs. Mammals meanwhile have a highly conserved set of 14 NCS genes. These encode a single NCS-1, five class B proteins, one recoverin, three GCAPs and four KChIPs, with sixteen alternatively spliced variants of the KChIPs currently identified (Pruunsild & Timmusk, 2005). In all species, the GCAPs and recoverin are expressed in the retina whilst the remaining NCS proteins are expressed to varying degrees in neurons, sometimes with very specific expression patterns in particular sub-populations of neurons (Kobayashi *et al.*, 1993; Paterlini *et al.*, 2000; Pruunsild & Timmusk, 2005).

Structurally, all of the NCS proteins contain four Ca^{2+}-binding EF-hand domains in common with calmodulin, although the similarity to calmodulin is limited (no more than 20% identity) (Burgoyne, 2007). Each EF-hand domain consists of a helix-loop-helix structure, first characterised for the protein parvalbumin, where helices E and F are positioned like the forefinger and thumb of the right hand and form the

Mammalian Protein	Subgroup	First Evolutionary Appearance	Expressed where?	Human splice variants	N-terminal myristoylation?	Proposed Functions
NCS-1	A	Yeast	Brain and retina	1	Yes	Regulation of neurotransmission, stimulation of exocytosis, learning, Ca2+ and Kv channel regulation, phosphoinositide metabolism, dopamine D2 receptor endocytosis, GDNF signalling, neuronal growth and survival, short-term synaptic plasticity
Hippocalcin	B	Nematodes	Brain	1	Yes	Anti-apoptotic, AMPA receptor recycling, MAPK signalling, learning
Neurocalcin-			Brain and retina	1	Yes	Guanylyl cyclase activation
VILIP1			Brain and retina	1	Yes	Guanylyl cyclase activation and recycling, traffic of nicotinic receptors, increase of cAMP levels and secretion
VILIP2			Brain	1	Yes	Regulation of P/Q-type Ca^{2+} channels
VILIP3			Brain	1	Yes	Unknown
Recoverin	C	Fish	Retina	1	Yes	Light adaptation by inhibition of rhodopsin kinase
GCAP1	D	Fish	Retina	1	Yes	Regulation of retinal guanylyl cyclases
GCAP2			Retina	1	Yes	Regulation of retinal guanylyl cyclases
GCAP3			Retina	1	Yes	Regulation of retinal guanylyl cyclases
KChIP1	E	Insects	Brain	3	Yes	Regulation of Kv channels, repression of transcription
KChIP2			Brain and heart	5	No	Regulation of Kv channels, repression of transcription
KChIP3			Brain and testes	2	No	Regulation of Kv channels, repression of transcription, presenilin processing, pro-apoptotic
KChIP4			Brain	6	No	Regulation of Kv channels, repression of transcription, presenilin processing

Figure 2. The NCS proteins.
A summary table of the NCS proteins and their known or proposed functions

Ca^{2+} binding motif, hence the name *EF-hand* (Kretsinger & Nockolds, 1973). The helix-loop-helix structure consists of 29 amino acids and binds a single calcium ion. There are six residues involved in Ca^{2+} binding at positions 1, 3, 5, 7, 9, and 12 within the EF-hand, with position 1 being a highly conserved aspartate and position 12 either aspartate or glutamate. The first EF-hand (nearest the N-terminal) in all NCS proteins is unable to bind Ca^{2+} due to the presence of a conserved cysteine-proline substitution in the binding loop (Hwang *et al.*, 2004), whilst EF-hand 4, in some NCS proteins such as recoverin, is also unable to bind Ca^{2+} due to an internal lysine-glutamate salt bridge (Ames *et al.*, 1997). Thus either two or three Ca^{2+} ions will bind to the NCS proteins and induce the appropriate conformational changes relative to their function (figure 3). EF-hand-containing proteins can have fast on-rates, with Ca^{2+} binding limited only by the rate of Ca^{2+} diffusion. However, the equilibrium dissociation constant can be variable due to differences in the Ca^{2+} off-rate between different EF-hands – this depends upon the nature of the amino acid at position 9 of the EF hand (Falke *et al.*, 1994). The slowest off-rates are found in proteins that act as Ca^{2+} buffers.

Another common feature of most of the NCS proteins (with the exception of KChIPs 2-4) is the N-terminal myristoyl tail (figure 3) (Burgoyne & Weiss, 2001). N-terminal myristoylation is the post-translational attachment of myristate, a 14-carbon saturated fatty acid, to the N-terminal glycine of the protein upon removal of the initiator methionine residue (Farazi *et al.*, 2001). Some isoforms of KChIPs 2, 3 and 4 have putative palmitoylation sites instead (Takimoto *et al.*, 2002). Myristoylation

and indeed palmitoylation would be predicted to enable interaction with membranes or hydrophobic domains of other proteins, and play an important role in the intracellular targeting of the proteins. Biochemical and structural analyses of myristoylated recoverin gave rise to the idea of the Ca^{2+}-myristoyl switch, where in the Ca^{2+}-free form of recoverin, the myristoyl group is located within a hydrophobic pocket, meaning that the protein remains cytosolic. However, upon Ca^{2+} binding a conformational change occurs which results in the extrusion of the myristoyl group thus enabling membrane association of the protein (figure 4) (Ames *et al.*, 1995; Tanaka *et al.*, 1995; Ames *et al.*, 1997). Several NCS proteins have also been shown to possess a Ca^{2+}-myristoyl switch including hippocalcin (O'Callaghan *et al.*, 2002; O'Callaghan *et al.*, 2003b), neurocalcin δ (Ivings *et al.*, 2002), VILIP-1 (Spilker *et al.*, 2002a; Spilker *et al.*, 2002b), and VILIP-3 (Spilker & Braunewell, 2003). Other NCS proteins however do not possess this switch mechanism (O'Callaghan & Burgoyne, 2003). NCS-1 and KChIP1 for instance do not exhibit a Ca^{2+}-myristoyl switch mechanism but have their myristoyl tails constantly exposed and are anchored to membranes independently of Ca^{2+} (O'Callaghan *et al.*, 2002; O'Callaghan *et al.*, 2003a) Further to the membrane association of the NCS proteins by the myristoyl group, residues within the myristoylation motif can also determine intracellular localisation of the NCS proteins, as is the case with KChIP1 which is localised to post-ER transport vesicles (O'Callaghan & Burgoyne, 2004).

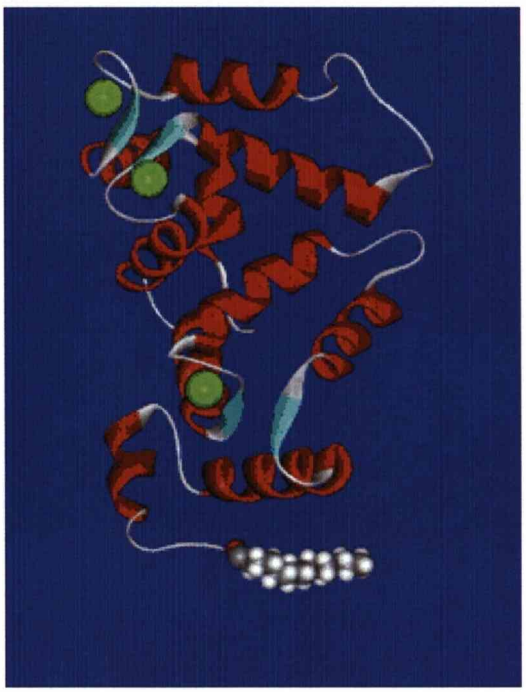

Figure 3. The crystal structure of NCS-1, a member of the NCS protein family. The green spheres represent Ca^{2+} ions bound to EF hand domains 2-4. The N–terminal myristoyl group is shown at the base of the protein and it is this group that can interact with cell membranes and hydrophobic domains of proteins. KChIPs 2-4 lack this myristoyl tail as they do not have the necessary glycine at position two and instead they contain palmitoylated cysteines in their N-terminal domains which contribute to their cell surface localisation.

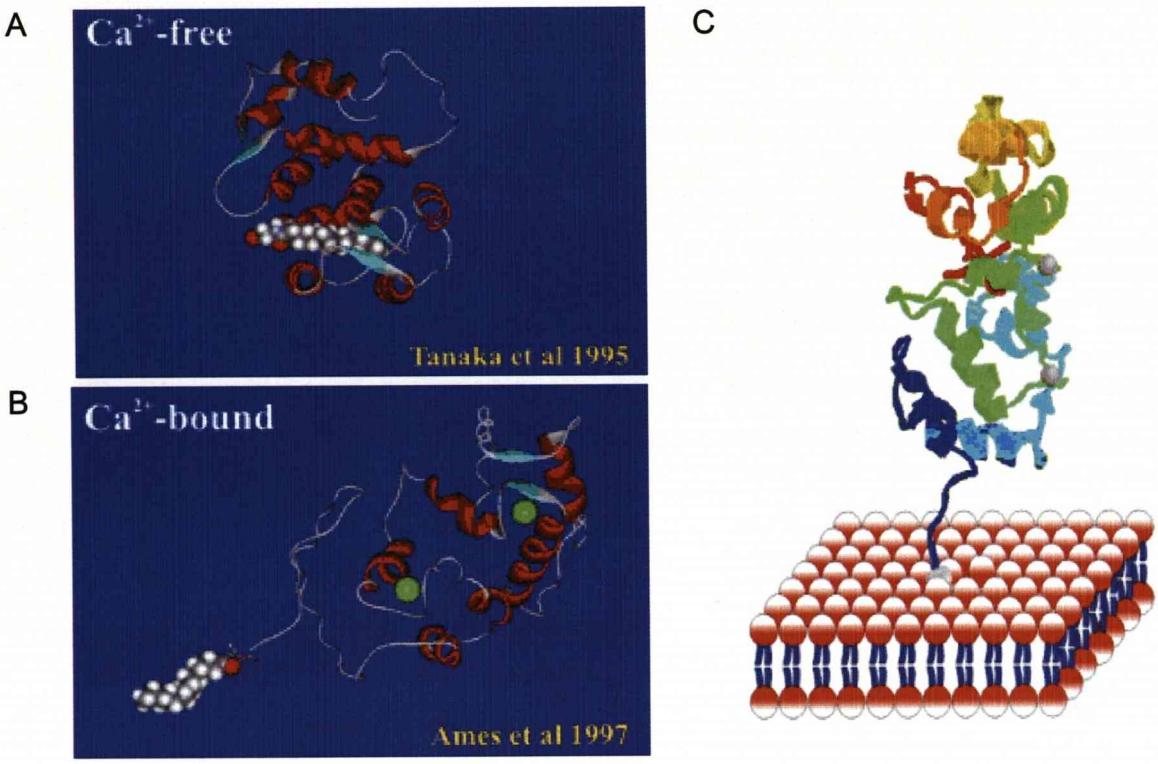

Figure 4. The Ca^{2+}/myristoyl switch in recoverin.
(A) Ribbon structure of recoverin without Ca^{2+} bound. In the absence of Ca^{2+} the N-terminal myristoyl group is folded into the protein. **(B)** Structure of recoverin with Ca^{2+} binding to EF-hands 2 and 3 (Ca^{2+} ions represented as green spheres). A conformational change is induced by Ca^{2+} binding, allowing the extrusion of the myristoyl tail. **(C)** The exposed myristoyl group can then interact with the lipid bilayer making up membranes.

1.2.2 Class A: Neuronal Calcium Sensor (NCS) -1

NCS-1 was originally discovered as frequenin in *Drosophila melanogaster* (Pongs *et al.*, 1993) and named after the effect seen due to its overexpression – it caused an enhanced, frequency dependent facilitation of neurotransmitter release at the neuromuscular junctions of transgenic fruit flies that resulted in a Shaker-like phenotype, characterised by abnormally large responses to repeated stimulation. Frequenin was subsequently named NCS-1 as it was thought to only be expressed in neuronal cell types (Nef *et al.*, 1995). This is not the case however, and it is, in fact, the most widely expressed of all the NCS proteins (McFerran *et al.*, 1998; Kapp-Barnea *et al.*, 2003; Gierke *et al.*, 2004). It is, in evolutional terms, the most ancient of the NCS proteins, with an orthologue of NCS-1 existing in *Saccharomyces cerevisiae.* This orthologue shares 60% identity to the human protein, and is essential for survival in yeast (Hendricks *et al.*, 1999).

NCS-1 has been implicated in multiple cellular functions from studies in both lower organisms and in mammalian cells (Burgoyne *et al.*, 2004), including stimulation of constitutive (Haynes *et al.*, 2005) and regulated exocytosis (McFerran *et al.*, 1998), regulation of neurotransmission (Pongs *et al.*, 1993), learning (Gomez *et al.*, 2001), short-term plasticity (Sippy *et al.*, 2003), regulation of both Kv4 (Nakamura *et al.*, 2001; Guo *et al.*, 2002) and Ca^{2+} channels (Weiss *et al.*, 2000; Tsujimoto *et al.*, 2002), neuronal growth (Hui *et al.*, 2006) and survival (Nakamura *et al.*, 2006), phosphoinositide metabolism (Hendricks *et al.*, 1999; Zhao *et al.*, 2001; Koizumi *et*

al., 2002; Haynes *et al.*, 2005) GDNF signalling (Wang *et al.*, 2001a), and dopamine D2 receptor endocytosis (Kabbani *et al.*, 2002). The latter in particular has been linked to schizophrenia and bipolar disorder, where levels of NCS-1 have been found to be significantly elevated, although other functions of NCS-1 may also underlie these pathophysiological conditions (Bergson *et al.*, 2003; Koh *et al.*, 2003). Furthermore, NCS-1 has been found to interact with interleukin-1 receptor accessory protein-like (IL1RAPL) protein, which has been suggested to have a role in X-linked mental retardation (Bahi *et al.*, 2003).

A significant contribution to the understanding of frequenin/NCS-1 function was the discovery that in yeast it is essential for survival because it is involved in the activation of phosphatidylinositol-4-OH (PI(4)K) Pik1, which is responsible for the synthesis of phosphatidylinositol-4-phosphate [PtdIns(4)P], the precursor of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] (Hendricks *et al.*, 1999). Subsequently it was shown that NCS-1 interacts with the mammalian Pik1 homologue PI(4)Kβ type III (Weisz *et al.*, 2000). By enhancing the activity of these enzymes, frequenin/NCS-1 can up-regulate $InsP_3$-dependent signalling and Ca^{2+} mobilisation, which is thought to be the underlying mechanism for its ability to increase exocytosis or synaptic transmission (Koizumi *et al.*, 2002; Rajebhosale *et al.*, 2003; Zheng *et al.*, 2005). Knockout of the gene for NCS-1 is not lethal in other organisms, but does lead to developmental phenotypes, including changes in the rate of development in *Dictyostelium discoideum* (Coukell *et al.*, 2004), impairment of memory and learning in *C-elegans* (Gomez *et al.*, 2001), and abolishment of the

formation of the semicircular canals in the inner ear in zebrafish (Blasiole *et al.*, 2005).

However, whilst the regulation of PI(4)Kβ type III can explain some of the functional roles of NCS-1, another key aspect of NCS-1 is its ability to bind to, and regulate, multiple target proteins that are unrelated to one another (Haynes *et al.*, 2006). Interactions of NCS-1 with different target proteins can involve distinct regions of NCS-1 (Kabbani *et al.*, 2002; Bahi *et al.*, 2003), and some of the interactions can be independent of Ca^{2+} binding (Kabbani *et al.*, 2002; Haynes *et al.*, 2006). The significance of these interactions and the various physiological roles of NCS-1 are still yet to be fully elucidated.

1.2.3 Class B: Visinin-like proteins (VILIPS), neurocalcin δ and hippocalcin

The class B NCS proteins are all closely similar in sequence, sharing between 66 and 94% sequence identity between one another (figure 5). Originally, VILIP-3 and neurocalcin δ were actually considered to be the same protein expressed in different species until they were identified as two distinct proteins, though sharing a great deal of sequence homology (91% homology between sequences) (Wang *et al.*, 2001b; Spilker *et al.*, 2002b). All have been demonstrated to exhibit a Ca^{2+}/myristoyl switch, with the exception of VILIP-2, and as such will translocate from the cytosol to membranes in a Ca^{2+}-dependent manner (Ivings *et al.*, 2002; O'Callaghan *et al.*, 2002; Spilker *et al.*, 2002a; Spilker *et al.*, 2002b; O'Callaghan *et al.*, 2003b; Spilker & Braunewell, 2003). However, the physiological functions of most of these proteins are still somewhat unclear.

Perhaps the most studied of the class B proteins is hippocalcin, which has a relatively specific distribution to hippocampal neurons and was suggested to be involved in long term potentiation (LTP) (Kobayashi *et al.*, 1993). Work in hippocalcin knock-out mice has backed up this theory, with the mice found to have deficiencies in learning (Kobayashi *et al.*, 2005). However, this is not the only physiological role it has been implicated as being involved in. Hippocalcin has also been suggested to be anti-apoptotic, having neuroprotective effects when, at high Ca^{2+} concentrations, it interacts with neuronal apoptosis inhibitory protein (NAIP) enhancing its inhibitory effects on cell death (Mercer *et al.*, 2000; Lindholm *et al.*,

2002). Furthermore, it has been demonstrated to influence caspase-12 activation (Korhonen *et al.*, 2005). A role in long term depression (LTD) has been suggested, where hippocalcin is believed to be involved in the recycling of AMPA receptors through an interaction with the β2 clathrin adapter protein (Palmer *et al.*, 2005), as well as having an involvement in Ca^{2+}-dependent phospholipase D (PLD) activation (Hyun *et al.*, 2000) and mitogen-activated protein kinase (MAPK) signalling pathways (Nagata *et al.*, 1998; Oh *et al.*, 2006). More recently, it has been demonstrated to interact with Ca^{2+}-dependent activator protein for secretion 1 (CAPS1) and CaM-dependent cyclic nucleotide 3', 5', phosphodiesterase (Haynes *et al.*, 2006). However, the precise functional role of hippocalcin is still yet to be fully defined.

Relatively little meanwhile, is know about the VILIPs. Indeed, only a modest amount of research has been published on VILIPs 2 and 3, with VILIP2 thought to be involved in the regulation of P/Q type Ca^{2+} channels (Few *et al.*, 2005; Lautermilch *et al.*, 2005) and the functional role of VILIP3 still unknown. More research has however, been carried out on the remaining VILIP. VILIP1 has a proposed role in the pathophysiology of Alzheimer's disease due to its association with amyloid plaques and neurofibrillar tangles in the brains of Alzheimer sufferers, and its influence of Ca^{2+}-mediated neurotoxicity and phosphorylation of tau protein (Schnurra *et al.*, 2001). It has also been shown to interact with and activate guanylyl cyclases, affect the traffic of both nicotinic receptors (Lin *et al.*, 2002)and guanylyl

cyclase receptors (Brackmann *et al.*, 2005), and increase intracellular cAMP levels resulting in an effect on secretion (Dai *et al.*, 2006).

Similarly, very little is known about the functions of neurocalcin δ. A role in guanylyl cyclase activation has been proposed (Krishnan *et al.*, 2004), and interactions with several other proteins have been demonstrated including CAPS1 and CaM-dependent cyclic nucleotide 3',5', phosphodiesterase (Haynes *et al.*, 2006), clathrin heavy chain (Ivings *et al.*, 2002), and actin and tubulins (Mornet & Bonet-Kerrache, 2001; Ivings *et al.*, 2002). Clearly however, a great deal more investigation is required before the functional roles of the class B NCS proteins are fully understood.

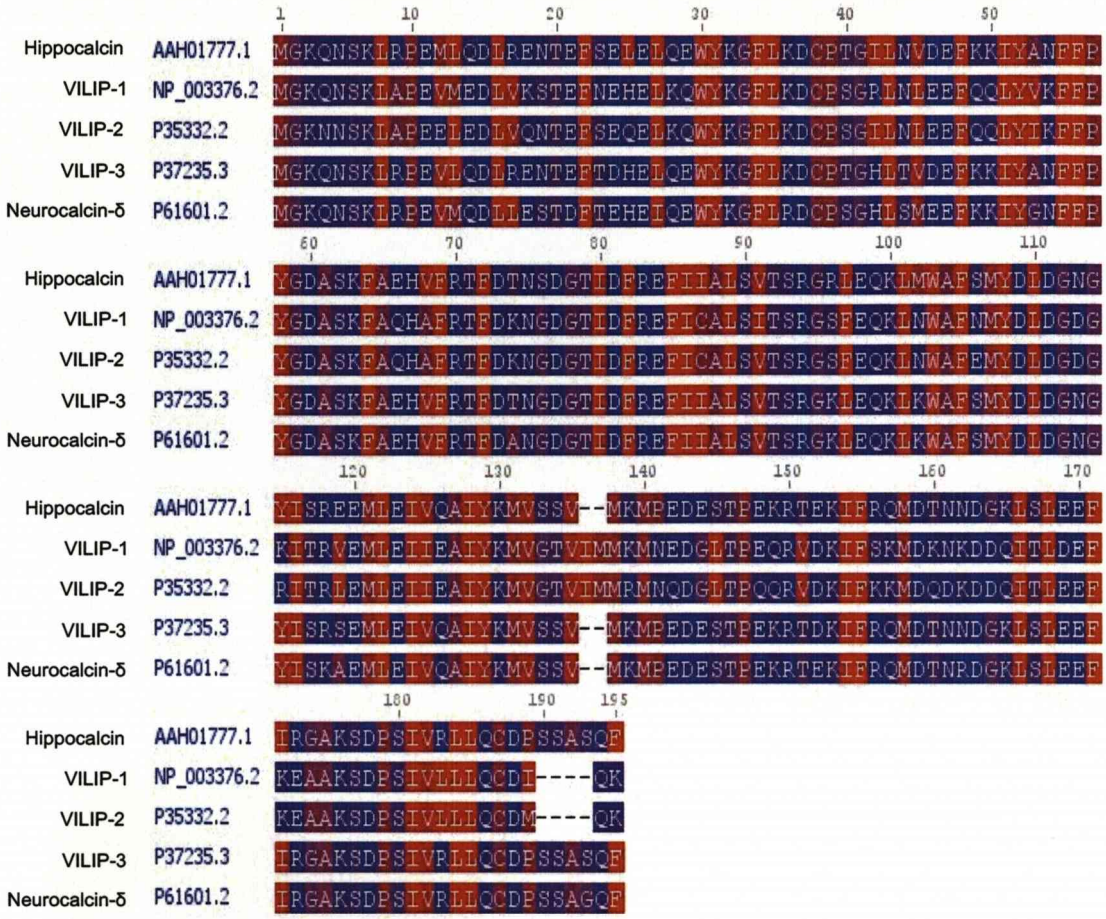

Figure 5. Alignment of the sequences of the Class B NCS family members. Sequence alignments were carried out utilising Geneious Pro v.3.5.6 software. Amino acids are colour coded based on their hydrophobicity.

1.2.4 Class C: Recoverin

Recoverin was the first of the NCS proteins to be discovered (Dizhoor *et al.*, 1991). It is expressed solely in retinal photoreceptors where it plays an important role in light adaptation, inhibiting rhodopsin kinase in the dark when Ca^{2+} levels are high and downregulating this inhibition when the Ca^{2+} levels fall (Kawamura *et al.*, 1993; Chen *et al.*, 1995; Palczewski *et al.*, 2000). By inhibiting rhodopsin kinase, phosphorylation of rhodopsin and its inactivation is effectively downregulated by recoverin. As a result, rhodopsin activation in the dark is prolonged by recoverin, allowing retinal photoreceptors to adapt to the lack of light and develop an increased sensitivity at low light levels (Makino *et al.*, 2004).

The structure of recoverin, in both its Ca^{2+}-bound and Ca^{2+}-free forms, has been extensively studied and led to the establishment of the Ca^{2+}/myristoyl switch model (Dizhoor *et al.*, 1992; Dizhoor *et al.*, 1993; Ames *et al.*, 1994; Ames *et al.*, 1995; Tanaka *et al.*, 1995; Ames *et al.*, 1997). In its Ca^{2+}-free form, the myristoyl group of recoverin is sequestered in a hydrophobic pocket within the protein, with the protein itself being cytosolic (Tanaka *et al.*, 1995). Sequential binding of Ca^{2+} to EF-hand 3 followed by EF-hand 2 (Ames *et al.*, 2002; Ames & Ikura, 2002; Senin *et al.*, 2002; Weiergraber *et al.*, 2003) results in a significant conformational change to the protein involving a 45-degree rotation of the amino-terminal domain relative to the carboxy-terminal domain, resulting in both the exposure of hydrophobic residues (enabling them to interact with a target) and the extrusion of the myristoyl group,

that is then available for interaction with membranes (figure 4) (Zozulya & Stryer, 1992; Ames *et al.*, 1997). The Ca^{2+}/myristoyl switch enables recoverin to associate with target membranes in a Ca^{2+}-dependent manner, and is particularly important for recoverin in the Ca^{2+}-dependent regulation of rhodopsin kinase (Senin *et al.*, 1995). Apart from recoverin, only the class B proteins also exhibit the Ca^{2+}/myristoyl switch, with other members of the NCS family using their myristoyl group for constitutive membrane association instead (Olshevskaya *et al.*, 1997; O'Callaghan *et al.*, 2002; O'Callaghan & Burgoyne, 2003; O'Callaghan *et al.*, 2003a).

1.2.5 Class D: Guanylate cyclase activating proteins (GCAPs)

As with recoverin, the guanylate cyclase activating proteins (GCAPs) are expressed only in photoreceptor cells, where they play a specific role in light adaptation during phototransduction, regulating retinal guanylyl cyclases 1 and 2 through a calcium feedback mechanism (Palczewski *et al.*, 2000; Palczewski *et al.*, 2004). There are three genes that encode GCAPs in the human genome (Palczewski *et al.*, 1994; Dizhoor *et al.*, 1995; Haeseleer *et al.*, 1999), with GCAPs 1 and 2 found expressed in both rod and cone photoreceptors in the human retina (Palczewski *et al.*, 2004). GCAP3 is expressed only in cones, although this is not the case in mice, suggesting that the protein is non-essential for normal vision (Imanishi *et al.*, 2002).

When photoreceptor cells are illuminated, the resultant lower Ca^{2+} level causes the GCAPs to act to increase the activity of retina-specific membrane-bound guanylate cyclases for resynthesis of cGMP. As the cGMP levels increase, cation channels re-open and this aids the recovery of the photoreceptor cell to the dark state. When retinal Ca^{2+} levels are elevated, GCAPs 2 and 3 act to inhibit guanylyl cyclase activity to below basal level (Gorczyca *et al.*, 1995; Dizhoor & Hurley, 1996).

The importance of the GCAPs has been illustrated by studies on mutations in human GCAP1 that found that these mutations can lead to the degeneration of photoreceptors (Dizhoor *et al.*, 1998; Newbold *et al.*, 2001). GCAPs 1 and 2 are both present in the retina at similar concentrations (Hwang *et al.*, 2003), and whilst

both have been demonstrated to regulate both of the guanylyl cyclases, they seem to interact with them in different ways (Hwang *et al.*, 2003), share only a 49% sequence homology and differ significantly in their Ca^{2+}-binding affinities for retinal guanylyl cyclase activation (Hwang *et al.*, 2003; Peshenko & Dizhoor, 2004; Peshenko *et al.*, 2004). However, expressing *Gcap1*in mice that lack both *Gcap1* and *Gcap2* recovers almost all normal function in rods and cones (Howes *et al.*, 2002; Pennesi *et al.*, 2003).

Like most other NCS protein family members, the GCAPs are N-terminally myristoylated, although a clear role for this myristoylation has only recently been established. Myristoylation is apparently important for the optimal interaction of GCAP1 with its membrane-bound target guanylyl cyclase 1 (Schrem *et al.*, 1999). GCAP2 on the other hand, whilst bound to membranes at low Ca^{2+} levels and dissociated from them at higher concentrations, does not require myristoylation for either its membrane association or its stimulation of guanylyl cyclases, and it does not possess the Ca^{2+}/myristoyl switch mechanism (Olshevskaya *et al.*, 1997). Thus it is believed that the myristoyl group present on the GCAPs is important in the fine tuning of guanylyl cyclase activity and in target recognition (Hwang & Koch, 2002b, a). Recent work has shown that the myristoyl group of GCAP1 is always buried within the protein even in the Ca^{2+}-bound state and its role is in the maintenance of the overall folding and stability of GCAP1 (Stephen *et al.*, 2007).

1.2.6 Class E: Potassium Channel Interacting Proteins (KChIPs)

The most recently discovered of the NCS proteins, the potassium channel interacting proteins (KChIPs), were found as a sub-family through a yeast two hybrid screen using the N-terminus of the Kv4.3 potassium channel protein as bait (An *et al.*, 2000) with one KChIP found independently in other studies and named DREAM (Carrion *et al.*, 1999) or calsenilin (Buxbaum *et al.*, 1998). The KChIP class has become the most diversified of the NCS protein family throughout evolution, with four KChIP genes present in mammals and a large number of potential splice variants. Currently, 16 different KChIP isoforms have been found to be detectably expressed, and whilst most are found in the brain, some of the KChIP2 isoforms are only found in the heart and KChIP3 has also been found to be expressed in the testes (An *et al.*, 2000; Kuo *et al.*, 2001; Patel *et al.*, 2002; Pruunsild & Timmusk, 2005).

The C-terminal EF-hand domains of the human isoforms of the KChIPs are at least 70% identical to each other, with their variable N-terminal extensions forming the key difference between the splice variants (figure 6). KChIP1 is the only member of the subfamily that has the glycine at position two required for myristoylation. The myristoyl group of KChIP1 is exposed irregardless of Ca^{2+} binding status and this myristoyl tail anchors the protein to intracellular membranes (O'Callaghan *et al.*, 2003a). The other three KChIP subtypes are not myristoylated, though the three longer isoforms of KChIP2 (KChIP 2.1, 2.2 and 2.3), and the longest isoforms of both KChIP3 (KChIP3.1) and KChIP4 (KChIP4.1) are palmitoylated through

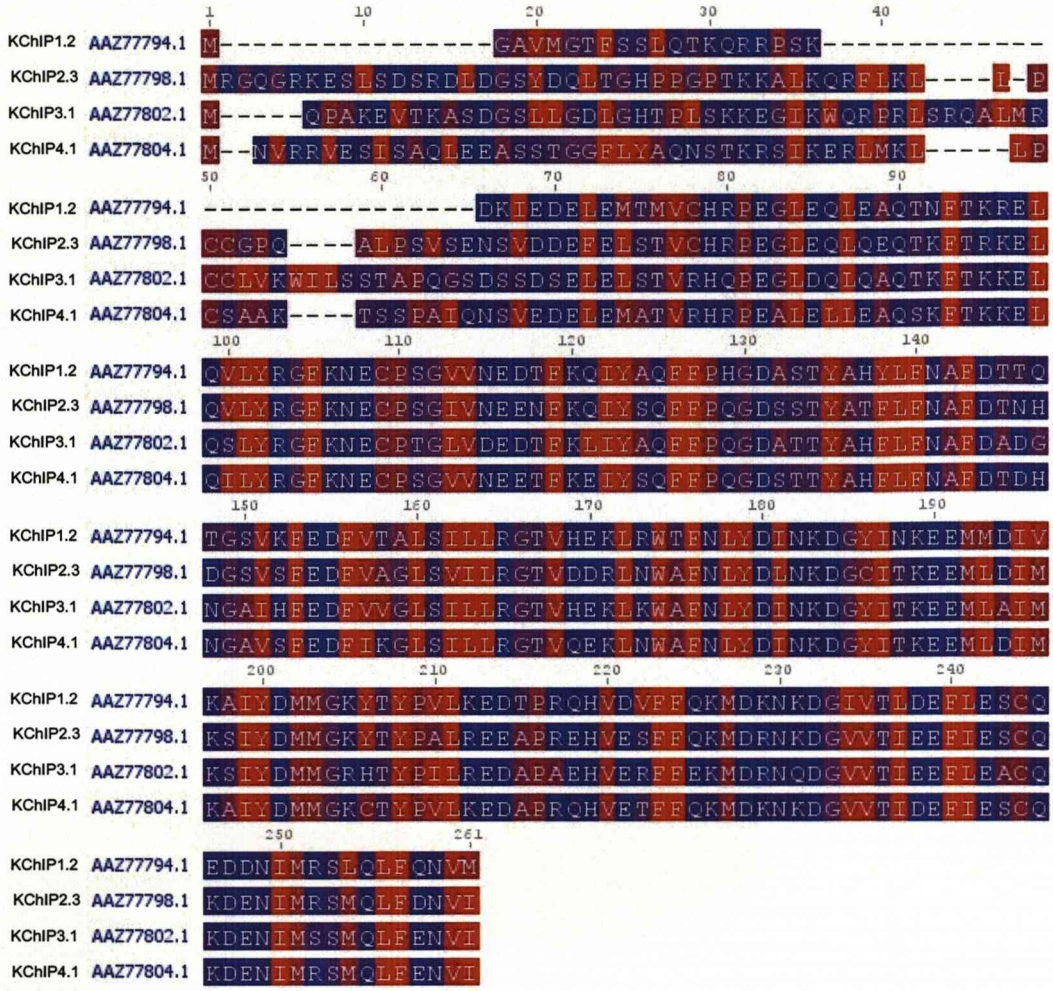

Figure 6. Alignment of the sequences of isoforms of the four KChIP protein members examined in this project.
The isoforms shown in the alignment are KChIP1.2, KChIP2.3, KChIP3.1 and KChIP4.1. Sequence alignments were carried out utilising Geneious Pro v.3.5.6 software. Amino acids are colour coded based on their hydrophobicity.

cysteines in their N-terminal domains. Palmitoylation of KChIPs 2.3 and 3.1 is required for efficient trafficking of Kv4 channels, and only palmitoylated KChIP2 associates with the plasma membrane when expressed alone (Rosati *et al.*, 2001; Takimoto *et al.*, 2002; Shibata *et al.*, 2003). Each of the KChIPs appear to have a cell-type specific expression in the brain suggesting that each neuronal cell type expresses only a limited number of KChIP isoforms (Rhodes *et al.*, 2004).

One subfamily of proteins, multiple functions

Whilst the KChIPs were originally discovered as a group of proteins that interact with the Kv4 potassium channels (An *et al.*, 2000), subsequent research has found that the KChIPs can actually carry out multiple cellular functions. This multiple functionality was identified initially following the realisation that KChIP3 had been identified previously and assigned other functions which have now been substantiated.

KChIP3/Calsenilin/DREAM

Amongst the KChIPs, KChIP3 is of particular interest as it has three well established but totally unrelated functions. As a result, this protein has been given three different names. It was named KChIP3 based on its ability, along with KChIPs 1, 2 and 4, to regulate certain K^+ channels (An *et al.*, 2000), calsenilin due to its interaction with the presenilin proteins (Buxbaum *et al.*, 1998) and DREAM based on its ability to repress gene transcription through binding to the DRE DNA motif

(Carrion *et al.*, 1999). For the purposes of this investigation and to avoid confusion, I will henceforth refer to this protein as KChIP3.

As KChIP3 is involved in three distinct biological pathways, it is likely that this protein performs multiple functions that are mediated through specific interactions with proteins in different cell compartments. In cultured cells and mammalian brain extracts, the majority of KChIP3 is found in the cytoplasmic fraction although it is also tightly associated with the membrane fraction (Choi *et al.*, 2001; Zaidi *et al.*, 2002) This localisation is in agreement with the ability of KChIP3/calsenilin to interact with the presenilins and the Kv4 channel proteins located in the ER, Golgi and/or the plasma membrane. It has also been shown that KChIP3 can also translocate from the cytoplasm to the nucleus in order to act as a transcriptional repressor, with this translocation from the cytoplasm to the nucleus modulated by the levels of intracellular calcium, though this effect does not appear to be mediated through the direct binding of calcium to KChIP3 (Zaidi *et al.*, 2004). Given that KChIP3 is localised to several different compartments of the cell, many questions regarding the mechanisms regulating its activity still need to be addressed.

Recent work has concerned KChIP3 in a variety of roles, including its involvement in pain modulation(Cheng *et al.*, 2002), apoptosis (Jo *et al.*, 2001; Jo *et al.*, 2003), epileptic seizure (Hong *et al.*, 2003), development (Stetsyuk *et al.*, 2007) and long term potentiation (Lilliehook *et al.*, 2003), although the precise mechanisms involved in each are yet to be fully elucidated, particularly with respect to its

interaction with the presenilins. KChIP3 has also been shown to stimulate apoptosis (Jo *et al.*, 2001; Jo *et al.*, 2004) and processing of the β-amyloid precursor protein (Lilliehook *et al.*, 2003), both of which are linked to Alzheimer's disease.

Furthermore, KChIP3 has been implicated in having a role in epilepsy, as after kainic acid-induced seizure in mice and using a cell-culture model of seizure-like activity, KChIP3 expression was markedly decreased, suggesting that it may functionally be associated with the pathophysiology of status epilepticus (Hong et al., 2003).

So whilst KChIP3 has been implicated as having an important role in multiple neurological functions and disorders including Alzheimer's Disease, its precise function and role within the cell has not been well characterised. It is the ability of KChIP3 to perform multiple cellular functions, and the potential for the other KChIPs to act in a similar manner that form the major basis for this investigation.

1.3 The Known Functions of the KChIPs

1.3.1 Kv4 Potassium Channels and their regulation by the KChIPs

The mammalian Kv4 subfamily of channels is one of the nine currently identified subfamilies of voltage gated K^+ channels (Kv1-9) (O'Grady & Lee, 2005), and was originally discovered as the 'Shal' gene in Drosophila (Butler *et al.*, 1990; Covarrubias *et al.*, 1991; Pak *et al.*, 1991). The Kv4 channel carries a transient, A-type K^+ current, controlling the excitability of neurons and cardiac myocytes by regulating the neuronal firing frequency, preventing the back propagation of action potentials into neuronal dendrites and underlying the early phase of repolarisation in cardiac myocytes (Serodio *et al.*, 1994; Dixon *et al.*, 1996; Hoffman *et al.*, 1997; Serodio & Rudy, 1998; Dilks *et al.*, 1999). At the present time, three mammalian Kv4 genes have been identified by molecular cloning, Kv4.1-4.3 (Pak *et al.*, 1991; Serodio *et al.*, 1996; Isbrandt *et al.*, 2000), with two isoforms of Kv4.3 present in mammals due to alternative splicing (Kv4.3 long and Kv4.3 short) (Ohya *et al.*, 1997; Dilks *et al.*, 1999).

Voltage gated K^+ channels consist of a tetrameric core of pore forming α-subunits, with their individual functional properties, turnover rates and intracellular traffic modulated by interactions with other proteins (Jan & Jan, 1997; Choe *et al.*, 1999). Each α-subunit is composed of 6 transmembrane domains, S1-S6, and cytoplasmic amino- and carboxy-terminal domains (figure 7) (Birnbaum *et al.*, 2004).

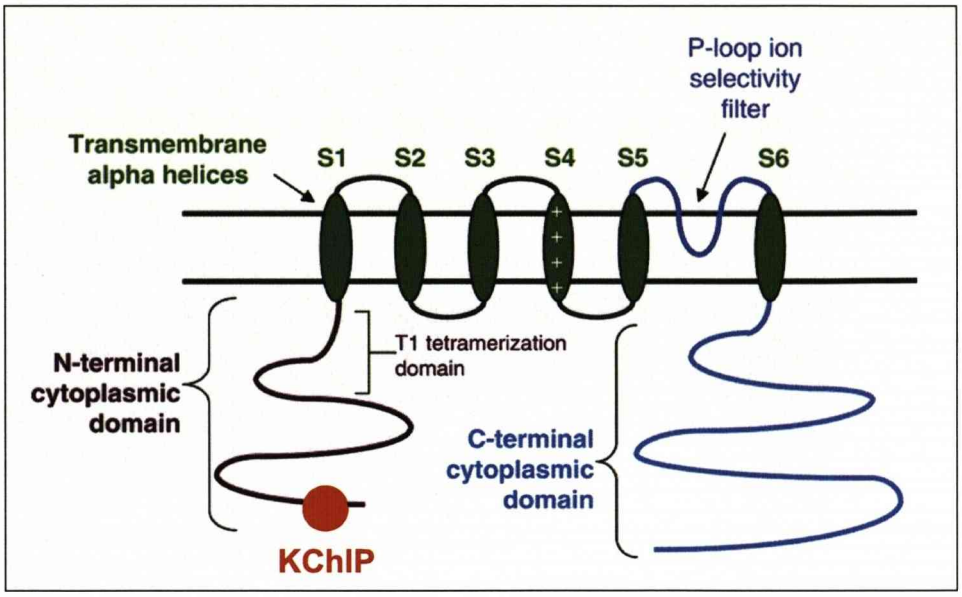

Figure 7. Schematic drawing of a Kv4 channel subunit.
Each α-subunit is composed of 6 transmembrane domains, S1-6, and cytoplasmic amino- and carboxy-terminal domains. KChIP binds to the N-terminus. The hydrophobic P segment, together with the adjacent S5 and S6 segments form the channel pore through which potassium ions are conducted. S4 carries positive charges and acts as the voltage sensor. Four subunits assemble to form a functional channel. Adapted and modified from Birnbaum *et al*, 2004.

When four α-subunits are arranged to form a tetrameric Kv channel, each hydrophobic P-loop between regions S5 and S6 forms a quarter of the ion conduction pathway, with the P-domain from each individual α-subunit facing one another to form the channel pore. As the S5 and S6 segments also align the pore of the channel, this allows the formation of an electrochemical gradient that allows K^+ ions to travel along this conduction pathway and across the cell membrane (Jerng *et al.*, 2004). The S4 domain acts as the voltage sensor of the channel, with membrane depolarisation triggering movement in the S4 segment leading to activation or opening of the channel. Similarly, during deactivation, the segments rearrange to close the channel (Robertson, 1997). The cytoplasmic N- and C-terminal regions of the α-subunits also carry out important roles. For example, the proximal N-terminal region (amino acids 71-90) mediates the specific binding of the KChIPs (Bahring *et al.*, 2001b; Scannevin *et al.*, 2004; Zhou *et al.*, 2004) whilst the C-terminus mediates interactions with a variety of regulatory proteins, including PSD-95 (Wong *et al.*, 2002) and Kvβ subunits (Yang *et al.*, 2001). In addition, the N-terminus is involved in mediating the specificity of subfamily association through its so-called tetramerisation domain, meaning that four α-subunits from the Kv4 subfamily associate with one another, but not with other Kv subfamilies (Kukuljan *et al.*, 1995). Furthermore, the N-terminus is involved in the inactivation of the Kv4 channel (N-type inactivation) (Bahring *et al.*, 2001a; Gebauer *et al.*, 2004), with the C-terminus also suggested to be involved in inactivation gating (Jerng *et al.*, 1999).

Kv4 channels are highly expressed in the brain, as well as in heart and smooth muscle (Isbrandt *et al.*, 2000). They can be found throughout the central nervous system (CNS), with their expression apparently neuron specific, where they are involved in regulating membrane excitability and postsynaptic signal transduction (Alonso & Widmer, 1997; Serodio & Rudy, 1998). Kv4 channels inhibit the ability of the dendrites to initiate action potentials, effectively dampening dendritic excitability and preventing the back propagation of action potentials into neuronal dendrites, with this control of dendritic excitability implicated in the induction of NMDA receptor-dependent LTP (Watanabe *et al.*, 2002). Kv4 channels have also been demonstrated to have a key role in tuning pacemaker activity in dopaminergic midbrain neurons, and as a result are involved in control of dopamine release, something that has made them potential therapeutic targets for neuronal disorders such as Parkinson's disease and schizophrenia. Kv4 channels are also of interest with respect to epilepsy research, as there is a markedly reduced expression of Kv4.2 associated with this condition that is thought to contribute to the increased neuronal excitability that causes the initiation and propagation of seizures (Castro *et al.*, 2001; Bernard *et al.*, 2004).

The KChIPs interact directly with Kv4 α-subunits and were first identified through a yeast two hybrid screen using the N-terminal region of Kv4.3 as bait. Subsequent immunohistochemical and co-immunoprecipitation studies have demonstrated that the KChIPs are integral components of native Kv4 channels in the brain (An *et al.*, 2000; Holmqvist *et al.*, 2002; Morohashi *et al.*, 2002; Rhodes *et al.*, 2004). They

form an octomeric structure made up of four KChIPs and four Kv4 channel subunits (Kim *et al.*, 2004a; Kim *et al.*, 2004b; Pioletti *et al.*, 2006; Wang *et al.*, 2007), as illustrated in figure 8.

The first comparative study of the KChIPs and their effects on Kv4 channels analysed KChIPs1.2, 2.3 and 3.1 (An *et al.*, 2000) which were all found, albeit with some quantitative differences, to demonstrate the same effects: increasing the expression of Kv4 at the plasma membrane, shifting the voltage-dependency of activation, slowing inactivation and recovering from the inactivation at a faster rate. Further work in heterologous expression systems where the Kv4 current was measured electrophysiologically, both in the presence and absence of KChIPs, confirmed these findings, namely a marked increase in peak amplitude (current density) and a slowing of Kv4 inactivation when the channel was co-expressed with the KChIPs (figure 8c) (Bahring *et al.*, 2001a; Decher *et al.*, 2001; Beck *et al.*, 2002; Hatano *et al.*, 2002; Holmqvist *et al.*, 2002; Morohashi *et al.*, 2002; Decher *et al.*, 2004). In summary, the KChIPs restore the native-like properties to the Kv4 current that are not seen when the Kv4 subunits are expressed alone. And whilst the interaction with the Kv4 channels is not reliant on Ca^{2+} binding, the functional activities exhibited by the KChIPs are, as they have been demonstrated to require intact EF-hands (An *et al.*, 2000; Morohashi *et al.*, 2002). The interaction of the KChIPs with the Kv4 channels is mediated through the C-terminal region of the KChIPs, with the variable N-terminal region demonstrated as being non-essential for the interaction with the channel and not required for the basic effects on channel

traffic and gating properties (An *et al.*, 2000). However, it has subsequently become clear that the variable N-terminal region of the different KChIP isoforms is important in generating the variable effects on Kv4 channels. For example, a comparative study on the rate of recovery from inactivation of Kv4 channels when co-expressed with the KChIP1 isoforms 1.1 and 1.2 found significant differences in the effects upon the channel (Boland *et al.*, 2003; Van Hoorick *et al.*, 2003). Similar differences in the efficiency of traffic of Kv4 channels to the plasma membrane and in the modulation of gating properties have also been demonstrated for several different KChIP2 and KChIP4 isoforms (Deschenes *et al.*, 2002; Holmqvist *et al.*, 2002; Morohashi *et al.*, 2002; Patel *et al.*, 2002; Takimoto *et al.*, 2002; Decher *et al.*, 2004; Patel *et al.*, 2004).

The physiological significance of the KChIPs as modulators of Kv4 channels has been illustrated by studies on KChIP knock-out mice. The KChIP2 knock-out resulted in a complete loss of the transient outward K^+ current that is mediated by the Kv4 potassium channels in ventricular myocytes, producing a phenotype highly susceptible to cardiac arrhythmias (Kuo *et al.*, 2001). The authors deduced that the absence of KChIP2 resulted in a trafficking defect where Kv4 channels no longer reached the plasma membrane, meaning that the myocardium became hyperexcitable.

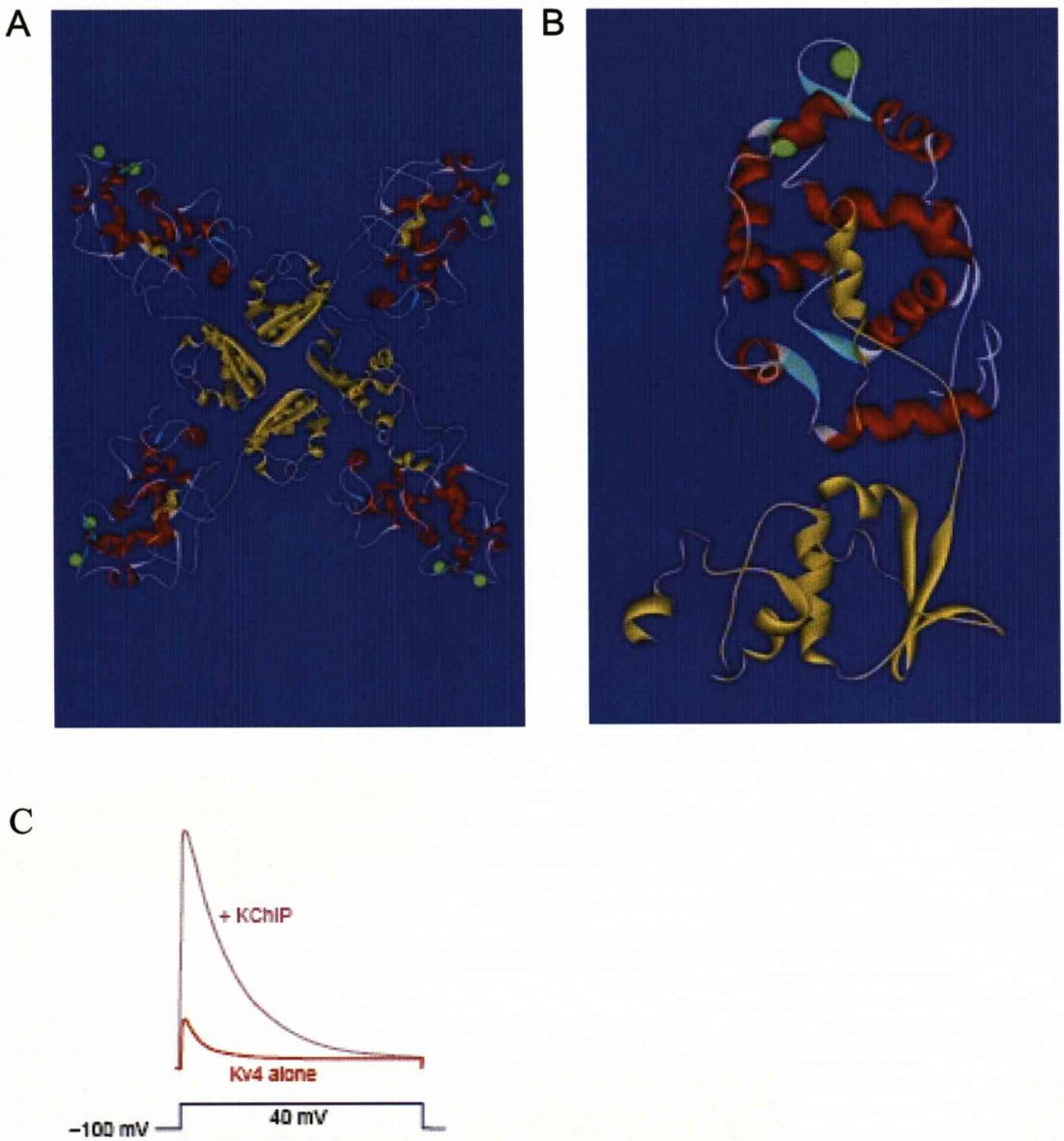

Figure 8. The interaction between the KChIPs and Kv4 channel subunits. **(A)** View of the octomeric structure formed between four KChIPs and four Kv4.3 channel subunits. **(B)** The interaction between KChIP1 and the N-terminus of Kv4.3. The N-terminus of Kv4.3 is shown in yellow and the green spheres represent Ca^{2+} ions bound to EF-hands 3 and 4. The structure was solved by Pioletti *et al*, 2006. **(C)** Kv4 currents as measured by whole-cell patch clamp recording. Co-expression of KChIP1 increases the current amplitude and slows the inactivation of the K^+ channels (from Burgoyne *et al*, 2004).

Two studies have been carried out using KChIP3 knock-out mice, although only one of the studies investigated the effects on Kv4 channels, where an increase in LTP (long term potentiation) was discovered which coincided with a downregulation of Kv4 current in the hippocampus (Lilliehook *et al.*, 2003). It is believed that the back propagation of action potentials into neuronal dendrites is prevented by activated Kv4 channels. However, when Kv4 channels are absent or inactivated, their block on the dendritic membranes is absent, allowing incoming EPSPs (excitatory postsynaptic potentials) to generate action potentials. An amplification of the dendritic action potentials results in the unblocking of NMDA receptors and influx of Ca^{2+} that induces LTP (Johnston *et al.*, 2000a; Johnston *et al.*, 2000b).

1.3.2 The DREAM function of the KChIPs

KChIP3 was identified as DREAM (downstream-regulatory-element-antagonist modulator) by Carrion and co-workers (Carrion *et al.*, 1999), who demonstrated that it can bind to DNA and act as a Ca^{2+} dependent regulator of transcription. DREAM was found to be identical to KChIP3/calsenilin except for the suggested presence of 30 additional residues at the N-terminus. This was later identified as being a mistake, with both sequences found to be identical. It binds as a tetramer to DREs (downstream regulatory elements) located downstream of the TATA box in target genes (Carrion *et al.*, 1998; Carrion *et al.*, 1999) where it acts to repress transcription. Ca^{2+} binding induces a conformational change in DREAM that prevents its binding to these DREs, demonstrated by work carried out on EF-hand mutants of DREAM/KChIP3, which were unable to bind Ca^{2+}. DRE residues are composed of a single copy or two inverted copies of the sequence GTCA (Ledo *et al.*, 2000b). So in summary, DREAM/KChIP3 binds to DREs to repress transcription in the absence of Ca^{2+}, whilst at increased intracellular Ca^{2+} concentrations, its DNA binding and repressor actions are abolished (Osawa *et al.*, 2001).

DREAM/KChIP3 can also bind Mg^{2+} in addition to Ca^{2+}, although the importance of Mg^{2+} binding is yet to be clearly defined. It had originally been concluded that it was calcium and not magnesium binding that was important in modulating DREAM/KChIP3 structure and function (Craig *et al.*, 2002). However, more recent

studies have suggested that binding of both Ca^{2+} and Mg^{2+} are able to induce distinct conformational changes, with Mg^{2+} binding at EF2 suggested to structurally bridge DREAM/KChIP3 to DNA targets (Osawa *et al.*, 2005).

A number of target genes have been identified for DREAM/KChIP3, including prodynorphin, which is involved in memory and pain perception, inducible cAMP early repressor (ICER), arylalkylamine N-acetyltransferase (AA-NAT), c-fos and Fos-related antigen-2 (fra-2), the apoptotic gene hrk and thyroid-specific transcription factors Pax8 and Foxe1 (Carrion *et al.*, 1999; Ledo *et al.*, 2000a; Sanz *et al.*, 2001; Ledo *et al.*, 2002; Sanz *et al.*, 2002; Link *et al.*, 2004; D'Andrea *et al.*, 2005). More recently it has been found to bind to the promoter of the NCX3 gene where it acts to repress transcription of NCX3 (Gomez-Villafuertes *et al.*, 2005). The Na^{+}/Ca^{2+} exchangers NCX1, NCX2 and NCX3 are vital for the control of cellular Ca^{2+} homeostasis in neurons.

The physiological relevance of DREAM/KChIP3 as a transcriptional repressor has been demonstrated by studies on DREAM knock-out mice, which were found to have increased prodynorphin levels and a decreased pain response. Cheng and colleagues found that KChIP3/DREAM acts as a transcriptional repressor involved in regulating pain, as KChIP3/DREAM$^{-/-}$ mice displayed markedly reduced responses in models of acute thermal, mechanical and visceral pain, as well as reduced pain behaviours in models of neuropathic and inflammatory pain. This led to the suggestion of DREAM as a therapeutic target for the management of arthritic

pain (Cheng & Penninger, 2004). At the same time however, these KChIP3/DREAM$^{-/-}$ mice showed no major defects in motor function or learning and memory (Cheng *et al.*, 2002). However, in another KChIP3/DREAM knock-out mouse line, long term potentiation (LTP) in the dentate gyrus of hippocampus, where KChIP3 is strongly and selectively expressed, is enhanced (Lilliehook *et al.*, 2003).

Interestingly, it has subsequently been shown that it is not just DREAM/KChIP3 that can bind to DRE sites. Link and co-workers demonstrated that KChIPs1, 2 and 4, in addition to KChIP3, can bind to DRE sites located in the regulatory regions of the ICER, fra-2 and AA-NAT genes which are involved in circadian rhythms (Link *et al.*, 2004). This adds further weight to the argument that the KChIPs can carry out multiple, overlapping functions within neuronal populations.

1.3.3 The KChIPs and the Presenilins

KChIP3 was originally isolated as a presenilin-interacting protein and named calsenilin (Buxbaum et al., 1998). It co-immunoprecipitates with both PS1 and PS2 in transfected cells, as well as at endogenous levels in brain (Buxbaum *et al.*, 1998; Choi *et al.*, 2001). KChIP3 binds to the C terminus of both PS1 and PS2, and for this reason it has been implicated as potentially having a role in presenilin-associated Alzheimer's disease.

The identification of calsenilin/KChIP3 as an interacting partner of presenilins 1 and 2 generated a lot of interest, as most early-onset familial Alzheimer disease cases are caused by mutations in the highly regulated genes presenilin-1 (PS1) and presenilin-2 (PS2) (Selkoe, 1997; Buxbaum *et al.*, 1998). Alzheimer's is the most common cause of senile dementia in humans, and is the fourth leading cause of death in the United States (Lendon *et al.*, 1997). These mutations lead to increased β-amyloid (Aβ) formation and apoptosis, with the deposition of Aβ in the extracellular compartments being one of the characteristics of Alzheimer's, along with the formation of neurofibrillary tangles in the neuronal cell body and massive neuronal death (Choi *et al.*, 2001; Lilliehook *et al.*, 2002; Jo *et al.*, 2003). To date six mutations in PS2 and 75 mutations in PS1 have been reported (Zaidi *et al.*, 2002) and although the presenilins have been implicated in having a role in the Notch signalling pathway, amyloid precursor protein (APP) processing, calcium homeostasis and programmed cell death, their normal physiological roles are still a topic of debate. It is hoped that the identification and characterisation of interacting

partners may ultimately provide a greater insight into the functional role of the presenilins.

KChIP3 has generated more interest than sorcin, another presenilin-binding protein that may play a role in modulating calcium signalling, as sorcin binds only to PS2 and not to PS1 (Pack-Chung et al., 2000). Therefore this molecule is unlikely to be involved in a general function of the presenilins, linked to the pathogenesis of Alzheimer's disease, whereas KChIP3, which binds to both PS1 and PS2, is in contrast more likely to play such a role (Leissring et al., 2000).

The link between calsenilin/KChIP3 and Alzheimer's disease has been enhanced by the discovery of increased levels of calsenilin/KChIP3 protein and mRNA in Alzheimer's disease brains (Jin *et al.*, 2005), and a calsenilin/KChIP3 knock-out mouse that exhibited reduced processing of the amyloid precursor protein (APP) (Lilliehook *et al.*, 2003). Processing of the APP leads to the formation of amyloid-β peptide (Aβ), the primary component of neuritic (senile) plaques that, along with neuronal cell death and the accumulation of neurofibrillary tangles (NFT), pathologically characterize Alzheimer's disease (Price & Sisodia, 1998). Aβ is generated by consecutive proteolytic cleavages mediated by β- and γ-secretase, with evidence suggesting that presenilins are either the catalytic subunit or a necessary co-factor of a high molecular weight multi-subunit aspartyl protease complex that exhibits γ-secretase activity (Li *et al.*, 2000; Esler *et al.*, 2002), and calsenilin/KChIP3 thought to play a role in modulating the presenilin activity

(Buxbaum, 2004). In cell cultures KChIP3 has been shown to enhance apoptosis by increasing the content of intracellular Ca^{2+} stores (Lilliehook *et al.*, 2002) and increasing presenilin-dependent γ-secretase cleavage of the Alzheimer disease amyloid protein precursor, thereby elevating Aβ formation (Jo et al., 2005).

The effect of KChIP3 on the content of intracellular Ca^{2+} stores is particularly interesting, as recent studies have suggested that the presenilins may act within the cell as ER Ca^{2+} leak channels (Tu *et al.*, 2006; Nelson *et al.*, 2007). Ca^{2+} signalling defects have been observed by numerous groups studying FAD, including studies with fibroblasts from FAD patients (Ito *et al.*, 1994), in FAD cellular and animal models (LaFerla, 2002; Smith *et al.*, 2005) and in cells from presenilin knockout mice (Herms *et al.*, 2003; Ris *et al.*, 2003; Takeda *et al.*, 2005). A number of hypotheses have previously been suggested to explain these phenomena, notably including regulation of expression of neuronal Ca^{2+} signalling proteins (Leissring *et al.*, 2002) and the proposal that the presenilins act as ER Ca^{2+} leak channels (Tu *et al.*, 2006). In the case of the latter, it is thought that in order to maintain a steady-state ER intraluminal Ca^{2+} level, an equilibrium develops between SERCA-mediated movement of Ca^{2+} from the cytosol into the ER lumen and the passive leak of Ca^{2+} from the ER into the cytosol through low-conductance divalent-cation-permeable ion channels formed by the presenilins. This is supported by the fact that the ER Ca^{2+} leak is abolished by the expression of presenilin mutants, with the authors suggesting that the disruption of the ER Ca^{2+} leak pathway may lead to Ca^{2+} overloading of the ER and that this disturbed Ca^{2+} homeostasis ultimately

contributes to Alzheimer disease pathogenesis (Nelson *et al.*, 2007). Thus both increased expression of KChIP3 and mutations in the presenilins, both found in Alzheimer's disease patients, may cause Ca^{2+} overloading of the ER resulting in cellular damage.

Presenilin activity itself has been shown to regulate calsenilin/KChIP3-mediated apoptosis, suggesting a combined role for the presenilins and calsenilin/KChIP3 in the neuronal cell death seen in Alzheimer's disease (Jo *et al.*, 2003). Furthermore, calsenilin/KChIP3 has been shown to be a substrate for caspase-3, and may not only play a role in increased ER Ca^{2+} loading, but also facilitate Ca^{2+} release from the ER, which is consistent with a role in apoptosis (Leissring *et al.*, 2000; Choi *et al.*, 2001; Lilliehook *et al.*, 2002; Choi *et al.*, 2003).

However, the exact contribution of apoptosis to the pathogenesis of the disease remains unclear, as does the role of KChIP3 and its interaction with the presenilins, although it has though been shown that in KChIP3 knock-out mice, there is a reduction in Aβ formation (Lilliehook et al., 2003). Aβ formation is believed to have a role in apoptosis, with KChIP3 thought to play an important role, possibly increasing Aβ formation by modulating γ-secretase activity through interaction with presenilin (Jo et al., 2001), although it has also been speculated that the pro-apoptotic effect of KChIP3 may be due to it acting as a potassium channel regulator, as efflux of K^+ has been suggested to contribute to neuronal apoptosis (Yu *et al.*, 1997). Blocking KChIP3 expression has also been shown to protect neuronal cells

from Aβ toxicity (Jo et al., 2003), again suggesting that its pro-apoptotic effect may be due to its role in Aβ formation. It is also possible that modifications in intracellular Ca^{2+} signalling by both KChIP3 and mutant presenilins may contribute to cellular apoptosis.

The precise significance of the interaction between calsenilin/KChIP3 and the presenilins, and how this relates to their functional roles is something that is yet to be completely understood. A summary of the current suggested role of the KChIP3-presenilin interaction is shown in figure 9. Again, as with the Kv4 channel regulation and DRE binding properties, this particular function does not appear to be limited to just one of the KChIPs, with at least KChIP4, in addition to KChIP3, able to interact with presenilin-2 (Morohashi *et al.*, 2002), once more suggesting the potential for the KChIPs to carry out similar cellular roles in different neuronal populations.

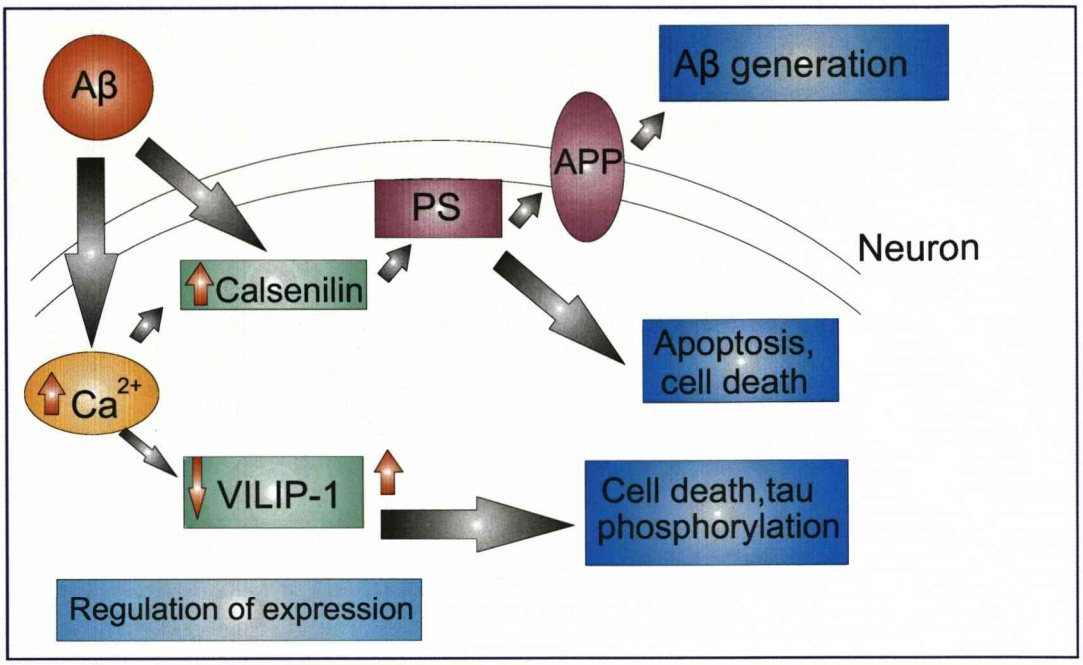

Figure 9. The current proposed mechanism for the role of calsenilin/KChIP3 in Alzheimer's disease.
Up-regulation of calsenilin/KChIP3 leads to increased binding with presenilin, which in turn stimulates cleavage of APP as well as causing cell death. APP cleavage leads to Aβ generation, which can then feedback in a positive loop, stimulating further calsenilin/KChIP3 production, increasing intracellular Ca^{2+} and down-regulating VILIP-1expression which in turn also leads to cell death and tau phosphorylation.

1.4 Aims and Objectives

The first major focus of this study was on the KChIP subfamily of the NCS proteins and their multiple functional roles. Do the KChIPs simply carry out the same functional roles in different neuronal populations, or do they maintain unique properties enabling them to carry out individual cellular roles? **In order to investigate this, I aimed to carry out a comparative study of selected isoforms of KChIPs 1-4 and some of their known functions.** One of these has been reported for all four KChIPs (regulation of Kv4 channels) whilst the others may be specific for particular KChIPs (regulation of Kv1.5 channels – KChIP2, interaction with presenilins – KChIPs 3 and 4). This work utilised fluorescently-tagged versions of the proteins, with the intrinsic targeting of each of the proteins expressed individually determined first, followed by co-expression studies of the KChIPs with the various interacting proteins. The investigation also included NCS-1, as this protein is also known to carry out multiple cellular functions, some of which reportedly overlap with the functions of the KChIPs. **Given this reported overlap in function, another aim of my project was to see if the KChIPs can perform another established function of NCS-1, its ability to affect secretion, utilising a well established essay. It was also aimed to elucidate some of the intracellular roles the KChIPs carry out by investigating how they affect Ca^{2+} signalling within the cell.**

CHAPTER 2:

Materials and Methods

2.1 General Reagents

Unless stated differently, all general chemicals were obtained from Sigma-Aldrich (Dorset, UK). Consumerables such as tips, cell culture flasks and plasticware were provided by Appleton Woods (Birmingham, UK). Glass coverslips and poly-D-lysine coated plates were obtained from VWR (Lutterworth, UK).

2.2 Plasmids

Unless otherwise stated, the KChIP splice variants used in the following constructs were KChIP1.2, KChIP2.3, KChIP3.1 and KChIP4.1. All KChIP isoforms used are human, with the GenBank accession numbers as follows: DQ148477 (1.2), DQ148481 (2.3), DQ148485 (3.1) and DQ148487 (4.1). Fluorescent vectors were obtained from Clontech (California, USA), the pFLAG-CMV-4 vector from Sigma (USA) and the pcDNA3.1 (+) vector from Invitrogen (Paisley, UK).

2.2.1 Fluorescent Constructs

The KChIP2-ECFP construct (pKChIP2-ECFP) was made by inserting the human KChIP2 sequence into the pECFP-N1 vector (Clontech, California, USA). The human KChIP2 sequence was amplified from an existing KChIP2-pcDNA construct, kindly provided by Dr. Robert Bähring (Bahring *et al.*, 2001b), with the primers used to facilitate this cloning as follows (endonuclease sites underlined): the sense

primer was 5'-ACTCAGATCTCAGGATGCGGGGCCAGGGCCGC-3' (BglII) whilst the antisense primer was 5'-TACCGTCGACTGGATGACATTGTCAAA GAG-3' (SalI).

The KChIP3-ECFP construct (pKChIP3-ECFP) was made by inserting the human KChIP3 sequence into the pECFP-N1 vector (Clontech, California, USA). The human KChIP3 sequence was amplified from an existing EYFP tagged KChIP3 fusion (pKChIP3-EYFP) construct kindly provided by D.O'Callaghan. The primers contained endonuclease sites to facilitate this cloning (underlined). The sense primer used was 5'-ACTCAGATCTCAGGATGCAGCCGGCTAAGGAA-3' (BglII) and the antisense primer was 5'-TGCAGAATTCCGATGACATTCTCAAA CAGC-3' (EcoRI). Subsequently a new KChIP3-EYFP (pKChIP3-EYFP) construct was made by replacing the ECFP tag of the KChIP3-ECFP fusion construct (pKChIP3-ECFP) with the EYFP tag of the pEYFP-N1 vector (Clontech, California, USA).

The KChIP4-ECFP construct (pKChIP4-ECFP) was made by insertion of the KChIP4 sequence, amplified by PCR from a human KChIP4 cDNA clone (OriGene, Rocksville, USA), into the pECFP-N1 vector (Clontech, California, USA). The primers used contained the following endonuclease sites (underlined) to enable the cloning: the sense primer was 5'-ACTCAGATCTCAGGATGAATGTGAGGAGG GTG-3' (BglII) whilst the antisense primer was 5'-TACCGTCGACTGAATCACA TTTTCAAAGAG-3' (SalI).

2.2.2 pcDNA constructs

The pcDNA-KChIP3 construct was made by the insertion of the KChIP3 sequence, amplified from the pKChIP3-EYFP plasmid by PCR, into the pcDNA3.1 (+) vector (Invitrogen). The primers used contained the following endonuclease sites (underlined): the sense primer used was 5'-CTTCGAATTCAGGATGCAGCCGG CTAAGGAA-3' (EcoRI) and the antisense primer was 5'-CAACGCGGCCGCTAG ATGACATTCTCAAACAGC-3' (NotI).

2.2.3 FLAG-tagged constructs

The pFLAG-KChIP1 construct was made by the insertion of the KChIP1 sequence into the pFLAG-CMV-4 Expression vector (Sigma, USA), with the KChIP1 sequence amplified from an existing KChIP1-ECFP construct (Dr. Burcu Hasdemir). The primers contained endonuclease sites (underlined) in order to facilitate the cloning. The sense primer was 5'-GCTTGCGGCCGCGAGGATGGGGGCCGTCA TGGGC-3' (NotI) whilst the antisense primer was 5'-TATCAGATCTATTTACAT GACATTTTGAAACAG-3' (BglII).

The pFLAG-KChIP2 construct was made by the insertion of the KChIP2 sequence, amplified from an existing KChIP2-pcDNA construct (a kind gift from Dr. Robert Bahring) by PCR, into the pFLAG vector. The primers used were as follows, with the endonuclease sites underlined: the sense primer used was 5'-GCTT

GCGGCCGCGAGGATGCGGGGCCAGGGCCGC-3' (NotI) and the antisense primer was 5'-TATCAGATCTATCTAGATGACATTGACAAAGAG-3' (BglII).

The pFLAG-KChIP3 construct was made by the insertion of the KChIP3 sequence, amplified from the pKChIP3-EYFP plasmid by PCR, into the pFLAG vector. The primers contained endonuclease sites (underlined) to facilitate this cloning. The sense primer used was 5'-GCTTGCGGCCGCGAGGATGCAGCCGGCTAAG GAA-3' (NotI) and the antisense primer was 5'-TATCAGATCTCCCTAGAT GACATTCTCAAACAGC-3' (BglII).

The pFLAG-KChIP4 construct was made by insertion of the human KChIP4 sequence, amplified from a human KChIP4 cDNA clone (OriGene, Rockville, USA) by PCR, into the pFLAG vector. The primers used were as follows, with the endonuclease sites used to facilitate the cloning underlined: Sense 5'-GCTT GCGGCCGCGAGGATGAATGTGAGGGTG-3' (NotI), antisense 5'-TATC AGATCTATTTAAATCACATTTTCAAAGAG-3' (BglII).

2.2.4 Other constructs used

The EGFP-tagged presenilin-1 fusion construct (PS1-EGFP/pCDNA3.1 Zeo) was a kind gift from Dr. Christoph Kaether (Kaether *et al.*, 2002). The PS1 wt in pcDNA3.1 Zeo (+) was a gift from Prof. Christian Haass. The EGFP tagged mouse Kv1.5 fusion construct (mKv1.5-EGFP) was a kind gift from Dr. Jeanne Nerbonne,

as were the human Kv1.4 and Kv1.5 fusion constructs (Kv1.4-EGFP, Kv1.5-EGFP) (Li *et al.*, 2001; Li *et al.*, 2005).

The KChIP1-ECFP and ECFP-Kv4.2 (pECFP-Kv4.2) constructs were provided by Dr. Burcu Hasdemir (Hasdemir *et al.*, 2005). The NCS-1-ECFP construct was provided by Dr. Lee Haynes (Haynes *et al.*, 2005).

All primers used in PCR amplification were produced by Genosys Biotechnologies (Cambs, UK) whilst the sequences of all these constructs were confirmed by sequencing by DBS Genomics (Durham, UK).

2.3 Cell Culture

2.3.1 Culture and transfection of HeLa and COS-7 cells

HeLa and COS-7 cell cultures were grown in 20 ml Dulbecco's modified Eagle's medium (Gibco, Paisley, UK) containing 5% foetal calf serum (FCS) (Gibco, Paisley, UK) and 1% non-essential amino acids (Gibco, Paisley, UK) and maintained at ~1,000,000 cells per 75cm^2 flask at 37°C in an atmosphere of 5% CO_2. 16-24 hours prior to transfection, cells were seeded onto glass cover slips in a 24-well plate at approximately 40,000 cells per well or approximately 100,000 cells per well of a 6 well plate. Cells were then transfected at approaching 50% confluency using 100µl transfection reaction mixture. The transfection mix typically contained 1µg plasmid DNA (2µg for double transfections), with either 3µl (per µg plasmid DNA) Fugene (Roche, UK) or 3µl (per µg plasmid DNA) GeneJuice (Novagen, USA) and was made up to 100µl with Dulbecco's modified Eagle's medium (Gibco, Paisley, UK). The transfection reaction mixture was incubated at room temperature for 30 minutes before being added drop-wise to the cells. Cells were then maintained at 37°C for 16-48 hours before being used in experiments.

2.3.2 Culture and transfection of PC12 cells

PC12 cells were grown in suspension in 20 ml RPMI 1640 media (Gibco, Paisley, UK) containing 5% foetal calf serum (FCS) (Gibco, Paisley, UK), 10% horse serum (HS) (Gibco, Paisley, UK), 100 units/ml penicillin and 0.1mg/ml streptomycin (Sigma) at 37°C in an atmosphere of 5% CO_2. 16-24 hours before transfection, freshly trypsinised (Gibco, Paisley, UK) cells were plated onto collagen (Sigma) – coated glass cover slips in a 24 well plate at a density of ~200,000 cells/well or ~350,000 cells/well for larger coverslips. Cells were then transfected using 100µl transfection reaction mixture consisting of 1µg plasmid DNA, 3µl LipofectAMINE 2000 transfection reagent (Invitrogen, Paisley, UK) per µg of plasmid DNA and made up to 100µl in opti-mem (Gibco, Paisley, UK). The transfection mixture was incubated at room temperature for 30 minutes before being added drop wise to the cells. The cells were then incubated for 4-6 hours at 37°C with the transfection reaction mixture, before removing the transfection reaction mixture and replacing with growth media. Cells were then maintained at 37°C, 5% CO_2 for 16-48 hours before being used in experiments.

2.4 Fluorescence Imaging and Immunofluorescence

2.4.1 Immunofluorescence

For imaging of transfected cells, the cells were washed twice in phosphate buffered saline (PBS); 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 , 2mM NaH_2PO_4, and fixed in PBS containing 4% formaldehyde for 30 minutes. Cells were then washed in PBS before air-drying coverslips and mounted on glass slides using antifade Vectashield (Vector Laboratories Inc.) or ProLong Gold Antifade (Invitrogen, USA).

For immunostaining of cells attached to coverslips, these were washed twice in PBS before fixation in PBS containing 4% formaldehyde for 30 minutes. The cells were incubated in PBT (PBS, 0.1% Triton X-100, 0.3% BSA) for 30 min. The PBT was removed and replaced by the primary antibody at the appropriate dilution in PBT [anti-FLAG (mouse) 1 in 5000 (Sigma), anti-CSEN (rabbit) 1 in 500 (Chemicon International), anti-EGFP (mouse) 1 in 500 (BD Biosciences), anti-PS1 (goat) 1 in 100 (Abcam, Cambs, UK)]. The primary antibody was incubated for 1 hour or overnight at 4°C, removed and the cells washed three times in PBT. The cells were then incubated with the appropriate biotinylated antibody (Amersham, Bucks, UK) diluted to 1 in 100 with PBT for 1 hour at room temperature. The secondary antibody was removed, the cells washed three times in PBT and the cells then incubated in streptavidin-Texas Red (Amersham, Bucks, UK) diluted to 1 in 50 with

PBT for 30 minutes. Alternatively, after removal of the primary antibody and subsequent washing, cells were incubated with the appropriate Dylight 649 secondary (Pierce, Rockford, USA) diluted to 1 in 250 with PBT for 1 hour at room temperature. Cells were then washed three times with PBT. The coverslips were air-dried and mounted on glass slides using antifade Vectashield (Vector Laboratories Inc.) or ProLong Gold Antifade (Invitrogen).

2.4.2 Confocal laser scanning microscopy

For confocal laser scanning microscopy either a Leica AOBS microscope system (Leica, Heidelberg, Germany) or a Leica TCS-SP-MP microscope was utilised to examine transfected cells. A 22.11μm pin hole and 63x water immersion objective with a 1.2 numerical aperture was used. For imaging ECFP tagged constructs, cells were excited at 430nm and light collected at 460-510nm. EYFP was imaged using excitation at 514nm with collection at 534-580nm. EGFP was imaged using excitation at 488nm with collection at 500-550nm. Texas red was excited at 543nm with light collected at 575-700nm. Dylight 649 was excited at 649nm and light collected at 620-720nm. For imaging of live cells, cells were maintained in Krebs buffer (20mM HEPES (pH7.4), 145mM NaCl, 10mM glucose, 5mM KCl, 3mM $CaCl_2$, 1.3mM $MgCl_2$, 1.2mM NaH_2PO_4).

2.5 Immunoblotting

Cells to be used for Western blotting were washed in PBS before lysis in 100µl Laemmli buffer (Sigma). Cell samples were then boiled for 10 minutes. Typically 20µl of sample was then run on a 4%/10% SDS gel before overnight transfer onto nitrocellulose (Whatman, Dassel, Germany). Nitrocellulose blots were washed twice with PBS over 15 minutes before blocking with 3% milk in PBS for 45 minutes. The primary antibody was added at the appropriate dilution in 3% milk in PBS [anti-GFP (mouse) 1 in 1000 (BD Biosciences), anti-FLAG (mouse) 1 in 10000 (Sigma), anti-PS1 (goat) 1 in 5000 (Abcam), anti-Kv4.2 (rabbit) 1 in 500 (Exalpha Biologicals Inc.), anti-pan KChIP potassium channel subunit (mouse) 1 in 1000 (UC Davis/NINDS/NIMH NeuroMab Facility, CA, USA)]. The primary antibody was added for 1 hour at room temperature (or overnight at 4°C), removed and the blot washed three times in PBS. The blot was then incubated with the appropriate HRP-conjugated secondary antibody (Sigma) diluted to 1 in 400 in 3% milk, 0.5% Tween, in PBS for 1 hour. The blot was washed once in PBS containing 0.5% Tween, washed 3 times in PBS, once in 0.3M NaCl in PBS for 30 minutes and once in dH_2O for 15 minutes. The appropriate ECL reagents (Amersham, Bucks, UK) were then added for 1 minute, the blot dried and covered in cling film before exposure to hyperfilm ECL for 1 minute. The film was developed in D19 developer for 1 minute, rinsed and fixed for 1 minute in Amfix. Alternatively, in the case of later blots (2007), blots were visualised using the Chemi Hi sensitivity mode on the

BioRad Gel Doc system using Quantity One software version 4.6.3 (BioRad, California, USA).

2.6 Assay of Growth Hormone release from transfected PC12 cells

Freshly trypsinized PC12 cells were plated out onto pre-coated poly-D lysine 24 well plates (VWR International, UK) at a density of ~350,000 cells/well 16-24 hours prior to transfection. Cells were then transfected using 100µl transfection reaction mixture consisting of 1µg control/test plasmid DNA, 1µg human Growth hormone plasmid (PXGH5) (Nichols Institute Diagnostics, California, USA), 3µl LipofectAMINE 2000 transfection reagent (Invitrogen, UK) per µg of plasmid DNA, made up to 100µl in opti-mem (Gibco, Paisley, UK). The transfection mixture was incubated at room temperature for 30 minutes before being added drop wise to the cells. The cells were then incubated for 4-6 hours at 37°C with the transfection reaction mixture before removing the transfection reaction mixture and replacing with growth media supplemented with 10µM $CdCl_2$. Cells were then maintained at 37°C, 5% CO_2 for 48 hours before use in experiments.

The secretion assay was performed on either permeabilised or intact cells depending upon the experiment. In the case of the former, cells were washed once with KGEP buffer (20mM PIPES (pH 6.5), 139mM Kglutamate, 5mM EGTA) before permeabilisation with KGEP buffer containing 20µM digitonin (20mM PIPES (pH 6.5), 139mM Kglutamate, 5mM EGTA, 20µM digitonin) for 6 minutes. Cells were then stimulated with 300µl of either 0 or 10µM free Ca^{2+} KGEP buffer for 15 minutes. For experiments on intact cells, cells were washed with Krebs Ringer buffer (20mM HEPES (pH7.4), 145mM NaCl, 10mM glucose, 5mM KCl, 3mM

$CaCl_2$, 1.3mM $MgCl_2$, 1.2mM NaH_2PO_4) and then stimulated for 15 minutes with 300µl of either 0 or 300µM ATP containing Krebs buffer.

After stimulation, supernatants were collected and stored (these samples contain secreted growth hormone) before lysis of the remaining adherent cells in distilled water containing 0.5% (v/v) Triton X-100 (300µl/well for 15 minutes, room temperature) to give corresponding samples containing unsecreted growth hormone to allow calculation of total cellular growth hormone levels.

Released growth hormone was assayed according to the instructions in the hGH ELISA kit (Roche Diagnostics, Mannheim, Germany). Final absorbance values were read at a wavelength of 405nm, with a reference wavelength of 490nm. The amount of secreted growth hormone was calculated and expressed as a percentage of total cellular growth hormone for each well. This procedure followed a well-established protocol (McFerran *et al.*, 1998).

2.7 Imaging of intracellular [Ca^{2+}] in PC12 cells

PC12 cells (350,000 cells per well) were plated onto collagen coated glass coverslips and transiently transfected with 2μg of either ECFP-N1 control plasmid or 2μg of KChIP1-ECFP, KChIP2-ECFP, KChIP3-ECFP, KChIP4-ECFP, NCS1-ECFP or PS1-EGFP using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's protocol. 48 hours post transfection, cells were washed in Krebs buffer (20mM HEPES (pH7.4), 145mM NaCl, 10mM glucose, 5mM KCl, 3mM $CaCl_2$, 1.3mM $MgCl_2$, 1.2mM NaH_2PO_4) and then incubated with 1μM X-rhod-1 AM (Molecular Probes Europe BV, Leiden, The Netherlands) in Krebs buffer for 30 minutes at room temperature. Cells were washed in Krebs buffer before imaging using laser-scanning confocal microscopy. An ECFP image was taken prior to each experiment to allow distinction between transfected and untransfected cells. X-rhod-1 was imaged using the Leica microscope with excitation at 543nm and light collection at 600-650nm. The X-rhod-1 fluorescence was diffusely and uniformly distributed, suggesting cytosolic localization of the probe. Through the application of 300μM ATP as an agonist, the effects of the overexpression of the aforementioned constructs on ATP-evoked intracellular Ca^{2+} transients in PC12 cells were then studied by examining the changes in fluorescence of X-rhod-1. This approach followed previously published methodology (Haynes *et al.*, 2004).

2.8 Statistical Analysis

Data in Chapter 4 are generally presented as mean ± SEM of *n* determinations. A student's two-tailed t test was performed for unpaired groups, whereas a one-way analysis of variance (ANOVA) was performed for three or more groups. Statistical significance was accepted at the $p<0.05$ level.

CHAPTER 3:

Analysis of the potential for KChIPs to carry out multiple functions within the cell

3.1 Introduction

Of the NCS proteins, the KChIP class has become the most diversified through evolution from one KChIP in *D.melanogaster* to the four KChIP genes and large number of potential splice variants that are present in mammals (Pruunsild & Timmusk, 2005). At least 16 KChIP isoforms have been detectably expressed in human tissue, although some of these KChIP2 isoforms are only expressed in the heart (Patel *et al.*, 2002) where KChIP2 plays a role in Kv4 channel regulation (Kuo *et al.*, 2001).

A key question regarding the KChIPs is the extent to which they carry out similar functions and to what extent their roles differ – do the individual KChIPs carry out specific individual roles, or do they perform similar roles in different cells? There are several reasons to believe either scenario. They are certainly expressed in different sub-populations of neurons within the brain, as demonstrated through the use of PCR after reverse transcription of RNA (RT-PCR) and *in situ* hybridization (Pruunsild & Timmusk, 2005). Furthermore, the C-terminal EF hand-containing domains of the human isoforms are at least 70% identical to each other with the main difference between the KChIP isoforms lying in the N-terminal domains (Burgoyne, 2007). So does this mean that as similar proteins they are simply carrying out the same functions but in different neuronal populations? Or does the fact that different KChIPs and different isoforms are expressed in varying regions of the brain mean that they are carrying out differing functions?

With respect to the KChIPs performing similar functional roles, previous work has demonstrated that the four different KChIPs do indeed have the ability to carry out the same function within the cell – KChIPs1-4 all have the ability to regulate Kv4 K^+ channels (An *et al.*, 2000; Morohashi *et al.*, 2002), and all four reportedly share the DRE binding property allowing them to repress transcription (Link *et al.*, 2004). By contrast, they have also been reported to have functions that may be distinct to each particular KChIP – KChIP3 for example has been implicated in APP processing (Buxbaum *et al.*, 1998), and has been shown to be pro-apoptotic (Jo *et al.*, 2001). KChIPs 3 and 4 have been reported to be involved in presenilin processing (Buxbaum, 2004), whilst KChIPs 1 and 2 have been shown to have an effect on Kv1.5 channel regulation (Li *et al.*, 2005). Not all four KChIPs have been tested in these assays however. This area is further complicated by the fact that certain functions may be modulated differently by different splice variants from the same gene – for example whilst KChIP4.1 is reported to stimulate traffic of the Kv4 channel to the plasma membrane (Morohashi *et al.*, 2002), by contrast KChIP4.4 does not stimulate traffic and leads to almost complete abolition of the fast inactivation of Kv4 currents (Holmqvist *et al.*, 2002).

The argument for distinct functions would appear to be given weight by the fact that the knock-out mice that have been studied for KChIPs 2 and 3 have observable phenotypes indicating that the KChIPs can not compensate for one another (Kuo *et al.*, 2001; Cheng *et al.*, 2002; Lilliehook *et al.*, 2003). In addition, while the multiple

KChIP isoforms have a common EF-hand containing domain, they also possess distinct N-termini suggesting the possibility of distinct interactions and functions. Here I set out to investigate the four individual KChIPs and their involvement in two of their most studied functional roles, namely the regulation of the traffic of Kv K^+ channels and their interaction with the presenilins. I investigated the intrinsic targeting of GFP-variant tagged isoforms of human KChIPs1-4, when expressed alone or together with GFP-variant tagged constructs of human Kv1.4, human Kv1.5, human Kv4.2 or human presenilin-1, and these observations provided some answers as to whether or not the KChIPs share these functions. Furthermore, I also investigated another NCS protein, NCS-1, which has been suggested to have similar effects to KChIPs with respect to certain functions, and is also known to carry out multiple distinct cellular roles.

3.2 Results

3.2.1 The KChIPs localise to different regions of the cell

In order to study the multiple functions of the KChIPs, I first set out to investigate their individual cellular localisations by using GFP-variant tagged KChIP constructs expressed in COS-7 cells and subsequently visualising them under the confocal microscope. This initially involved tagging the C-terminus of KChIPs 2-4 with ECFP and using an existing, similarly tagged KChIP1 construct (Figure 10), then confirming that these proteins were expressed by the cells, once transfected, by Western Blotting analysis (Figure 11). Bands for the fusion proteins were detected at the expected size of ~52kDa for KChIP1-ECFP and ~57kDa for the other ECFP tagged KChIPs, using both an anti-GFP antibody and an anti-pan KChIP antibody. The KChIPs were tagged at the C-terminus so as to minimise potential interference with post-translational modifications that might be important for their intrinsic targeting and localisation. KChIP1.2 is myristoylated, whilst KChIPs 2.1, 2.2, 2.3, 3.1 and 4.1 all possess potential palmitoylation sites (Burgoyne, 2007), with these post-translational modifications thought to be relevant for their intracellular targeting and some functional activity. Palmitoylation of KChIPs 2.3 and 3.1 is necessary for the efficient trafficking of Kv4 channels for example (Takimoto *et al.*, 2002).

At this point the diverse nature of the KChIP class should be noted. Analysis in human tissue has indicated that at least 16 KChIP isoforms are detectably expressed, with the major difference between these splice variants lying at the N-terminus. As current literature on these splice variants has given different variants multiple names (Decher *et al.*, 2004; Jerng *et al.*, 2004), I will use the terminology shown in figure 12 (Burgoyne, 2007), with the work carried out in this study using KChIPs 1.2, 2.3, 3.1 and 4.1. When I refer to KChIPs1-4, this corresponds to the aforementioned splice variants.

COS-7 cells were used in contrast to previous localisation studies in this laboratory using HeLa cells, as KChIP3 has been shown to induce significant levels of apoptosis when overexpressed in HeLa cells (Jo *et al.*, 2001). Initial experiments confirmed this finding (data not shown). COS-7 cells have been used by several other groups studying KChIP3, and are a widely used research tool as they grow adherently to both glass and plastic in a monolayer, enabling their ease of use for imaging experiments. COS-7 cells were developed from an African green monkey kidney fibroblast cell line, and exhibit typical fibroblast morphology. When expressed in COS-7 cells, the four KChIPs show different localisation patterns which may be relevant to their functional roles, particularly that of stimulating traffic of Kv4 channels to the plasma membrane (figure 13). KChIP1.2 was present on vesicular structures, with this localisation previously having been shown to be dependent upon its myristoylation (O'Callaghan *et al.*, 2003a). It has been shown that this targeting to intracellular vesicles enables an increase of efficiency of

trafficking Kv4 channels to the plasma membrane (Hasdemir *et al.*, 2005). KChIP2.3 was expressed on the plasma membrane, due to its palmitoylation – previous work has shown that only palmitoylated KChIP2 associates with the plasma membrane (Shibata *et al.*, 2003; Burgoyne, 2007). It is therefore surprising that both KChIPs 3.1 and 4.1 differ from KChIP2.3 in their localisation. Both also possess a palmitoylation site, yet KChIPs 3.1 and 4.1 remained diffuse and present in the cytosol and nucleus when expressed alone.

To examine whether the findings made in COS-7 cells were specific to this cell type, the localisations of the various fusion proteins were also investigated in PC12 cells as well. PC12 cells are widely used as a neuronal and neuroendocrine cell model and are known to possess many proteins that are believed to be neuronal specific. PC12 cells are tumour cell derivatives of adrenal chromaffin cells, which are derived embryonically from the neural crest like sympathetic neurons. Although they have been shown to express endogenous NCS-1 (McFerran *et al.*, 1998), which is localised to the TGN and plasma membrane, it is not known whether PC12 cells express any of the KChIP splice variants endogenously. However, when transiently transfected, KChIP1.2 was targeted to punctate structures, KChIP2.3 was localised to the plasma membrane and KChIPs 3.1 and 4.1 were diffuse and cytosolic, similar to transiently transfected COS-7 cells (figure 14). Again, expression was confirmed by Western blot analysis and bands for the fusion proteins were detected at the expected size of ~52kDa for KChIP1-ECFP and ~57kDa for the other ECFP tagged KChIPs, using both an anti-GFP antibody and an anti-pan KChIP antibody (figure

11C). The pan-KChIP antibody did not appear to pick up any endogenous KChIPs in untransfected cells, though given its inefficiency in detecting KChIP3, I could not determine if PC12 cells express KChIP3 endogenously. It appears that the situation in transfected COS-7 cells appears to be representative of that in more physiological cell types, such as PC12 cells, where the fusion proteins localised in a similar way.

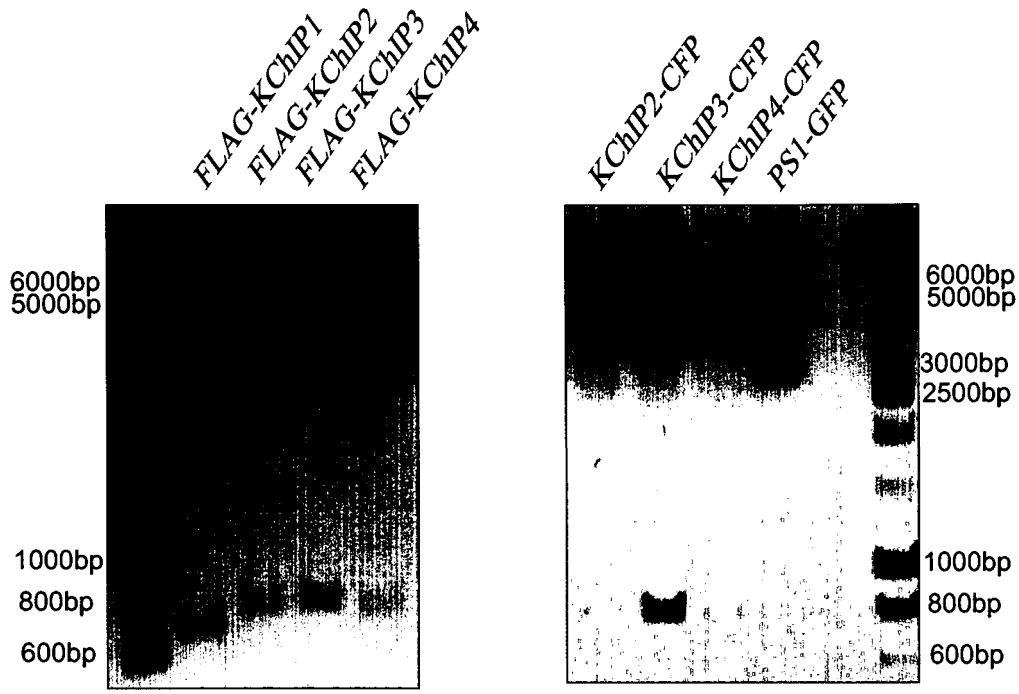

Figure 10. Agarose gels showing plasmids prepared for the project.
(A) FLAG-tagged KChIP constructs. In each case plasmids were digested with the restriction enzymes used to clone in the desired insert (n=3). (B) ECFP-tagged KChIP constructs. In each case plasmids were again digested with the appropriate restriction enzymes (n=3). EGFP-tagged presenilin-1 is also shown for comparison. In this case the presenilin insert contains an EGFP tag in the cytoplasmic loop giving a larger insert of 2.7kb.

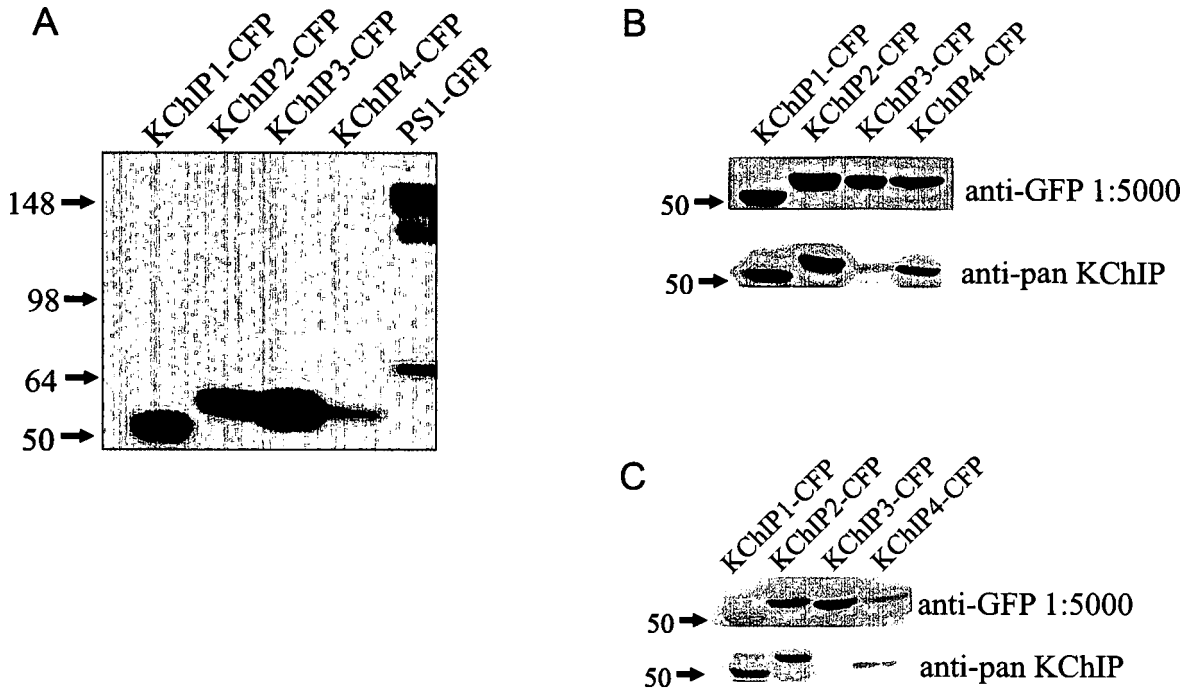

Figure 11. Western blots to show expression of fluorescent-tagged proteins in COS-7 and PC12 cells.
(A) Expression of ECFP-tagged KChIPs and EGFP-tagged presenilin-1 in COS-7 cells, detected by Western blotting using an anti-GFP antibody (1:5000). Bands for presenilin-1 are detected at both the expected molecular weight and at a higher weight, indicating an aggregation of the protein (n=2). (B) Detection of ECFP-tagged KChIPs using an anti-GFP antibody (1:5000) and an anti-pan-KChIP antibody (1:1000). The pan-KChIP works well for KChIPs 1, 2 and 4, but is not efficient at detecting KChIP3. The upper panel shows an anti-GFP blot for ECFP tagged versions of the proteins, the lower shows an anti-pan-KChIP blot from a gel run using identical amounts of the same cell lysates from COS-7 cells (n=3). (C) Detection of expression of ECFP-tagged KChIPs by Western blotting using anti-GFP and anti-pan-KChIP antibodies using cell lysates from transfected PC12 cells (n=3).

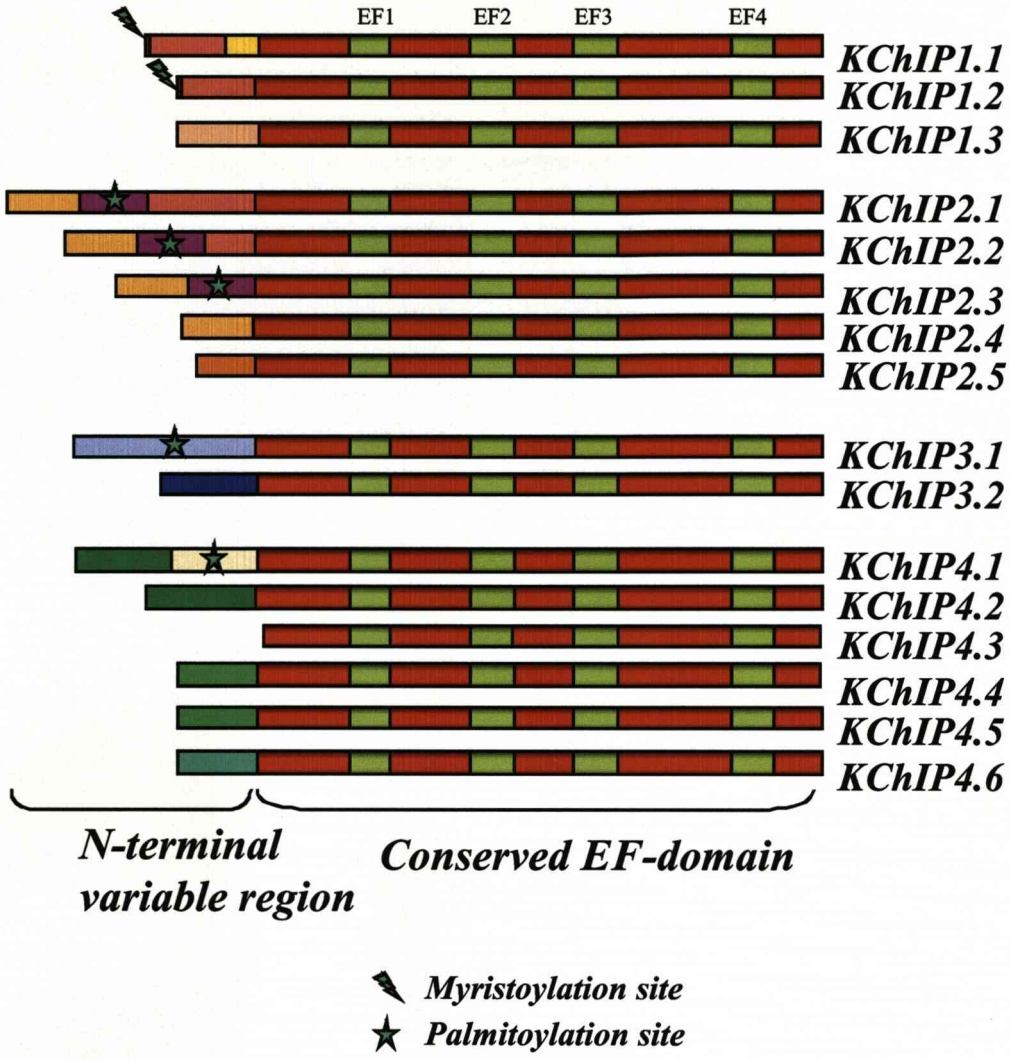

Figure 12. The KChIP class of NCS proteins and their splice variants (adapted from Burgoyne 2007).
The alternative splicing of the four human KChIP genes generates a series of splice variants with distinct amino (N)-terminal domains. Some of the isoforms possess myristoylation or palmitoylation sites (as indicated) that could confer membrane targeting and localisation. There is no generally agreed terminology for KChIP isoforms, and those shown are based on =the following sequences: human KChIP1 isoforms: GenBank accession number DQ148478 (1.1), DQ148477 (1.2), DQ148476 (1.3). Human KChIP2isoforms: NM_01491 (2.1), DQ148480 (2.2), DQ148481 (2.3), DQ148482 (2.4), DQ148483 (2.5). Human KChIP3 isoforms DQ148485 (3.1), DQ148486 (3.2). Human KChIP4 isoforms:DQ148487 (4.1), DQ148488 (4.2), DQ148491 (4.3), DQ148489 (4.4), DQ148490 (4.5), DQ148492 (4.6).

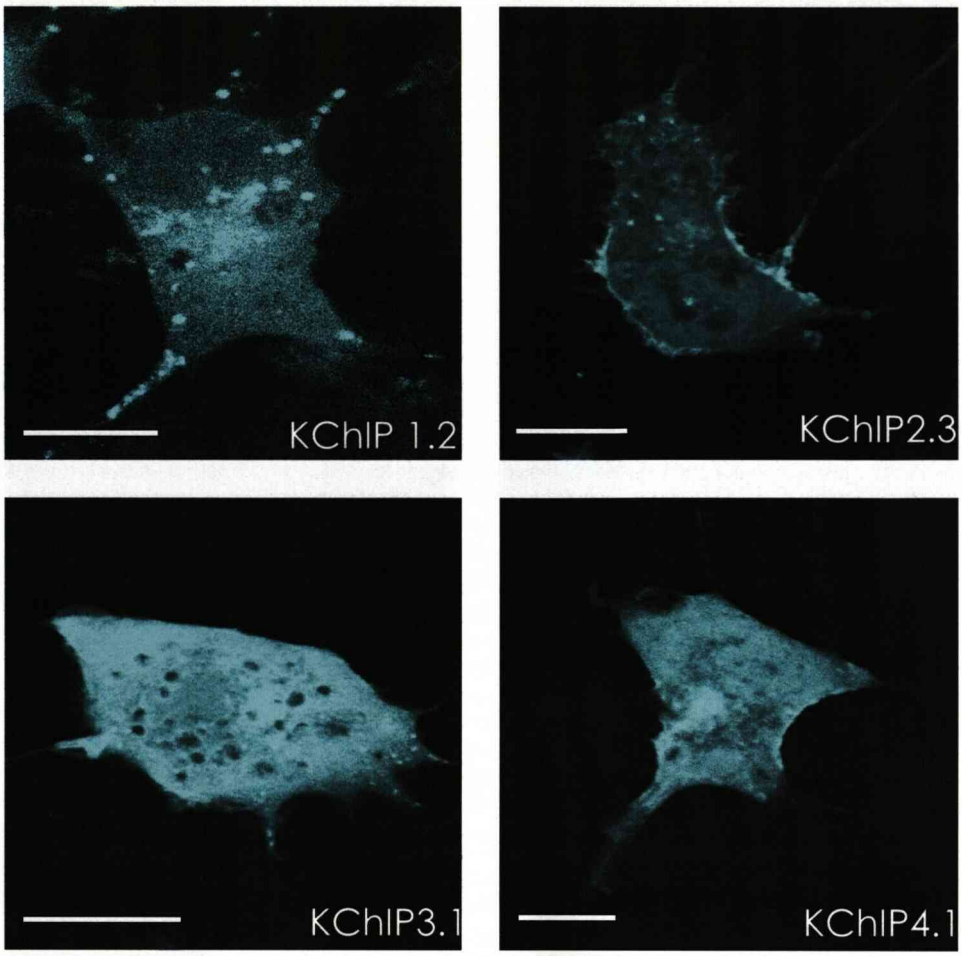

Figure 13. Differential localisation of four KChIPs when expressed in COS-7 cells.
COS-7 cells were transfected to express ECFP-tagged KChIPs from each of the four KChIP genes and then cells were imaged live on the confocal microscope 2 days post-transfection. KChIP1.2 localises to distinct punctate vesicles within the cell, whilst KChIP2.3 is found predominantly on the plasma membrane. KChIPs 3.1 and 4.1 appear to be mainly cytosolic, though were also present in the nucleus. In each case the scale bar represents 10μm.

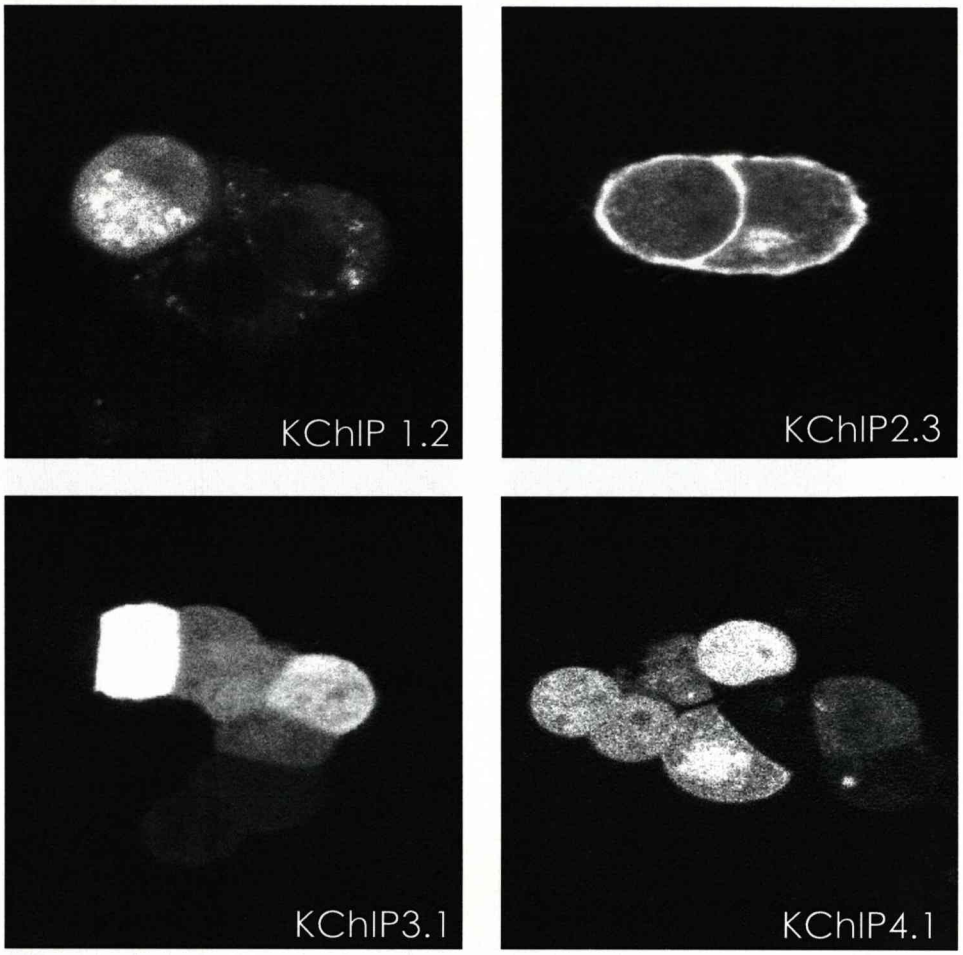

Figure 14. Differential localisation of four KChIPs when expressed in PC12 cells.
PC12 cells were transfected to express ECFP-tagged KChIPs from each of the four KChIP genes and then cells were imaged live on the confocal microscope 2 days post-transfection. In a similar manner to COS-7 cells, KChIP1.2 localises to distinct punctate vesicles within the cell, whilst KChIP2.3 is found predominantly on the plasma membrane. KChIPs 3.1 and 4.1 appear to be mainly cytosolic, though were also present in the nucleus. Images are representative of 82% (23/28) of KChIP1.2 transfected cells, 89% (17/19) KChIP2.3 transfected cells, 96% (26/27) of KChIP3.1 transfected cells and 92% (23/25) of KChIP4.1 transfected cells.

3.2.2 The KChIPs do not possess a Ca^{2+}/myristoyl switch mechanism

The first of the NCS proteins to be characterised at a structural level, recoverin, possesses a reversible Ca^{2+}/myristoyl switch mechanism, where Ca^{2+} -binding leads to exposure of the myristoyl group and subsequent membrane association (Ames *et al.*, 1997). This Ca^{2+}/myristoyl switch is present in other NCS proteins, but not all. For example, hippocalcin, neurocalcin δ, VILIP-1 and VILIP-3 have all been shown to possess a Ca^{2+}/myristoyl switch and will translocate to the plasma membrane and TGN upon an increase in cytosolic Ca^{2+} concentration (Spilker *et al.*, 2002a; O'Callaghan *et al.*, 2003b; Spilker & Braunewell, 2003). By contrast, NCS-1 has its myristoyl group constitutively exposed, even in the absence of Ca^{2+}, meaning that it is already associated with the plasma membrane and the TGN at resting or lowered Ca^{2+} levels (Ames *et al.*, 2000; Bourne *et al.*, 2001; Burgoyne *et al.*, 2004; O'Callaghan & Burgoyne, 2004).

Here I confirm previous work from this laboratory demonstrating that KChIP1, despite being myristoylated, does not possess a Ca^{2+}/myristoyl switch mechanism but is constitutively membrane associated (O'Callaghan & Burgoyne, 2003; O'Callaghan *et al.*, 2003a), and confirm that KChIPs2, 3 and 4, whilst not myristoylated, do not possess a switch mechanism or display any translocation to specific cellular compartments upon cytosolic Ca^{2+} elevation.

In order to check for Ca^{2+} -dependent changes in localisation, COS-7 cells were co-transfected to express a KChIP-ECFP construct together with a positive control, hippocalcin-EYFP, as this is known to show a Ca^{2+}/myristoyl switch mechanism. When treated with the Ca^{2+} ionophore ionomycin, there was no change in the distribution of KChIP1-ECFP over time following Ca^{2+} elevation in living COS-7 cells. By contrast, the co-transfected hippocalcin-EYFP translocated from the cytosol to membranes most likely the plasma membrane and the Golgi complex as previously reported (figure 15). Similarly, KChIPs2, 3 and 4 remained in their particular cellular localisations upon Ca^{2+} elevation – KChIP2 was located to the plasma membrane whilst KChIPs 3 and 4 remained cytosolic. Again, the co-transfected hippocalcin-EYFP translocated as expected from the cytosol to the plasma membrane and the Golgi complex in response to Ca^{2+} elevation (figures 16-18).

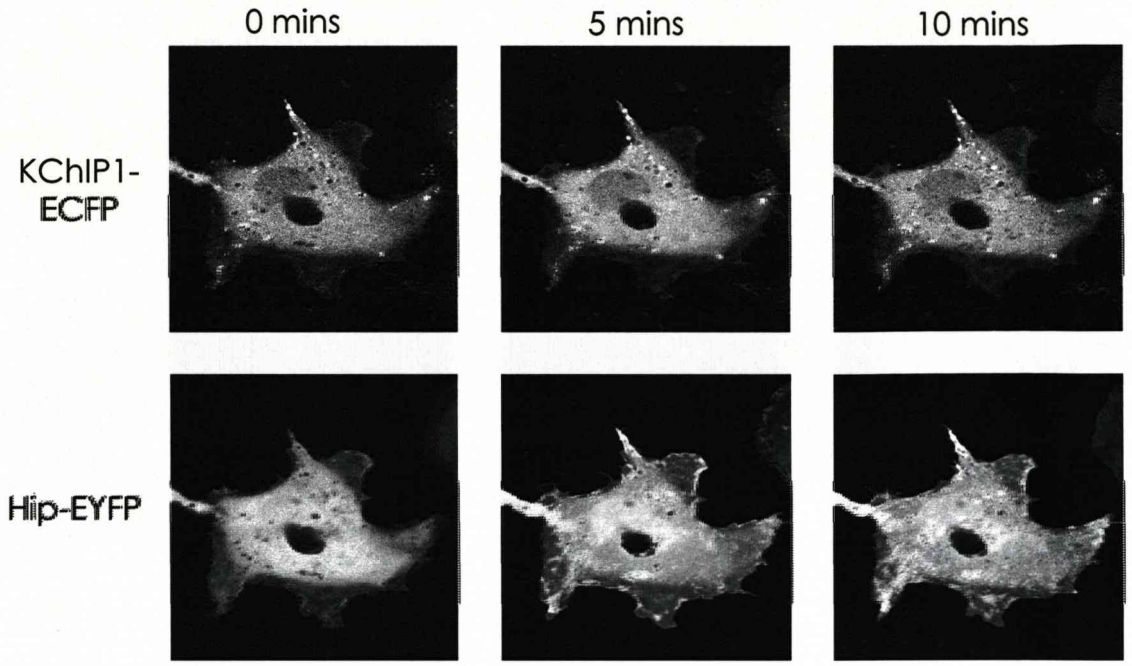

Figure 15. Localisation of KChIP1 and hippocalcin and the effects of Ca^{2+} elevation with the Ca^{2+} ionophore ionomycin.
COS-7 cells were transfected to co-express hippocalcin-EYFP together with KChIP1-ECFP. Cells were then imaged live via confocal microscopy 2 days post-transfection. Hippocalcin is cytosolic under resting conditions (0 min), but following treatment with 5μM ionomycin, the subsequent Ca^{2+} elevation causes translocation due to its Ca^{2+}/myristoyl switch. By contrast, the KChIP1 does not display a similar translocation following Ca^{2+} elevation. KChIP1 has its myristoyl group exposed regardless of Ca^{2+} binding status (i.e. no switch) and is found associated on vesicles within the cell.

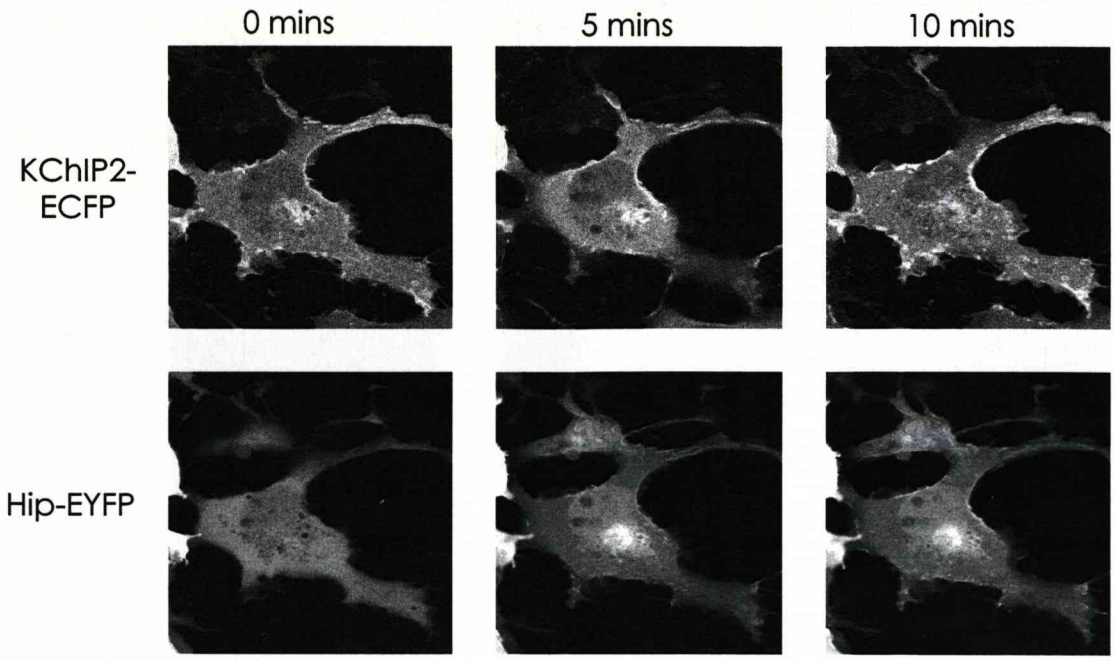

Figure 16. Localisation of KChIP2 and hippocalcin and the effects of Ca^{2+} elevation with the Ca^{2+} ionophore ionomycin.
COS-7 cells were transfected to co-express hippocalcin-EYFP together with KChIP2-ECFP. Cells were then imaged live via confocal microscopy 2 days post-transfection. Hippocalcin is cytosolic under resting conditions (0 min), but following treatment with 5μM ionomycin, the subsequent Ca^{2+} elevation causes translocation due to its Ca^{2+}/myristoyl switch (lower traces). KChIP2 however remains constitutively associated to the plasma membrane (upper traces).

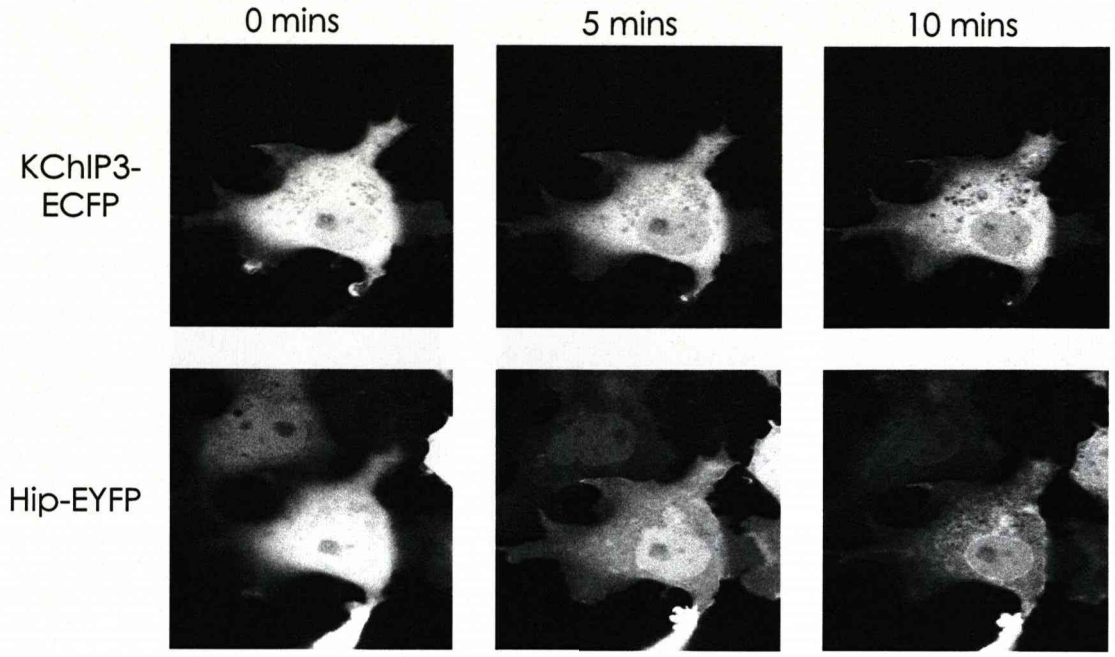

Figure 17. Localisation of KChIP3 and hippocalcin and the effects of Ca^{2+} elevation with the Ca^{2+} ionophore ionomycin.
COS-7 cells were transfected to co-express hippocalcin-EYFP together with KChIP3-ECFP. Cells were then imaged live via confocal microscopy 2 days post-transfection. Hippocalcin is cytosolic under resting conditions (0 min), but following treatment with 5μM ionomycin, the subsequent Ca^{2+} elevation causes translocation due to its Ca^{2+}/myristoyl switch. By contrast, the KChIP3 does not display a similar translocation following Ca^{2+} elevation and remains cytosolic (upper traces).

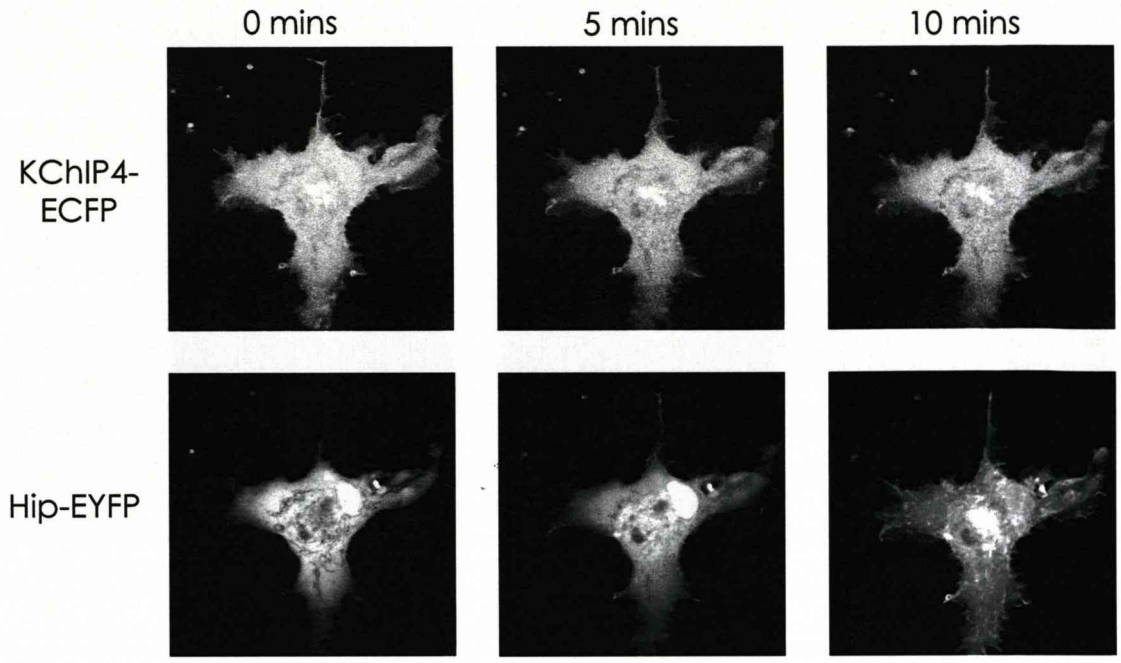

Figure 18. Localisation of KChIP4 and hippocalcin and the effects of Ca^{2+} elevation with the Ca^{2+} ionophore ionomycin.
COS-7 cells were transfected to co-express hippocalcin-EYFP together with KChIP4-ECFP. Cells were then imaged live via confocal microscopy 2 days post-transfection. Hippocalcin is cytosolic under resting conditions (0 min), but following treatment with 5μM ionomycin, the subsequent Ca^{2+} elevation causes translocation due to its Ca^{2+}/myristoyl switch (lower traces). KChIP4 however remains cytosolic (upper traces).

3.2.3 The KChIPs and their interaction with the Kv channels

3.2.3.1 The Kv channels localise to different regions of the cell

Having shown the differential localisation of the four KChIPs, I then set out to investigate differences in their functions. Perhaps the most widely studied function of the KChIPs is their interaction with the Kv potassium channels, and specifically their effects on the stimulation or inhibition of the traffic of these channels to the plasma membrane. In order to investigate this further, I utilised three existing GFP-variant tagged channels of the Kv family (Kv1.4, Kv1.5 and Kv4.2) that had previously been studied with one or more of the KChIPs (O'Callaghan *et al.*, 2003a; Hasdemir *et al.*, 2005; Li *et al.*, 2005). Interestingly, each of the three channels showed distinct intracellular localisation when expressed alone (figure 19).

When expressed in the absence of any of the KChIPs in COS-7 cells and visualised under the confocal microscope, the three channels all exhibited differing localisation. Kv1.4-EGFP was very clearly plasma membrane associated, whilst ECFP-Kv4.2 was found to localise to the perinuclear region of the cell (figure 19). Both of these findings were as expected on the basis of previous studies (O'Callaghan *et al.*, 2003a; Hasdemir *et al.*, 2005; Li *et al.*, 2005). Kv1.5-EGFP was found to display a reticular staining pattern, with little or no expression of GFP present on the cell surface. This is in contrast to the findings of H.Li *et al* (2005) who showed that when expressed in HEK-293 cells, whilst the majority of the

channel appeared to remain in the ER, there was some expression of GFP evident at the cell surface, which in some cases appeared to be aggregated. This may be a cell-type specific phenomenon, but it is important in the context of some of the experiments to follow, as KChIP2 was suggested to prevent the forward traffic of Kv1.5-encoded channels, resulting in Kv1.5 retention in the ER (Li *et al.*, 2005). However, in the case of COS-7 cells at least, the vast majority of the channel would already seem to be retained.

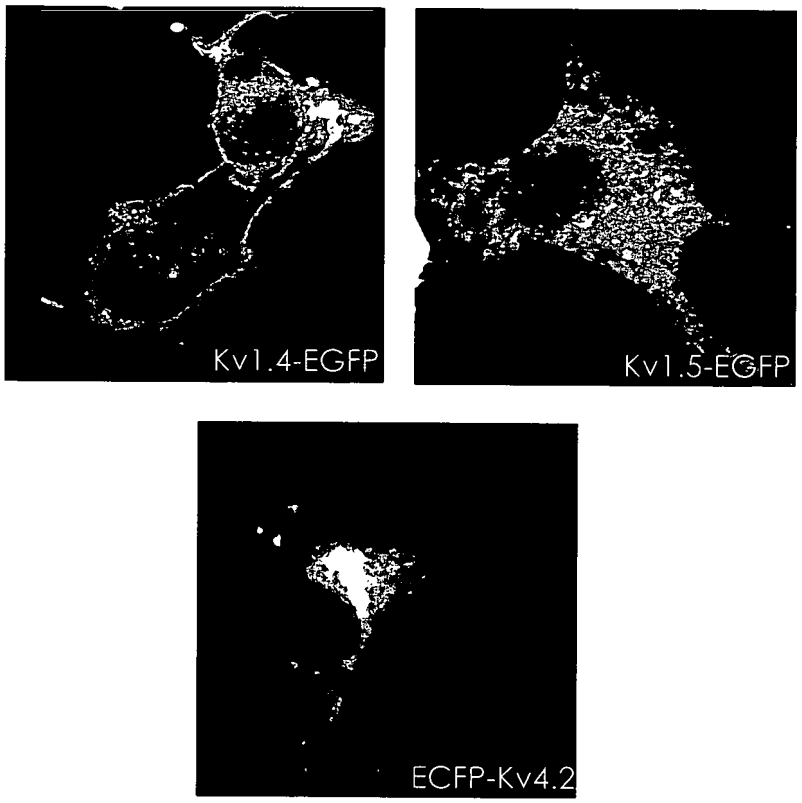

Figure 19. Localisation of Kv1.4-EGFP, Kv1.5-EGFP and ECFP-Kv4.2.
COS-7 cells were transfected with either Kv1.4-EGFP, Kv1.5-EGFP or ECFP-Kv4.2 and imaged live on the confocal microscope 48 hours post-transfection. Whilst Kv1.4-EGFP is found to be predominantly membrane associated on the plasma membrane, Kv1.5-EGFP displays a reticular localisation, with very occasional patches of plasma membrane expression. ECFP-Kv4.2 localises to the perinuclear region, previously identified as the Golgi apparatus.

3.2.3.2 ECFP and EGFP-labelled proteins can be co-expressed and are individually identifiable

Before co-expressing the KChIPs and the Kv channels, as all of the KChIPs were ECFP-tagged and two of the channels had were EGFP-tagged, it was important to confirm that such tagged proteins could be individually distinguished, as the excitation and emission wavelengths for ECFP and EGFP overlap to a certain extent (figure 20A, B).

To check that it was possible to co-express ECFP and EGFP-tagged proteins together, and identify each individually, I used two tagged proteins known to localise to different regions of the cell but not known to interact with each other, namely KChIP3-ECFP and another existing construct, a temperature sensitive mutant of the vesicular stomatitis virus G protein (ts045 VSVG-EGFP). For the purposes of this experiment, the temperature sensitive aspect was not important. Briefly however, at a restrictive temperature of 40°C, ts045 VSVG-EGFP has a thermo-reversible folding defect which retains it in the ER, whilst at a permissive temperature of 32°C, synchronous folding of the protein is enabled which allows the protein to exit from the ER and traffic to the Golgi apparatus and further to the plasma membrane (Bergmann, 1989; Hirschberg *et al.*, 1998). At 37°C, the temperature used for this experiment, ts045 VSVG-EGFP could be found localised to the Golgi apparatus and the plasma membrane, whilst KChIP3-ECFP was cytosolic (figure 13).

When the proteins were co-expressed in COS-7 cells, with each individual protein excited and light collected at appropriate wavelengths, both proteins were readily identifiable in their distinct cellular localisations (figure 20C). The fact that both proteins were distinctly localised showed that ECFP and EGFP-tagged proteins were suitable for their use in co-expression studies.

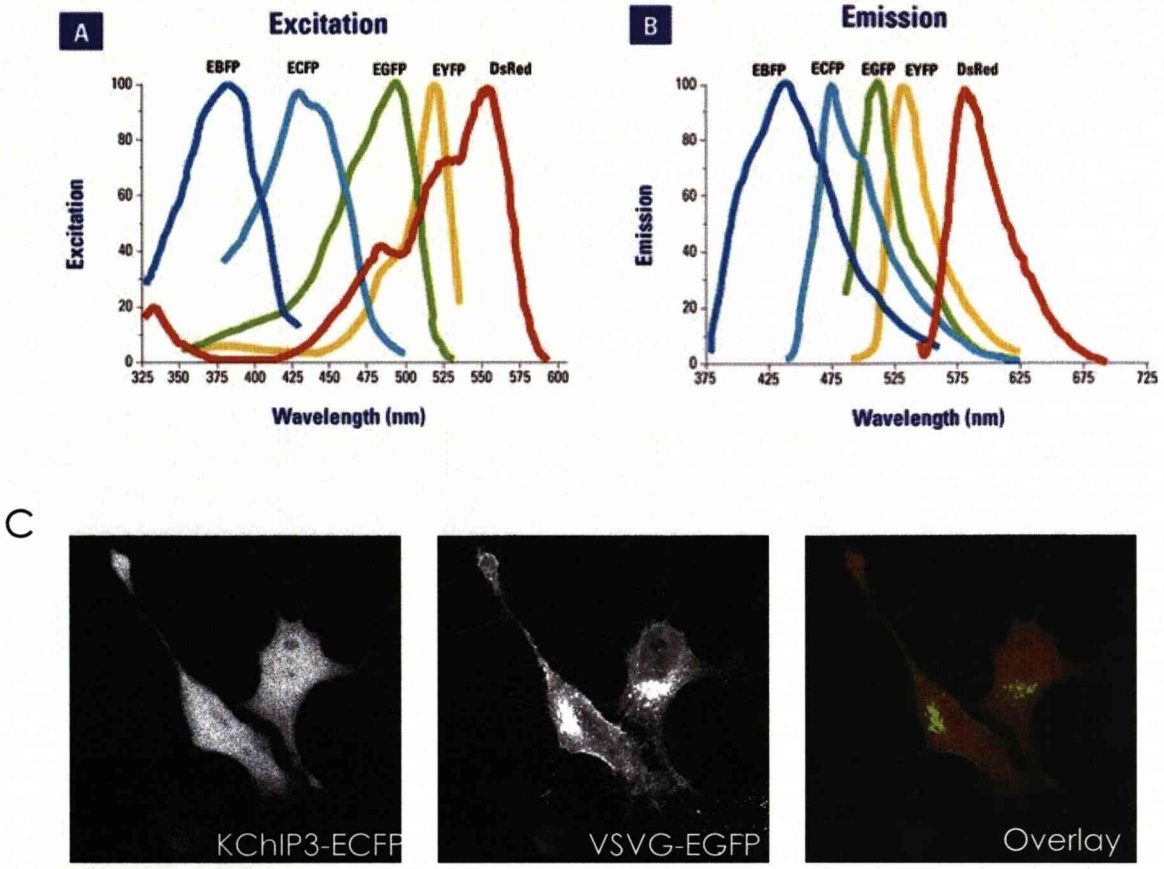

Figure 20. ECFP and EGFP labelled proteins can be co-expressed in cells and are individually identifiable.
ECFP and EGFP labelled constructs have excitation and emission spectra that share some degree of overlap **(A, B)**. In order to check that it is possible to co-transfect cells with ECFP and EGFP labelled proteins then identify each separately, COS-7 cells were co-transfected with KChIP3-ECFP, a mainly cytosolic protein, and VSVG-EGFP, a protein that is membrane associated to the plasma membrane and Golgi complex. Cells were fixed 48 hours post-transfection and imaged on the confocal microscope **(C)**. Distinct localisations of the two tagged proteins were readily resolved. The colour overlay shows KChIP3-ECFP in red and VSVG-EGFP in green.

3.2.3.3 KChIP2 inhibits the traffic of the Kv1.4 channel to the plasma membrane in COS-7 cells

Having demonstrated both the individual cellular localisations of the KChIPs and the Kv K^+ channels respectively, it was next examined whether co-expressing each different KChIP with each Kv K^+ channel would affect the localisation of either protein.

When expressed alone, Kv1.4-EGFP was found to be associated with the plasma membrane, with a small amount retained intracellularly (figure 19), and indeed, when co-expressed with the KChIP1-ECFP the channel can still be seen to have reached the plasma membrane with the KChIP retaining its individual cellular localisation (figure 21A). Similarly, when the channel was co-expressed with ECFP-tagged KChIPs 3 and 4, the GFP expression was clearly evident on the cell surface, suggesting that forward traffic of the channel to the plasma membrane had occurred, with both of the KChIPs retaining their cytosolic localisation. However, whilst forward traffic of the channel was still evident in these cases, it was also possible to observe some GFP-fluorescence in perinuclear regions of the cell, suggesting some retention of the channel (figure 21C, D).

When KChIP2-ECFP was co-transfected with Kv1.4-EGFP, it retained its association with the plasma membrane, as seen by the CFP fluorescence at the cell surface (figure 21B). By contrast, the channel itself was no longer seen to be

trafficked to the plasma membrane but was instead retained intracellularly, with hardly any GFP-fluorescence seen at the plasma membrane. The fluorescence seen exhibited a reticular pattern, suggesting Kv1.4 retention in the ER and in turn suggesting that co-expression of KChIP2 had prevented the forward trafficking of Kv1.4-encoded channels. Previous work by Li *et al* (2005) has shown that KChIP2 co-expression can reduce the cell surface expression of Kv1.5, and from these results it would appear that KChIP2 can play a similar inhibitory role in the forward traffic of Kv1.4.

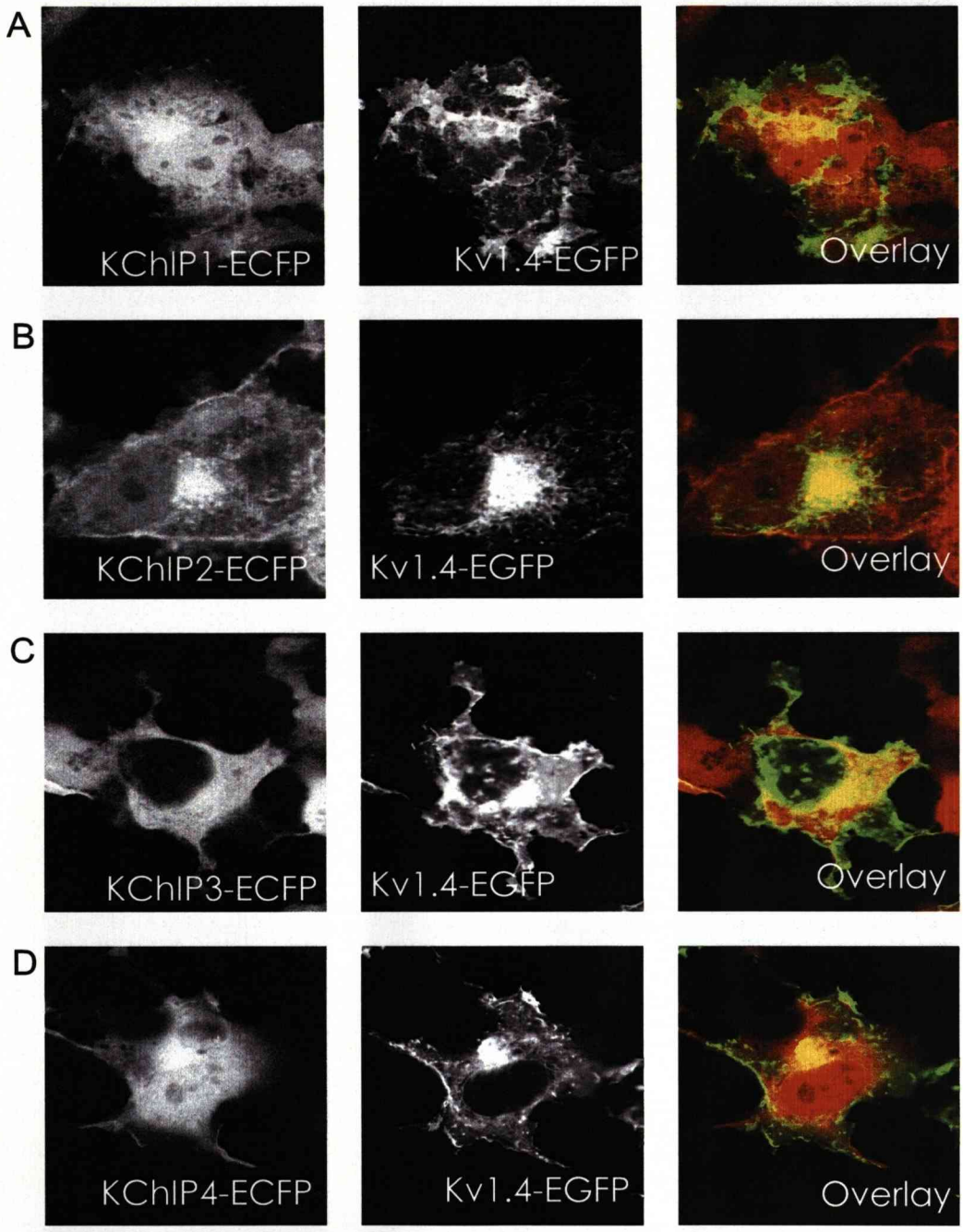

Figure 21. KChIP2 inhibits traffic of the Kv1.4 channel to the plasma membrane in COS-7 cells.
Cells were transfected to co-express KChIPs1-4 (as indicated **A-D**) together with Kv1.4-EGFP. In the case of KChIPs 1,3 and 4, the channel is still clearly localised to the plasma membrane **(A, C, D)**. However in the case of cells co-transfected with the channel and KChIP2-ECFP **(B)**, the traffic of the channel appears to be inhibited and the Kv1.4 remains trapped in the ER/Golgi region. The colour overlays show the KChIPs in red, Kv1.4 in green and areas of co-localisation in yellow.

3.2.3.4 The KChIPs do not appear to affect the traffic of the Kv1.5 channel in COS-7 cells

Previous studies have demonstrated that KChIP2 (and KChIP1) can modulate the functional cell surface expression of Kv1.5-encoded K^+ channels in transiently transfected HEK-293 cells, where both KChIP1 and 2 can reduce current densities and cell surface expression of Kv1.5. It is thought that this reduction in cell surface expression of Kv1.5 is due to the KChIPs inhibiting forward traffic of the channel from the endoplasmic reticulum (Li *et al.*, 2005).

In order to investigate this further, I co-expressed a GFP-variant tagged Kv1.5 (Kv1.5-EGFP) with each of the four CFP-tagged KChIPs in COS-7 cells and analysed changes in cellular localisation by utilising confocal microscopy. It is important to note at this point however, that in COS-7 cells at least, it was very difficult to visualise any Kv1.5-EGFP fluorescence at the plasma membrane (figure 19) with the vast majority of the protein retained intracellularly in the ER/Golgi region. Thus it was difficult to consider any inhibitory effects that the KChIPs may possess.

When KChIPs 1, 3 and 4 are co-expressed with Kv1.5-EGFP, neither the localisation of the KChIPs or the channel displayed any significant change, and the proteins did not appear to co-localise to any great extent (figure 22A, C, D). When co-expressed with KChIP2, the channel again maintained an intracellular

localisation, and although a larger proportion of KChIP2 appeared to be retained within the cell in the perinuclear region, it was still clearly visible on the plasma membrane and did not exhibit complete co-localisation with the Kv1.5 channel (figure 22B). Therefore it is difficult to conclude any effect on trafficking of the Kv1.5 channel, other than the KChIPs do not stimulate traffic of the channel to the plasma membrane. They may play an inhibitory role but this is difficult to conclude from my data, and it may be that effects seen are isoform or cell type specific.

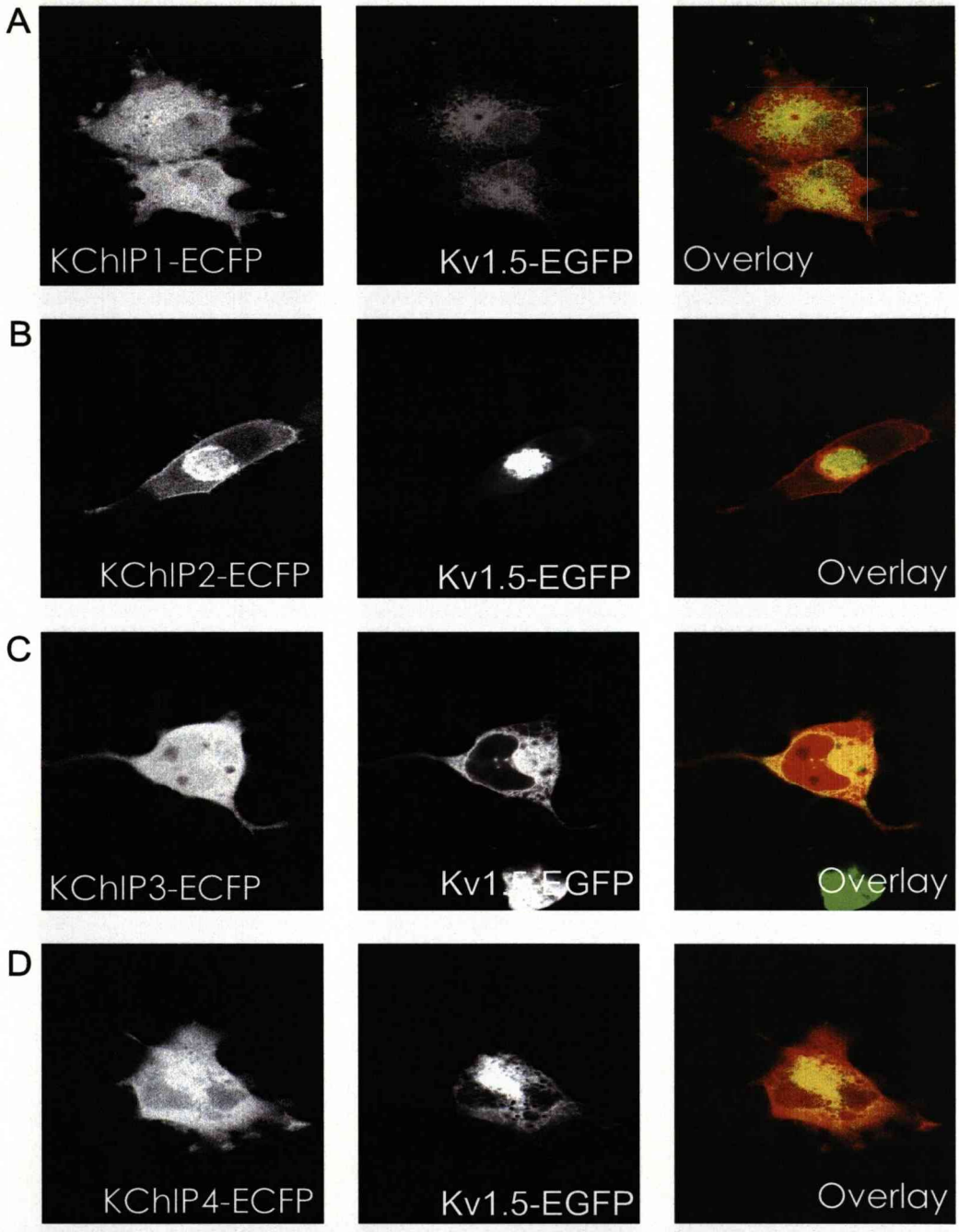

Figure 22. Co-expression of fluorescent KChIPs and Kv1.5-EGFP in COS-7 cells does not stimulate traffic of Kv1.5 to the plasma membrane.
COS-7 cells were transfected to co-express Kv1.5-EGFP and one of KChIPs 1**(A)**, 2**(B)**, 3**(C)** or 4**(D)** as indicated and imaged 48 hours post-transfection. In each case, the KChIP does not stimulate traffic of the channel to the plasma membrane, and the proteins do not co-localise to any extent, with the channel remaining in a perinuclear region of the cell and the KChIPs retaining their distinct cellular localisations. The colour overlay shows the KChIPs in red and the channel in green, with regions of overlap in yellow.

3.2.3.5 The KChIPs can stimulate traffic of the Kv4.2 channel to the plasma membrane

One of the most studied of the KChIP's functions is that of their interaction with the Kv4 channels. The first study of the effects of the KChIPs on Kv4 channels compared KChIPs 1.2, 2.3 and 3.1, which were all found to have the same effects (albeit with some quantitative differences): increasing cell surface expression of Kv4, shifting the voltage-dependency of activation, slowing inactivation and more rapidly recovering from inactivation (An *et al.*, 2000). Later studies on a KChIP4 isoform, KChIP4.1, showed similar effects in increasing traffic to the cell surface and slowing channel inactivation (Holmqvist *et al.*, 2002; Morohashi *et al.*, 2002).

I set out to confirm that these KChIP isoforms all promote traffic of Kv4.2 channels to the plasma membrane, again by using GFP-variant tagged KChIP constructs and a pcDNA-Kv4.2 construct. Here, the mention of KChIP isoforms is particularly relevant as some isoforms of KChIPs 2 and 4 have been shown to have differing effects – one KChIP2 isoform actually inhibits Kv4 surface expression (Patel *et al.*, 2002; Decher *et al.*, 2004), whilst the KChIP4.4 isoform does not stimulate traffic of the Kv4 channels and leads to the almost complete abolition of the fast inactivation of Kv4 channels (Holmqvist *et al.*, 2002; Morohashi *et al.*, 2002).

When expressed alone in cells, Kv4.2 can be found to be concentrated within a perinuclear region of the cell, previously identified as the Golgi (O'Callaghan *et al.*,

2003a) (figure 19). However, when the Kv4.2 channel was co-expressed in COS-7 cells with each of the KChIPs, with subsequent immunostaining against Kv4.2, the distribution of both the KChIPs and the channel within the cell was altered (figure 23A-D). In every case, ECFP fluorescence was detected at the plasma membrane, demonstrating a change in localisation of the KChIPs. This fluorescence almost perfectly co-localised with the immunostaining against the Kv4.2, which was now found at the cell surface. These findings confirm that the four KChIP splice variants tested can all stimulate forward traffic of the Kv4.2 channel to the plasma membrane.

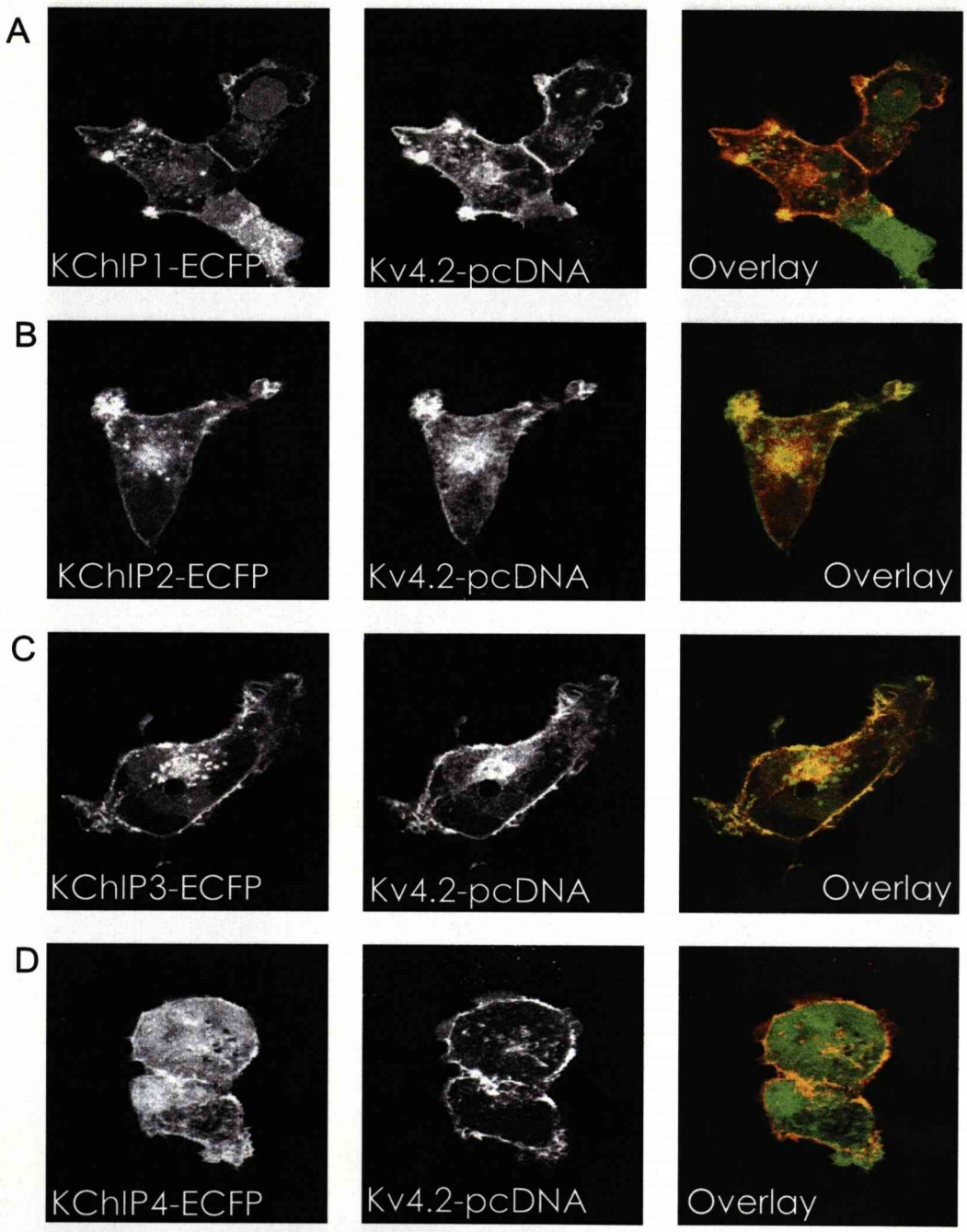

Figure 23. Co-expression of fluorescent KChIPs and Kv4.2-pcDNA in COS-7 cells stimulates traffic of Kv4.2 to the plasma membrane.
COS-7 cells were transfected to co-express Kv4.2 and one of KChIPs 1**(A)**, 2**(B)**, 3**(C)** or 4**(D)** as indicated and imaged 48 hours post-transfection. The cells were fixed and immunostained with anti-Kv4.2 antibody, using a Dylight secondary to detect the expressed channel. In each case, the KChIP clearly stimulates traffic of the channel to the plasma membrane, with the two proteins co-localising at this region of the cell. The colour overlays show the KChIPs in green and Kv4.2 in red, with co-localisation seen in yellow.

3.2.4 The KChIPs and their interaction with the presenilins

3.2.4.1 Presenilin-1-EGFP can be expressed in both COS-7 and PC12 cells and is localised to the endoplasmic reticulum (ER)

KChIP3 was first identified as a Ca^{2+} binding protein that binds to the last 103 amino acids of presenilins 1 and 2, and as such was originally named calsenilin (Buxbaum *et al.*, 1998). This generated significant interest as the presenilins and more specifically, mutations in the genes encoding them, are known to be involved in early onset familial Alzheimer's disease (FAD) (Tandon & Fraser, 2002). Subsequent work also indicated a similar interaction between the presenilins and KChIP4 (Morohashi *et al.*, 2002).

To examine whether this interaction is specific to particular KChIPs, I used the ECFP-tagged KChIPs in conjunction with an existing presenilin-1 construct (PS1-EGFP) that contained an EGFP tag in its cytoplasmic loop (Kaether *et al.*, 2002), and co-expressed these proteins within COS-7 cells to examine their intracellular localisations and potential interactions.

Firstly, it was important to confirm the intracellular localisation of PS1-EGFP when expressed alone in cells, as previous studies have provided contrasting results. Several groups described the presenilins as being predominantly located within early compartments such as the ER and the intermediate compartment (Annaert *et al.*,

1999; Cupers *et al.*, 2001), whilst others found them to be localised to post-Golgi compartments (Takashima *et al.*, 1996; Efthimiopoulos *et al.*, 1998; Ray *et al.*, 1999; Schwarzman *et al.*, 1999; Singh *et al.*, 2001). Furthermore, one study demonstrated the ability of the presenilins to reach the cell surface, though most of the protein is retained within the cell (Kaether *et al.*, 2002). Here I found that when PS1-EGFP is transiently transfected into both COS-7 and PC12 cells, it demonstrated a reticular localisation (figure 24A, B) with no obvious plasma membrane association visible.

To confirm expression, Western blot analysis was used in conjunction with an anti-PS1 antibody. These results were complicated by the fact that a high molecular weight aggregate of the protein can be detected (figure 24C). But, when cell lysates are left unboiled, a band corresponding to the full length protein together with the fused EGFP tag can be detected. Whether the high molecular weight band found represents an accumulation of proteolytic fragments, or indeed of the full length protein, is unknown. However, previous work has indicated that the insertion of the EGFP tag does not affect the physiological function of the protein (Kaether *et al.*, 2002).

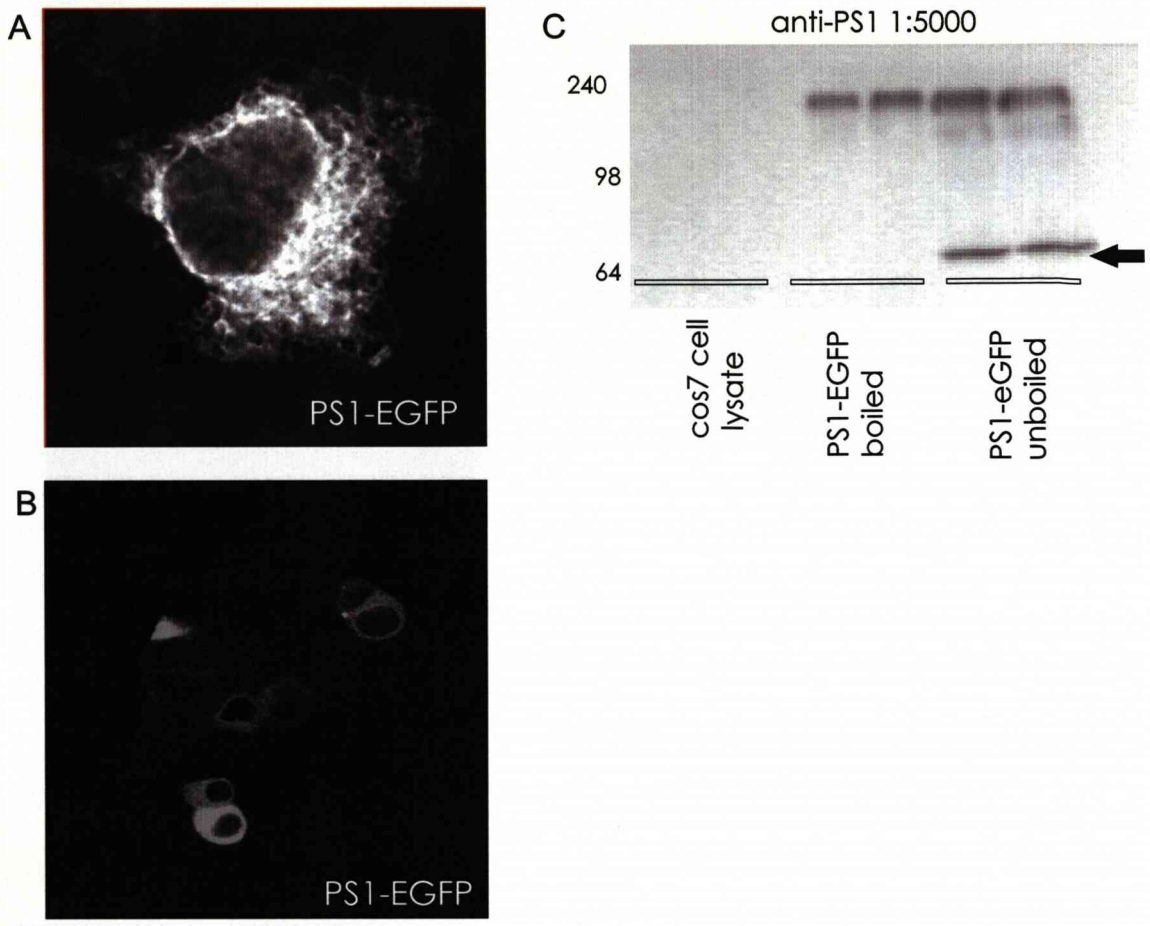

Figure 24. Expression of GFP-tagged presenilin-1 in COS-7 and PC12 cells. COS-7 cells were transfected with PS1-EGFP and imaged 48 hours post-transfection. Presenilin-1 displays a reticular localisation **(A)**. Similarly, in transfected PC12 cells, presenilin-1shows a reticular localisation **(B)**. When Western blotting for the protein, the protein forms a high molecular weight aggregate **(C)**. However, if cell lysates are left unboiled, a clear band corresponding to the correct molecular weight of the tagged protein can be seen.

3.2.4.2 KChIP3-ECFP co-localises with presenilin-1-EGFP when co-expressed in COS-7 cells

In this study, I investigated if the KChIPs could stimulate traffic of presenilin-1 to its site of action at the plasma membrane, or indeed whether they appear to interact with presenilin-1. It has previously been demonstrated that the KChIPs can act to stimulate traffic of the Kv4 channels from the Golgi complex to the plasma membrane (O'Callaghan *et al.*, 2003a) and here I studied the possibility that the KChIPs could perform a similar function with respect to the trafficking of presenilin-1.

KChIPs 2 and 4 did not appear to co-localise with presenilin-1, with each individual KChIP maintaining its own cellular localization (figure 25B and D). In the case of KChIP4 this was slightly unexpected as some work has suggested a role for KChIP4 in presenilin processing (Morohashi *et al.*, 2002). When co-expressed with KChIP1, whilst there was some apparent co-localisation with PS1-EGFP, the ECFP and EGFP detected did not form a perfect overlap (figure 25A). However, when KChIP3 was co-expressed with PS1-EGFP, a change in the localization of KChIP3 was observed, with a large proportion of the ECFP fluorescence detectable in the perinuclear region of the cell and overlapping somewhat with the EGFP fluorescence from the presenilin construct (figure 25C). This redistribution of KChIP3 to the perinuclear region is similar to the effect observed by Buxbaum et al (1998), who demonstrated a comparable effect when co-expressing KChIP3 with

presenilin-2. This data would suggest that KChIP3 can become associated with membranes in the presence of presenilin-1. Indeed, it was also possible to observe some small regions of co-expression at the plasma membrane. This data is consistent with an interaction between KChIP3 and the cytoplasmic domain of presenilin-1 that has previously been demonstrated by co-immunoprecipitation (Buxbaum *et al.*, 1998).

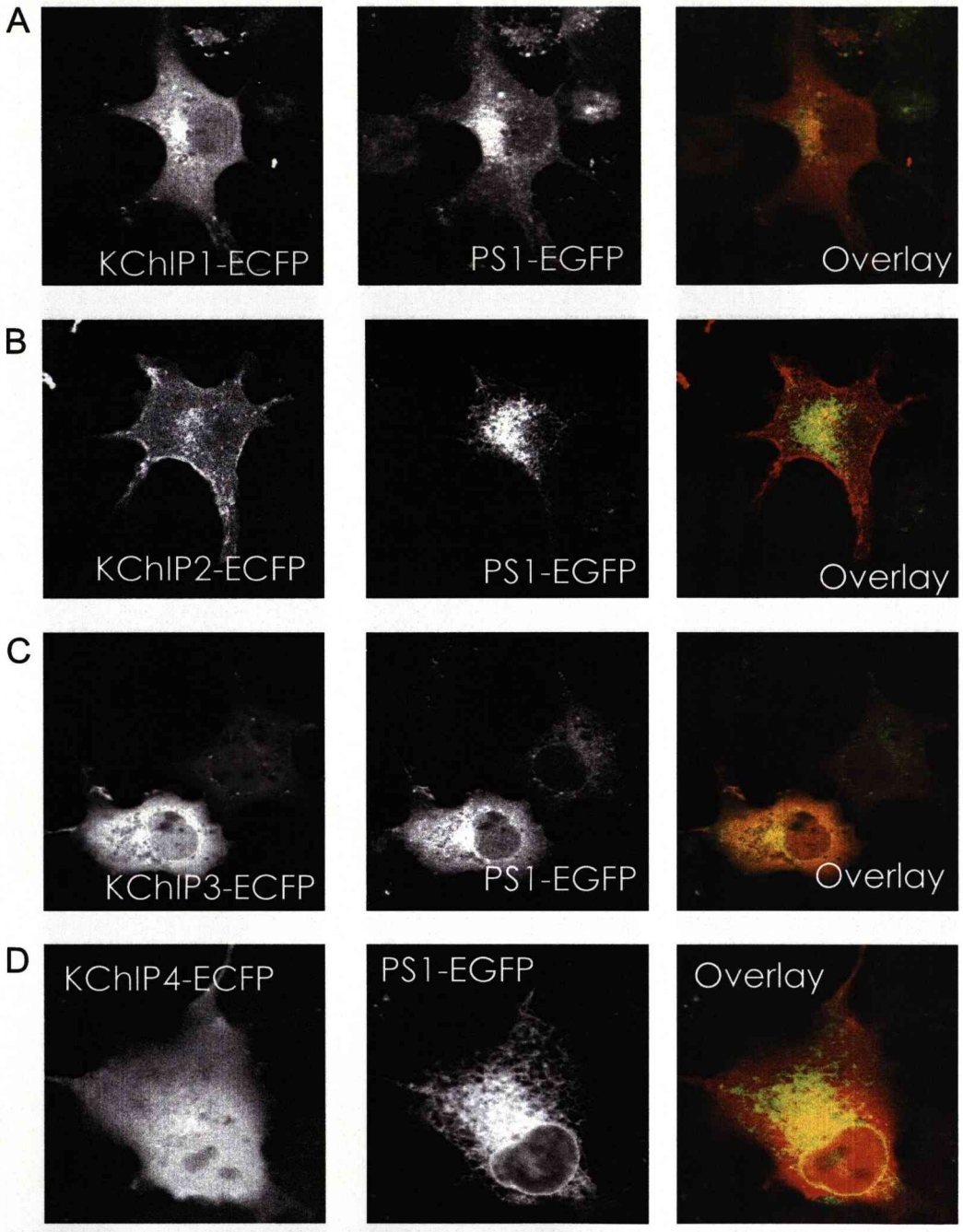

Figure 25. KChIP3 co-localises with presenilin-1 when co-expressed in COS-7 cells.

ECFP-tagged KChIPs 1-4 (**A-D** as indicated) were transfected along with PS1-EGFP into COS-7 cells. KChIPs 2 and 4 do not co-localise with the presenilin, with each protein retaining its distinct localisation **(B, D)**. In the case of KChIP1 there is some co-localisation with presenilin-1, but this is not a perfect overlap, and the localisation of the presenilin does not appear to change significantly **(A)**. Cells co-transfected with KChIP3-ECFP and PS1-EGFP exhibit a more significant change in localisation of the KChIP3-ECFP and it exhibits a more perinuclear localisation and partially co-localises with PS1-EGFP **(C)**. The colour overlays show PS1-EGFP in green, the KChIPs in red and areas of overlap in yellow.

3.2.4.3 KChIP3 does not appear to exhibit preferential binding for either presenilin-1 or Kv4.2

Having established that KChIP3 can apparently interact with both presenilin-1 and the Kv4.2 K^+ channel, I set out to try and establish if KChIP3 exhibited a preference for binding to either protein, and indeed whether co-expressing all 3 proteins would result in a change in their intracellular localisation. Again, in order to study this, COS-7 cells were transiently transfected with GFP-variant tagged KChIP3 and presenilin-1 constructs, together with Kv4.2, and the intracellular distribution of the three proteins then visualised using confocal microscopy.

A large proportion of the each of the expressed proteins remained trapped within the cell, with all three proteins appearing to co-localise in the perinuclear region of the cell. However, it was also observed that both KChIP3 and Kv4.2 were present on the plasma membrane of the cell, suggesting that the overexpression of presenilin-1 did not block traffic of the channel to the plasma membrane by KChIP3 (figure 26). Furthermore, small areas of EGFP fluorescence were visible on the cell surface, suggesting that presenilin-1 is also being trafficked to the plasma membrane, though the vast majority of the presenilin is retained within the cell.

These data would suggest that KChIP3 is able to interact independently with both Kv4.2 and presenilin-1, but does not exhibit preferential binding for either protein. Moreover, the presenilin does not prevent traffic of the channel to the plasma

membrane, nor does the channel appear to prevent presenilin-1 becoming membrane associated in the presence of KChIP3.

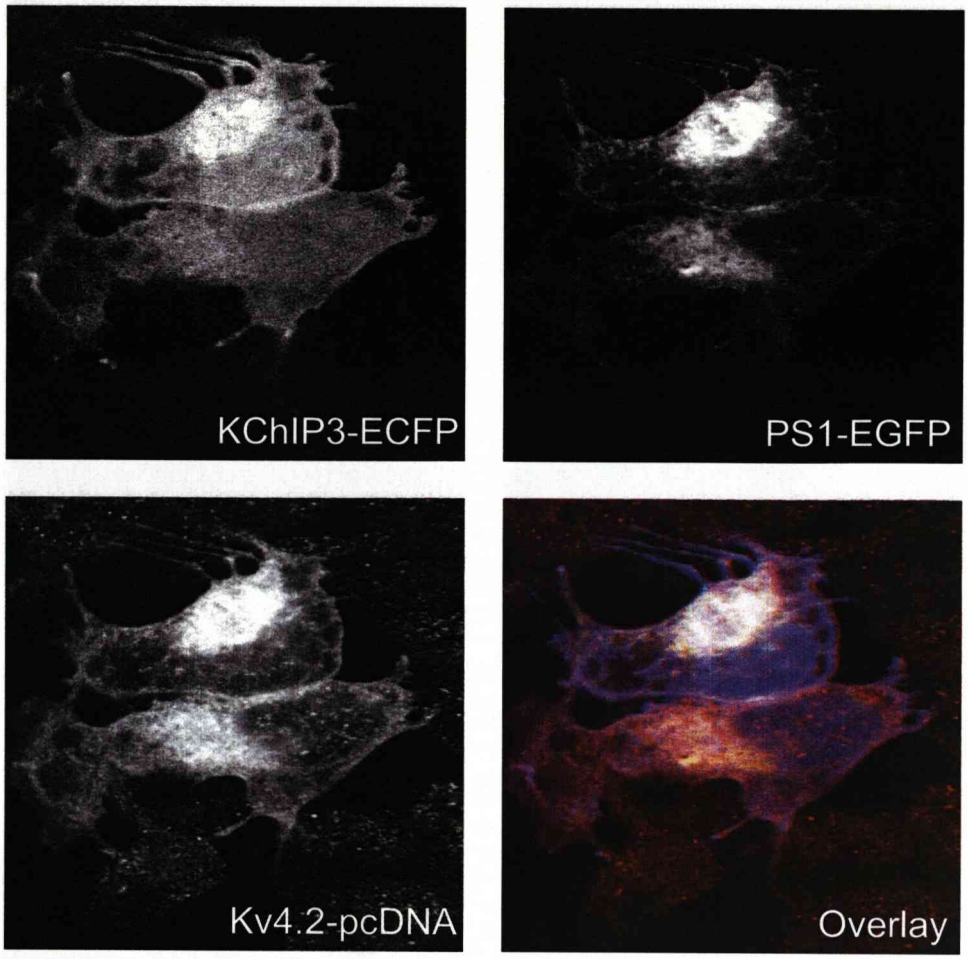

Figure 26. Triple transfection of COS-7 cells with KChIP3-ECFP, PS1-EGFP andKv4.2-pcDNA.
Cells were transfected with KChIP3-ECFP, PS1-EGFP and Kv4.2 then imaged 48 hours post-transfection. The cells were fixed and immunostained with anti-Kv4.2 antibody, using a Dylight secondary to detect the expressed channel. Whilst KChIP3 appears to co-localise with both presenilin-1 and Kv4.2, it does not appear to exhibit preferential binding to either protein and all three proteins appear to localise to the ER/Golgi region of the cell, with the channel also reaching the plasma membrane and PS1 not blocking its traffic. The colour overlay shows areas of overlap in white.

3.2.5 NCS-1 exhibits some properties similar to those of the KChIPs

The KChIPs are not the only NCS proteins that have been reported to modulate Kv4 channels. NCS-1 has also been reported to regulate Kv4 channels as well (Guo *et al.*, 2002; Nakamura *et al.*, 2003), although the discoveries made by previous investigations have been somewhat contradictory (Ren *et al.*, 2003; Zhang *et al.*, 2003). NCS-1 is known to possess multiple functions (Pongs *et al.*, 1993; McFerran *et al.*, 1998; Weiss *et al.*, 2000; Gomez *et al.*, 2001; Zhao *et al.*, 2001; Guo *et al.*, 2002; Kabbani *et al.*, 2002; Koizumi *et al.*, 2002; Tsujimoto *et al.*, 2002; Sippy *et al.*, 2003; Haynes *et al.*, 2005; Burgoyne, 2007), and is highly expressed not only in all brain regions but also in many non-neuronal cell types (McFerran *et al.*, 1998; Paterlini *et al.*, 2000; Gierke *et al.*, 2004). Here, I set out to investigate whether NCS-1 could indeed carry out some of the same functions as the KChIPs, again by utilising GFP-variant tagged constructs of NCS-1 (NCS-1-ECFP), the channels (Kv1.4-EGFP, Kv1.5-EGFP) and presenilin-1 (PS1-EGFP) as well as an untagged Kv4.2 construct (Kv4.2-pcDNA) and studying their co-expression in COS-7 cells using confocal microscopy.

To demonstrate correct expression of NCS-1-ECFP, the construct was transiently transfected into COS-7 cells and examined using both confocal microscopy and Western blot analysis (figure 27A, B). When expressed alone, NCS-1-ECFP can clearly be found associated to the Golgi and the plasma membrane as described previously (O'Callaghan *et al.*, 2002).

The results of the co-expression experiments were not clear-cut. As previously reported, when co-expressed with Kv4.2, NCS-1 appears to be able to stimulate traffic of the channel to the plasma membrane (figure 28A). However, this trafficking effect is not always evident in all cells examined (figure 28B). Similarly, when co-expressed with Kv1.4, a retention effect similar to that seen with KChIP2 was observed in some cells (figure 29A) with both NCS-1 and Kv1.4 apparently retained within the Golgi region of the cell, though this was not always the case, and in some other cells Kv1.4 clearly reached the plasma membrane with no apparent disruption in its traffic by NCS-1 (figure 29B). When NCS-1 was co-expressed with Kv1.5, in common with the KChIPs, NCS-1 did not demonstrate any affect on the traffic of the channel and the proteins did not appear to co-localise within the cell (figure 30).

Interestingly, when NCS-1 is co-expressed with presenilin-1, there is also the suggestion of an interaction taking place, but, again, the results are not clear cut. Some co-transfected cells exhibited both proteins in their expected individual cellular localisations, with NCS-1 found to be present on the Golgi and plasma membrane, and presenilin-1 in the ER (figure 31A). However, in other cells a change in localisation of presenilin-1 was observed, with presenilin-1 and NCS-1 now co-localised in a perinuclear compartment within the cell, and the suggestion of some traffic to the plasma membrane of the presenilin (figure 31B), an effect similar to that seen with KChIP3 (figure 25C).

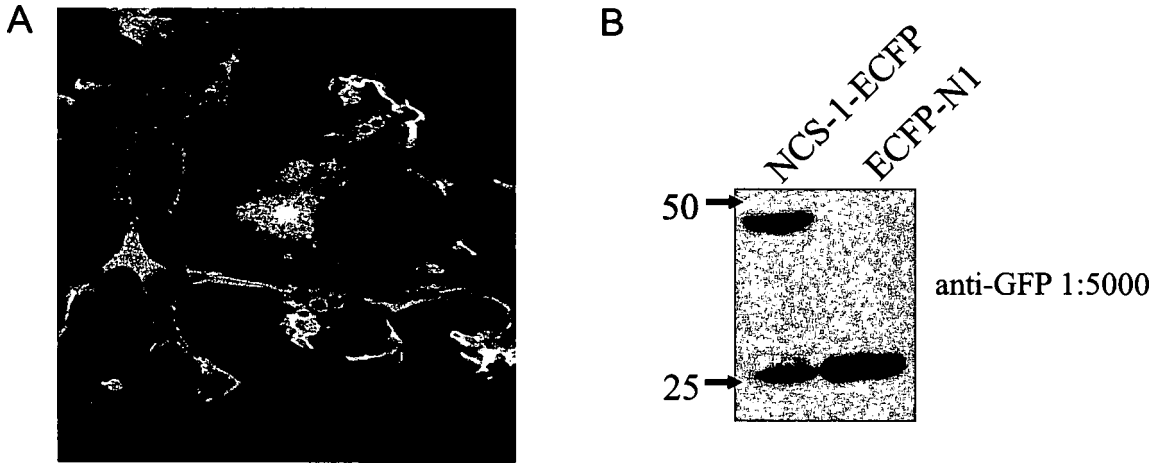

Figure 27. Localisation and expression of NCS-1-ECFP in COS-7 cells. COS-7 cells were transfected with ECFP-tagged NCS-1 and imaged 48 hours post-transfection **(A)**. NCS-1 is localised to the Golgi and plasma membrane. **(B)** Western blot to show NCS-1-ECFP expression in COS-7 cells. ECFP is shown as a comparison.

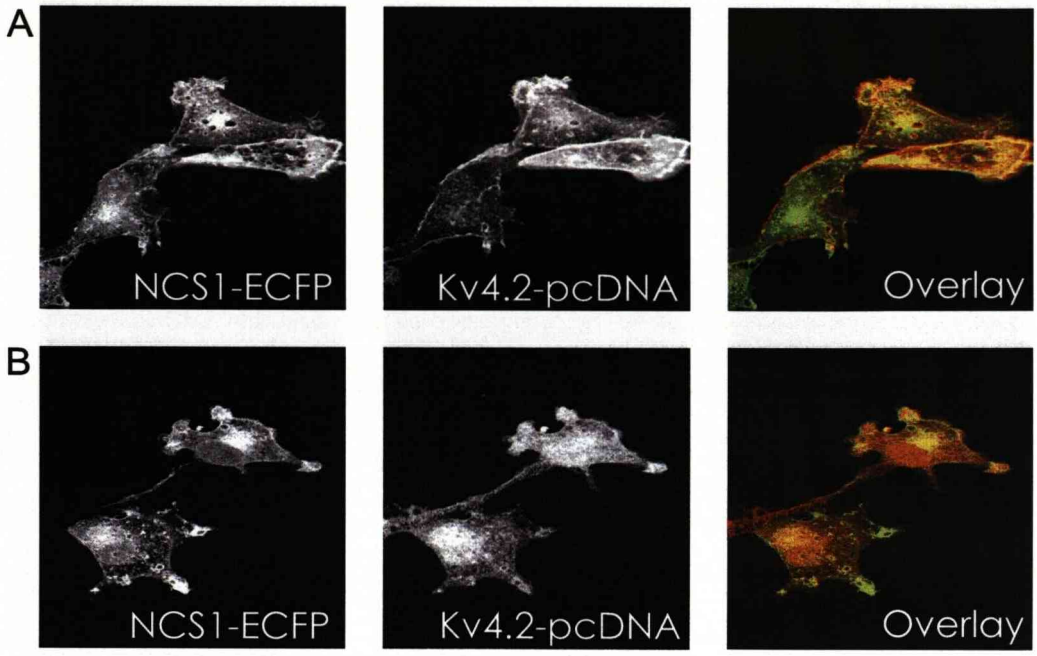

Figure 28. Effect of co-expression of NCS-1-ECFP and Kv4.2-pcDNA in COS-7 cells on their localisation.
COS-7 cells were co-transfected with NCS-1-ECFP and Kv4.2 before imaging 48 hours post-transfection. Cells were fixed and immunostained with anti-Kv4.2 antibody, using a Dylight secondary in order to detect the channel. Two examples are shown **(A and B)**. Co-expression of the proteins caused some association of Kv4.2 with the plasma membrane **(A)** although this trafficking effect is not always evident **(B)**. The colour overlays show NCS-1-ECFP in green and Kv4.2 in red, with overlap seen in yellow.

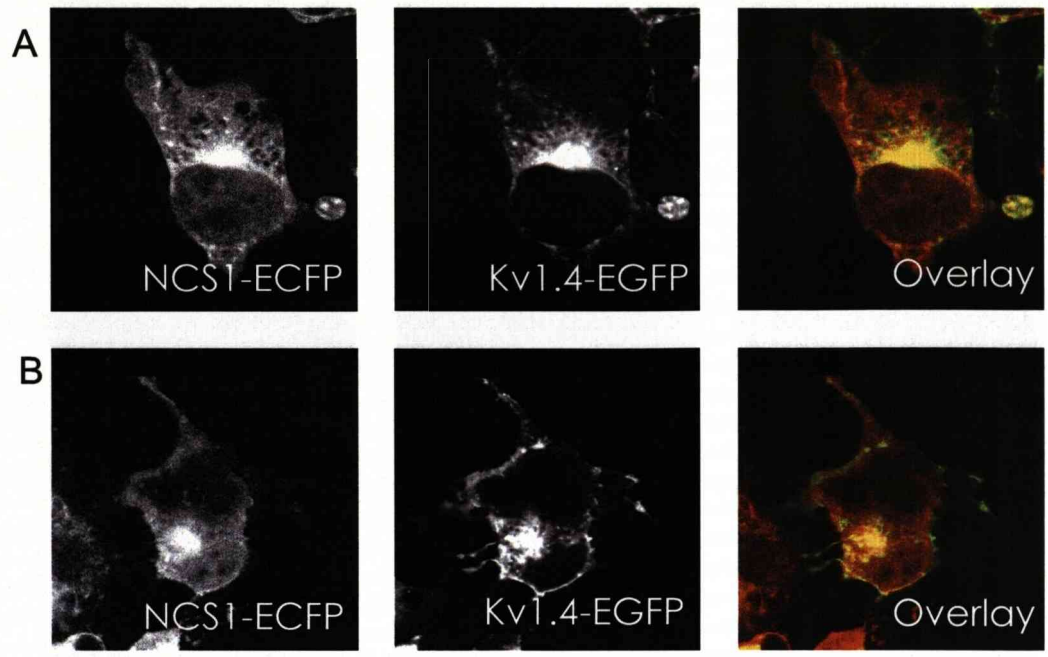

Figure 29. Effect of co-expression of NCS1-eCFP and Kv1.4-eGFP in COS-7 cells on their localisation.
COS-7 cells were co-transfected with NCS-1-ECFP and Kv1.4-EGFP. NCS-1 does not appear to have a clear effect on the localisation of Kv1.4. Two examples are shown **(A and B)**. In some cases, an effect can be seen, with the channel remaining trapped in the ER/Golgi region of the cell **(A)**. Conversely however, the channel can also be found to be clearly localised to the plasma membrane of some cells, suggesting no effect on its traffic due to NCS-1 **(B)**. The overlays show NCS-1 in red and Kv1.4 in green, with overlapping areas appearing yellow.

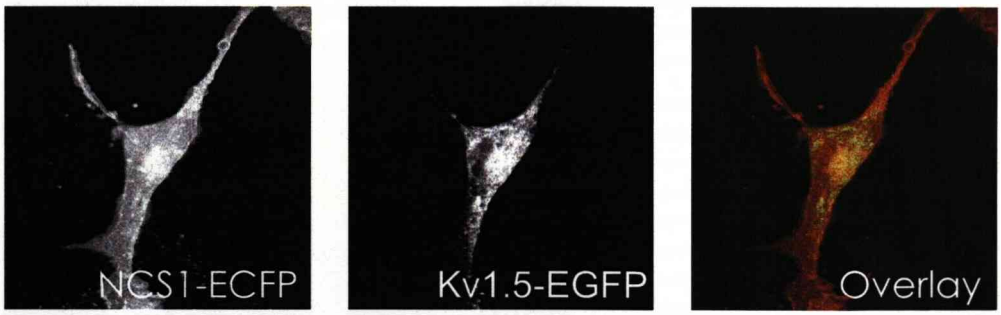

Figure 30. Co-expression of NCS-1-ECFP and Kv1.5-EGFP in COS-7 cells does not stimulate traffic of Kv1.5 to the plasma membrane.
COS-7 cells were transfected to co-express Kv1.5-EGFP and NCS-1-ECFP and imaged 48 hours post-transfection. NCS-1 does not stimulate traffic of the channel to the plasma membrane, with the channel remaining in the Golgi region of the cell and the NCS-1being localised to both the Golgi and the plasma membrane. The colour overlay shows the NCS-1-ECFP in red and the channel in green, with regions of overlap in yellow.

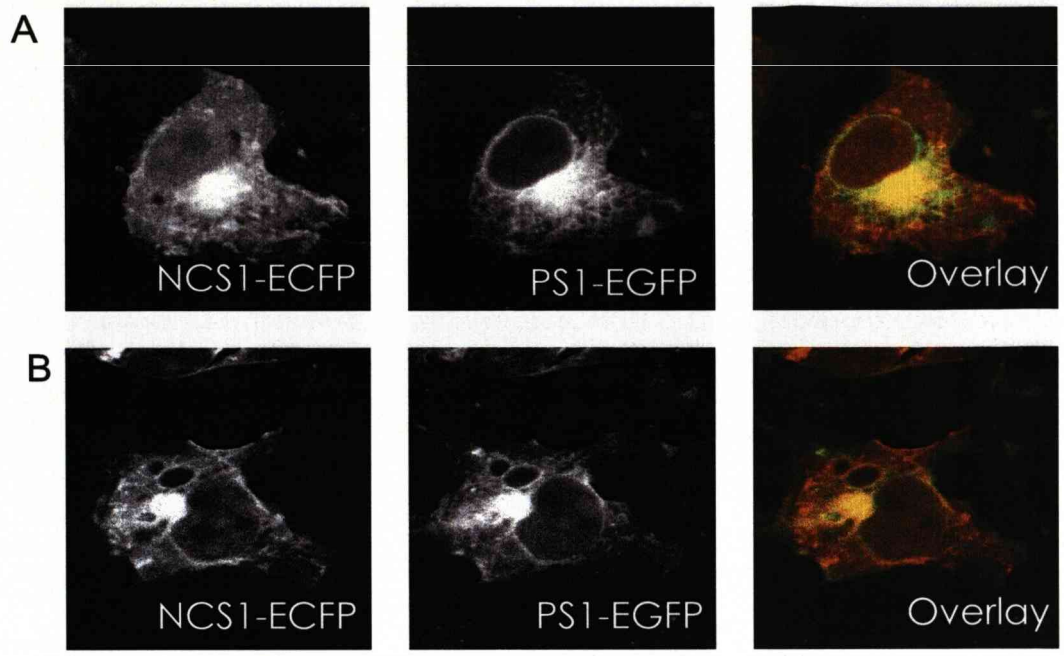

Figure 31. Effect of co-expression of NCS1-ECFP and PS1-EGFP in COS-7 cells on their localisation.
COS-7 cells were co-transfected to express both NCS-1-ECFP and PS1-EGFP. Two examples are shown **(A and B)**. Some cells co-expressing the proteins appear to show no change in the individual localisation of the individual proteins **(A)** with NCS-1 found in the Golgi region and on the plasma membrane and presenilin-1 appearing to be reticular. However, some cells appear to suggest a change in the localisation of presenilin-1 **(B)** with the presenilin co-localising with the NCS-1 in the TGN and the suggestion of some plasma membrane localisation. The colour overlay shows NCS-1 in red, presenilin-1 in green and areas of overlap in yellow.

3.3 Discussion

The results presented here demonstrate that whilst the KChIPs do possess the ability to perform multiple functions in common with each other, they also maintain distinct functions specific to themselves. Whilst all four KChIPs can stimulate the traffic of the Kv4.2 channel to the plasma membrane, only KChIP2 seems to have a regulatory role with respect to Kv1.4 channels whilst only KChIP3 (and to a lesser extent KChIP1) appears to interact with presenilin-1. The results on potential interactions are summarised on Table 1. It is important that it is noted that a significant limitation of this study is that it is based only on co-localisation or changes in localisation and was based on a non-quantitative assessment. It cannot be ruled out that interactions occurred that did not produce obvious effects detectable by imaging.

	Kv1.4	Kv1.5	Kv4.2	PS1
KChIP1	-	-	++	-
KChIP2	+	-	++	-
KChIP3	-	-	++	++
KChIP4	-	-	++	-
NCS-1	+/-	-	+/-	+/-

Table 1. Summary of potential interactions of the KChIPs and NCS-1 with K+ channels and presenilin based on localisation studies. ++ indicates a strong suggestion of interaction, + a potential interaction and – no evidence of interaction.

The importance of different splice variants of the KChIPs is something that remains unclear and yet something that might assume particular relevance with regards to their function. Literature on the KChIP splice variants is confusing due to differing nomenclature being used to describe the same variant which makes dissecting isoform specific effects difficult, especially when it is not clear which isoform has been used in a particular study. However, it is becoming apparent that different isoforms can display different effects. A study comparing the effects of KChIP1 splice variants upon the rate of recovery from inactivation of Kv4 channels found significant differences between KChIP1.1 and KChIP1.2 (Van Hoorick *et al.*, 2003), whilst several studies on KChIP2 isoforms have demonstrated differences in the efficiency of stimulating the traffic of Kv4 channels to the cell surface (Deschenes *et al.*, 2002; Decher *et al.*, 2004; Patel *et al.*, 2004). Furthermore, whilst most KChIP isoforms apparently act to stimulate traffic of Kv4 channels to the cell surface, one KChIP2 isoform has been shown to inhibit Kv4 surface expression (Patel *et al.*, 2002), and one KChIP4 isoform has no effect on channel trafficking and leads to the complete abolition of the fast activation of Kv4 channels (Holmqvist *et al.*, 2002).

The variable effects of these different isoforms become more intriguing when you consider the interaction of the KChIPs with the Kv4 channels. Interaction of the two proteins results in the formation of an octomeric structure that consists of four KChIP and four Kv channel subunits (Kim *et al.*, 2004b; Pioletti *et al.*, 2006). It is known that KChIPs can form homo- or hetero-oligomers (Osawa *et al.*, 2001), which thus raises the question of whether or not functional KChIP-Kv4 complexes

could contain multiple KChIP isoforms, thus resulting in subtle variations in channel expression and function. Analysis of the expression of the four KChIP genes showed major regional differences in their expression in the brain (Pruunsild & Timmusk, 2005), whilst the use of antisera specific for KChIP1-4 indicated a cell-type-specific expression of the four KChIPs in the cerebellum, cortex striatum and hippocampus (Rhodes *et al.*, 2004; Strassle *et al.*, 2005). It would be interesting however to discover the differing expression of each of the splice variants – it is possible that specific KChIP isoforms are limited to particular neuronal cell types together with specific Kv4 channel isoforms to modulate their function as desired for that individual cell type?

The differing intracellular localisation of the KChIPs is something that may also be important in maintaining subtle functional differences. As I demonstrated here, KChIP1.2 is targeted to a population of intracellular vesicles when expressed in COS-7 cells, and this is targeting is believed to increase the efficiency of trafficking Kv4 channels to the plasma membrane relative to the other KChIPs (An *et al.*, 2000). However, despite differential cellular localisation, it is clear that KChIP 2.3 (membrane associated), and KChIPs 3.1 and 4.1 (cytosolic) can all carry out this particular functional role. It is quite possible that these differences in intracellular targeting, together with cell-type specific expression of different KChIPs and different isoforms are important in terms of regulating specific channel isoforms in specific cell types. It was surprising that KChIPs 2, 3 and 4 were not all membrane associated when expressed alone as they all possess palmitoylation motifs.

Palmitoylation is required for the ability of KChIPs 2 and 3 to stimulate traffic of Kv4.2 channels to the plasma membrane (Takimoto *et al.*, 2002)

The importance of the interaction of the KChIPs with the presenilins is not entirely clear. Whilst I demonstrated a change in localisation of the two proteins when KChIP3 and presenilin-1 were co-expressed in COS-7 cells, I was unable to see a similar change in localisation with KChIP4, and whilst observing some intracellular changes in localisation with KChIP1, I was unable to confirm previous work suggesting an interaction between KChIP 4, and presenilin-1 (Morohashi *et al.*, 2002), neither by confocal microscopy nor by co-immunoprecipitation (data not shown). Undoubtedly, the significance of the interaction between KChIP3, and potentially the other KChIPs, with respect to the functional role of the presenilins is something that requires further investigation. Given the widespread expression of both presenilins 1 and 2 throughout the brain compared to the more cell type specific expression of the KChIPs, it might make sense that multiple KChIPs can interact with the presenilins if this interaction is functionally significant.

It is still unclear as to whether KChIPs have distinct or overlapping roles. Whilst I have demonstrated that isoforms of all KChIPs can modulate Kv4 channel trafficking, and it has been reported that isoforms of all four KChIPs have DREAM activity (Link *et al.*, 2004), I have also shown that not all KChIPs appear to interact with Kv1 channels, nor do they all appear to interact with presenilin-1. The KChIPs may not all have overlapping function as knock-outs of KChIP2 and KChIP3 both

lead to differing and non-compensated phenotypes (Kuo *et al.*, 2001; Cheng *et al.*, 2002; Lilliehook *et al.*, 2003). However, this non-redundancy may be due to differential expression of KChIPs in different cell types. The need for multiple KChIPs may be due to specific neuronal expression patterns to allow subtle differences in the fine tuning of channel function for example, in different neuronal cell types. Thus the extent to which KChIP functions overlap or are distinct is something that remains to be fully elucidated.

KChIPs are not the only NCS proteins that are able to modulate Kv4 channels, with NCS-1 previously reported to have the ability to regulate these channels. The findings of previous studies have, however, been somewhat contradictory. Some groups suggest that NCS-1 has similar effects to KChIPs, although the increase in current density prompted by NCS-1 is not as large as with the KChIPs (Guo *et al.*, 2002; Nakamura *et al.*, 2003). By contrast, another study found that in lobster pyloric neurons, NCS-1 was ineffective in increasing current density (Zhang *et al.*, 2003), whilst a further study was unable to confirm a direct interaction between NCS-1 and Kv4 channels (Ren *et al.*, 2003). Thus the precise role that NCS-1 may play in regulating Kv4 membrane traffic and any physiological relevance of this interaction remains controversial. I have found that in COS-7 cells, NCS-1 is not only able to stimulate Kv4 traffic, but may also have the ability to regulate Kv1.4 traffic, and furthermore, potentially interact with presenilin-1. Some of the observations made are however, less than clear cut, and it would appear that

although NCS-1 apparently has the ability to carry out functions similar to those of the KChIPs, but is less effective at doing so. Whether this lower effectiveness is a result of differential intracellular targeting, a lower affinity of NCS-1 for the aforementioned proteins, variable intracellular conditions or potentially as a result of the multiple functionality of NCS-1 is unknown. The significance of these potential interactions for the physiological functions of NCS-1 is unclear, and would require further work in order to be resolved.

The results presented here raise the possibility that KChIPs carry out multiple cellular functions that in some cases overlap amongst the KChIPs and also with NCS-1. Given the potential overlap between NCS-1 and KChIP function, and the reported ability of NCS-1 to stimulate regulated exocytosis in a well defined assay, the effect of KChIPs on exocytosis was subsequently examined.

CHAPTER 4:

Differential effects of KChIP3 on calcium signalling and regulated secretion in PC12 cells

4.1 Introduction

Given the potential for the overlap of functions between NCS-1 and the KChIPs, I set out to investigate another known function of NCS-1, its role in stimulating regulated exocytosis, and whether or not the KChIPs also shared this particular functional property using a well established assay.

Regulated exocytosis of neurotransmitters and hormones during neurosecretion is triggered by a rise in cytosolic Ca^{2+} concentration (Burgoyne & Morgan, 1995), and in the multiple stages of the exocytotic pathway (Martin, 1997; Burgoyne & Morgan, 1998), it is likely that both secretory vesicle recruitment and membrane fusion are regulated by Ca^{2+}. Whilst the main Ca^{2+} binding protein involved in exocytosis would appear to be the low affinity Ca^{2+} -binding protein synaptotagmin I (DiAntonio *et al.*, 1993; Littleton *et al.*, 1993; Geppert *et al.*, 1994), there are multiple stages throughout the exocytotic pathway where other Ca^{2+} -binding proteins may play an important role. The NCS proteins are well suited to respond to small increases in Ca^{2+} that enhance exocytosis from nerve endings due to their high affinity for Ca^{2+}, and indeed, overexpression of NCS-1 has been shown to enhance evoked neurotransmitter release, paired-pulse facilitation and exocytosis in several neuronal and neuroendocrine cell types (Pongs *et al.*, 1993; McFerran *et al.*, 1998; Haynes *et al.*, 2005). The mechanism by which this stimulation of release occurs is not entirely clear. The current data suggest that NCS-1 may potentiate secretion from a variety of cell types by enhancing the trafficking of TGN-derived transport

carriers via activation of PI4Kβ, with possible additional role(s) in modulating channels and/or receptors through direct and/or indirect interactions (Hilfiker, 2003).

To probe the effect of KChIP over-expression on secretion I made use of an assay in which cells were co-transfected so that they expressed human growth hormone (GH) as well as a test protein of choice. GH is not normally expressed by these cells but in transfected cells is packaged into dense core granules and secreted in response to Ca^{2+} elevation (Wick *et al.*, 1993). This system was used to first demonstrate the stimulatory effect on regulated secretion due to NCS-1 over-expression and utilises the PC12 cell line, which has previously proved to be a good model for the study of neurosecretion (McFerran *et al.*, 1998). Co-transfection with a plasmid encoding growth hormone has been widely used in the study of proteins involved in dense-core granule exocytosis in PC12 cells (Chung *et al.*, 1995; Orita *et al.*, 1996; Graham *et al.*, 1997; Orita *et al.*, 1997; Wang *et al.*, 1997). Transfection using LipofectAMINE (Invitrogen) results in transfection of up to 10% of the PC12 cells (Graham *et al.*, 1997), with the assay of GH release allowing analysis of only those cells that have taken up the plasmid. It has previously been demonstrated that essentially all cells expressing GH will also express the second transfected protein (McFerran *et al.*, 1998).

The potential for the KChIPs to play a role in neurosecretion is further enhanced by the knowledge that certain KChIPs have previously been shown to modulate Ca^{2+} signalling in other cell types (Lilliehook *et al.*, 2002), and thus may be ideally suited

to respond to and modulate Ca^{2+} levels during the process of exocytosis. Furthermore, the KChIPs share the DREAM function and are able to repress transcription, something that has been implied as being important in relation to Ca^{2+} -signalling through a reduction of the expression of the Na/Ca^{2+} exchanger NCX3 due to a direct repression of its transcription (Gomez-Villafuertes *et al.*, 2005).

Here I aimed to not only investigate effects of the overexpression of the KChIPs on neurosecretion, but subsequently their effects on the intracellular Ca^{2+} changes elicited during ATP stimulation of purinergic receptors on the cells.

4.2 Effects of the KChIPs on regulated secretion

4.2.1 The KChIPs express to comparable levels in PC12 cells

Before carrying out experiments to directly compare the KChIPs in a functional assay, it was important to confirm that the four KChIP constructs that would be used in the experiments were expressed to the same level once transiently transfected into PC12 cells so that any differences observed could not be down to a difference in expression of the proteins. Previous investigation of protein expression in transfected PC12 cells by Western blot analysis was inconclusive (figure 11). When blotted using an anti-GFP antibody, it would appear that KChIP2-ECFP and KChIP3-ECFP were expressed to a higher level than KChIP1-ECFP and KChIP4-ECFP. However, when identical volumes of the same cell lysates were run on a different gel and the subsequent blot probed with an anti-pan KChIP antibody, KChIPs1, 2 and 4 all expressed to a comparable level, with KChIP3 not really detected at a significant level.

In order to confirm expression levels were at a similar intensity in PC12 cells, cells were transfected with 2μg of either one of the four KChIP-ECFP plasmids or with 2μg of control ECFP-N1 plasmid and imaged using confocal microscopy 48 hours post transfection. All microscope settings were kept constant when carrying out the cellular imaging, and from the images obtained, the relative fluorescence of

transfected cells was calculated by drawing regions of interest around transfected cells and measuring the fluorescence from each cell.

For reasons that were unclear, transfection with ECFP-N1 alone resulted in a higher level of fluorescence expressed. Cells transfected with the four ECFP-tagged KChIPs fluoresced to an equivalent mean level (figure 32), and thus illustrated their suitability for use and direct comparison in the subsequent growth hormone and X-rhod-1 imaging.

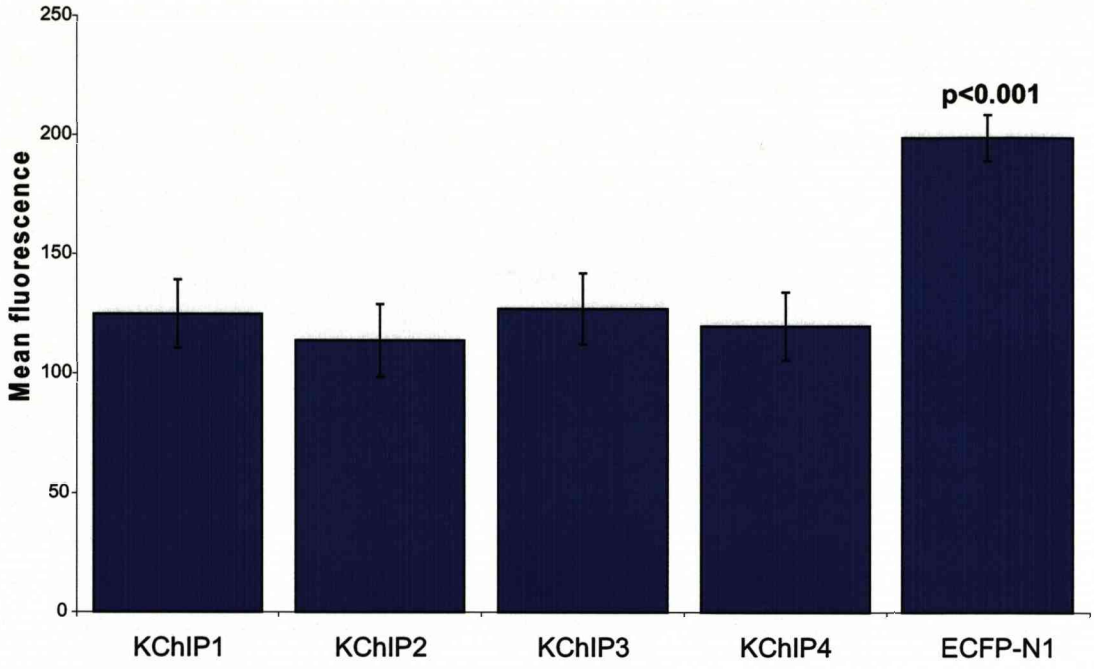

Figure 32. All KChIP ECFP-tagged constructs fluoresce to a comparable level in PC12 cells.
PC12 cells were transfected to express ECFP-tagged KChIPs 1-4 or a control ECFP-N1 plasmid. Live cells were imaged 48 hours post-transfection under identical microscope and laser conditions. The microscope settings for the experiment were as follows - the Leica CS-SP-MP microscope was utilised with cells excited at 430nm and light collected at 460-510nm. The laser power was maintained at 30.38%, the gain set at 931.4V, the beam expansion set at 6, the beam splitter used RSP455, the pinhole(M) at 143.02μm and the pinhole(AU) at 1.07 airy. After completion of the experiment, average values were collected for whole cell fluorescence for transfected cells. The number of cells for each condition were as follows: KChIP1 15 cells, KChIP2 12, KChIP3 24, KChIP4 18, ECFP-N1 23.

4.2.2 FLAG-KChIP3 increases secretion from PC12 cells in response to activation of purinergic receptors

PC12 cells were transfected to express GH (human growth hormone plasmid) and each of the KChIPs as FLAG-tagged constructs, and GH secretion was assayed under basal conditions and in response to activation of purinergic receptors by the addition of 300μM ATP, described by McFerran *et al,* 1998. Neither cells transfected with the control FLAG plasmid, nor cells transfected with any of the FLAG-tagged KChIPs exhibited a significant difference in the amount of basal GH release, expressed as a percentage of total cellular GH levels. However, whilst the control FLAG plasmid and FLAG-tagged KChIPs 1, 2 and 4 all displayed similar amounts of GH release in response to ATP-stimulation, FLAG-KChIP3 specifically and significantly increased the level of GH release (figure 33).

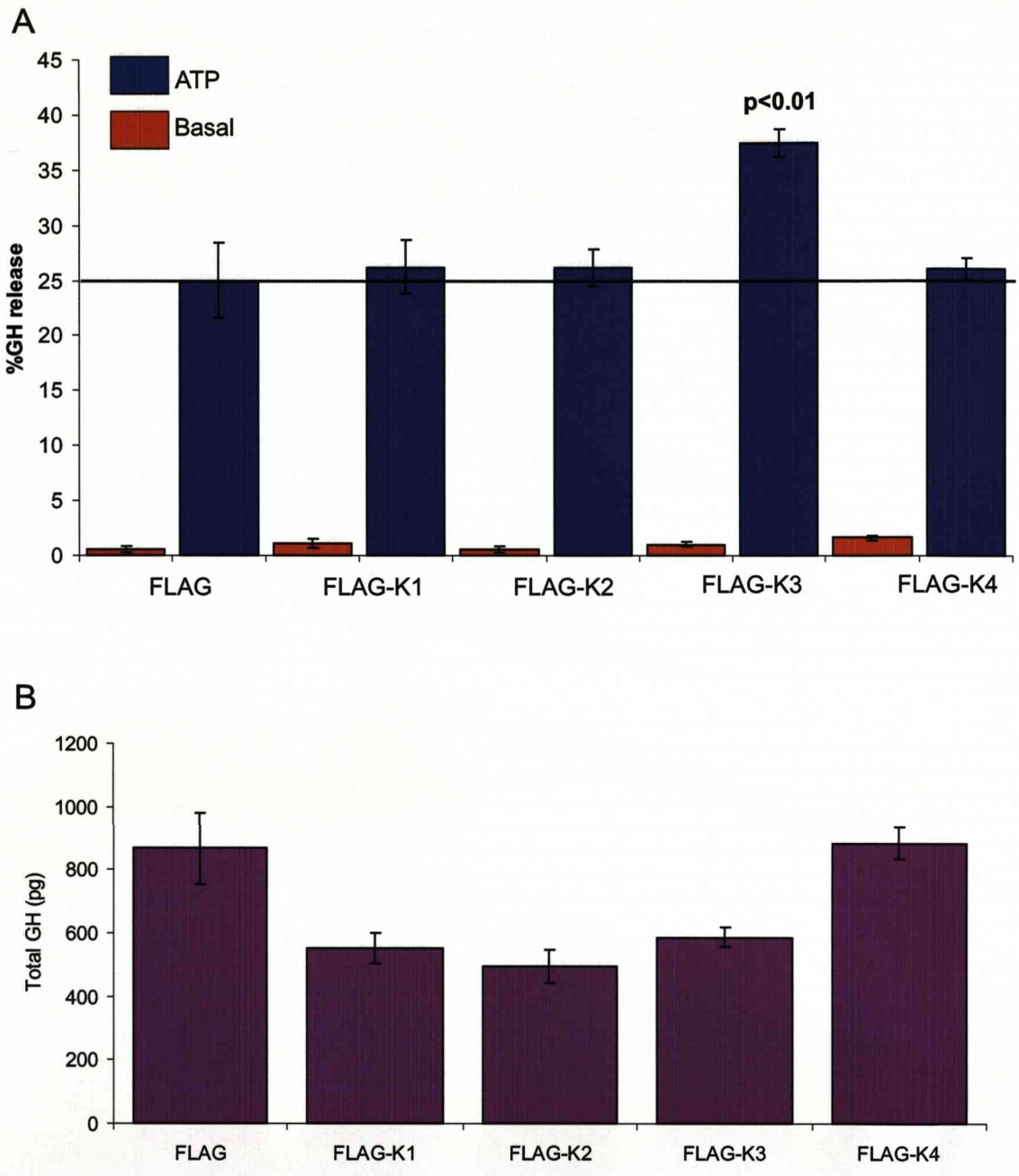

Figure 33. Overexpression of KChIP3, but none of the other KChIPs, increases reporter growth hormone release from PC12 cells.
PC12 cells were transfected with either FLAG plasmid as a control, or with one of the FLAG-KChIP encoding plasmids, together with GH-encoding plasmid. After 2 days, cells were washed and incubated with no additions or with 300μM ATP for 15 minutes. **(A)** Growth hormone release was then assayed and expressed as a percentage of total cellular GH levels (n=4). Compared to the control plasmid, KChIP3 significantly enhances GH release from the cells, and this effect is specific for KChIP3 with none of the other KChIPs inducing any change in GH release compared to the control plasmid. **(B)** Total cellular growth hormone content (pg).

4.2.3 Overexpression of KChIP3 increases secretion from PC12 cells in response to ATP-stimulation

Given that the FLAG-tag constructs have the tag at the N-terminus, I wanted to confirm that the FLAG-tag was not affecting the results obtained in any way, as the presence of the FLAG-tag at the N-terminus would prevent the myristoylation of KChIP1, something that is important for its membrane targeting (O'Callaghan *et al.*, 2003a), and could affect palmitoylation of KChIPs2-4, which although apparently not as important in the intrinsic targeting of the proteins (at least in the case of KChIPs 3 and 4), may have importance with respect to the functional affects of the proteins.

Secretion assays were therefore carried out using cells co-transfected with GH and either KChIP1-pcDNA or KChIP3-pcDNA respectively to allow expression of untagged proteins. Again, in the case of basal GH release, neither expression of KChIP1-pcDNA nor expression of KChIP3-pcDNA produced a change in basal GH release relative to the control plasmid (empty pcDNA vector). However, when the cells were stimulated with 300μM ATP, KChIP1-pcDNA failed to elicit a significant change in amount of GH secreted relative to the control, whilst KChIP3-pcDNA transfected cells again showed an enhanced level of GH secretion (figure 34).

For the purposes of use in later experiments, assays were then completed to confirm whether or not the presence of an ECFP-tag on the C-terminus of the protein would

have any effect on the effects seen on ATP-stimulated secretion. KChIP3-ECFP, KChIP3-pcDNA and FLAG-KChIP3 all exhibited a comparable escalation of GH release from PC12 cells when stimulated by 300μM ATP, whilst showing no change in basal release of GH (figure 35). This illustrated that the presence of an ECFP-tag on the C-terminus of the protein (or indeed the presence of a small FLAG-tag on the N-terminus) had no effect on the level of secretion, and thus ECFP-tagged proteins were suitable for use in further secretion assay experiments.

Multiple assays were carried out using the ECFP-N1 vector as a control plasmid and KChIP3-ECFP as the test plasmid and the data from all experiments were pooled. Whilst the basal levels of secretion did not significantly differ between the control and test plasmids, an increase in GH release was observed in cells transfected with KChIP3-ECFP in response to stimulation with 300μM ATP (figure 36), confirming that KChIP3 can act to elicit an increase in stimulated exocytosis in intact PC12 cells.

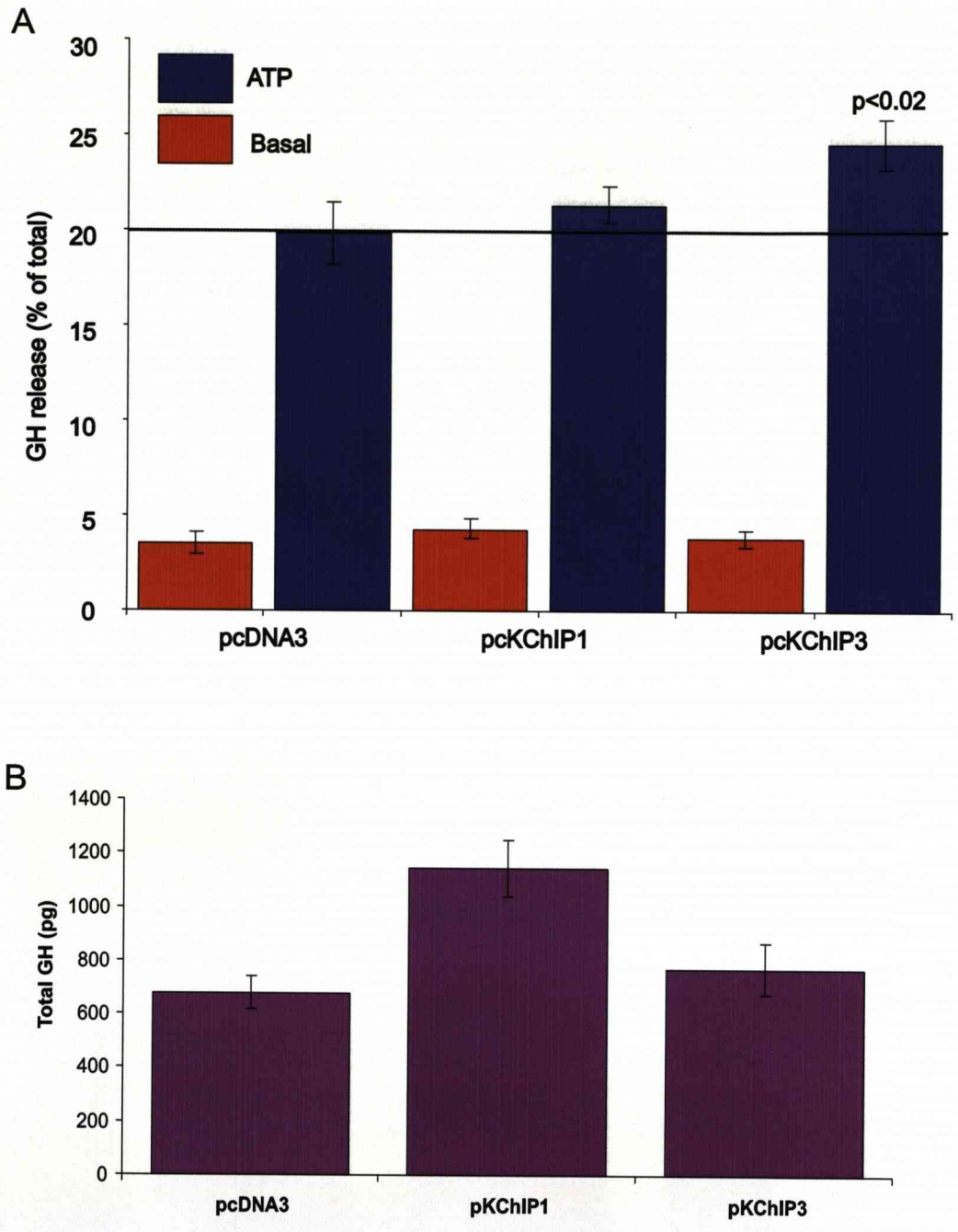

Figure 34. Overexpression of KChIP3 but not KChIP1 increases reporter growth hormone release from PC12 cells.
PC12 cells were transfected with either pcDNA3 plasmid as a control or with either KChIP3-pcDNA or KChIP1-pcDNA, together with GH-encoding plasmid. After 2 days, cells were washed and incubated with no additions or with 300μM ATP for 15 minutes. **(A)** Growth hormone release was then assayed and expressed as a percentage of total cellular GH levels (n=9). Compared to the control plasmid, KChIP3 but not KChIP1 significantly enhances GH release from the cells. **(B)** Total cellular growth hormone levels (pg).

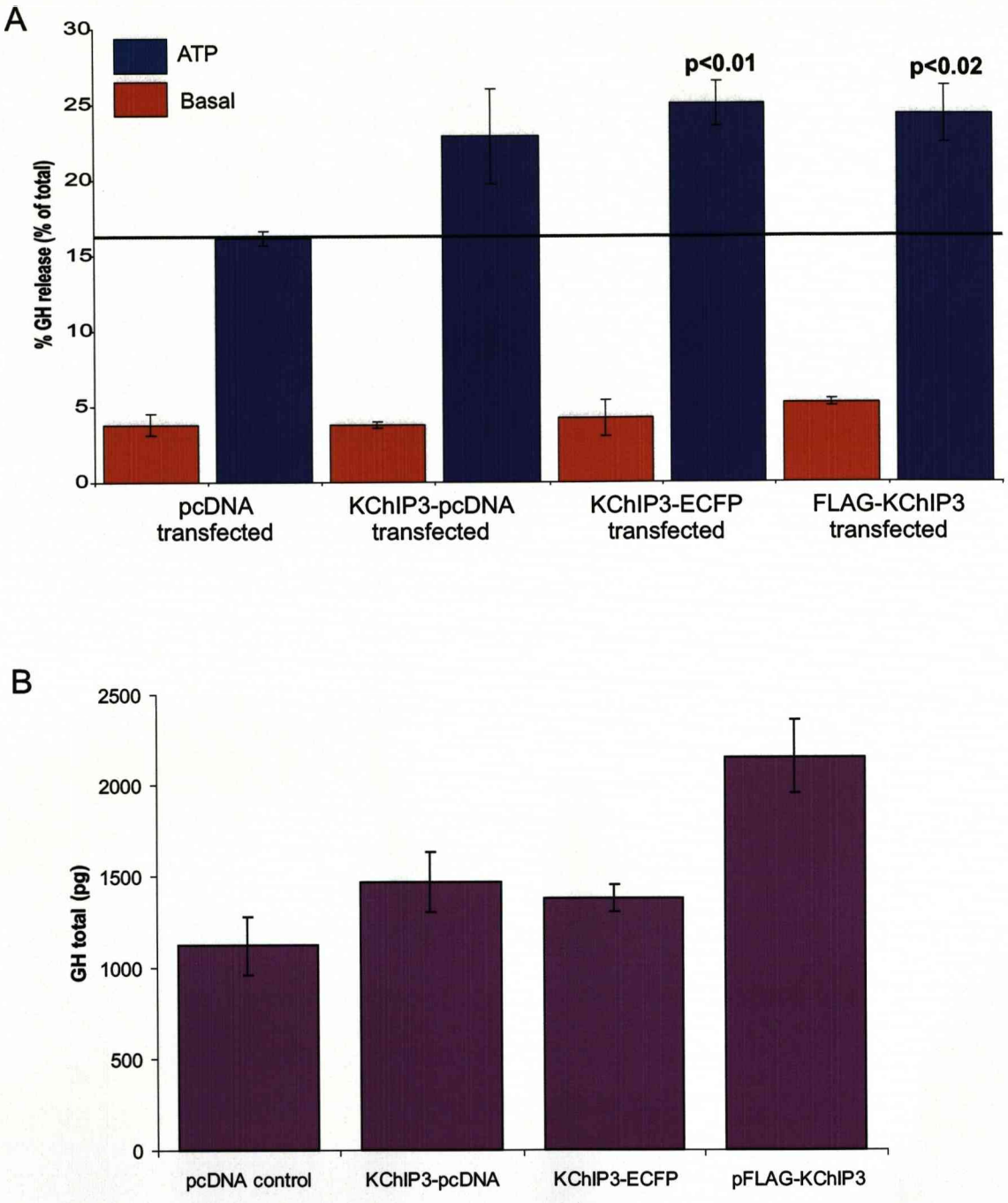

Figure 35. Overexpression of KChIP3 increases reporter growth hormone release from PC12 cells.

PC12 cells were transfected with either ECFP-N1 plasmid as a control, or with one of a variety of KChIP3 encoding plasmids, together with GH-encoding plasmid. After 2 days, cells were washed and incubated with no additions or with 300μM ATP for 15 minutes. **(A)** Growth hormone release was then assayed and expressed as a percentage of total cellular GH levels (n=3). Compared to the control plasmid, KChIP3 significantly enhances GH release from the cells, with this effect being similar for each KChIP3 encoding plasmid tested. **(B)** Total growth hormone levels (pg).

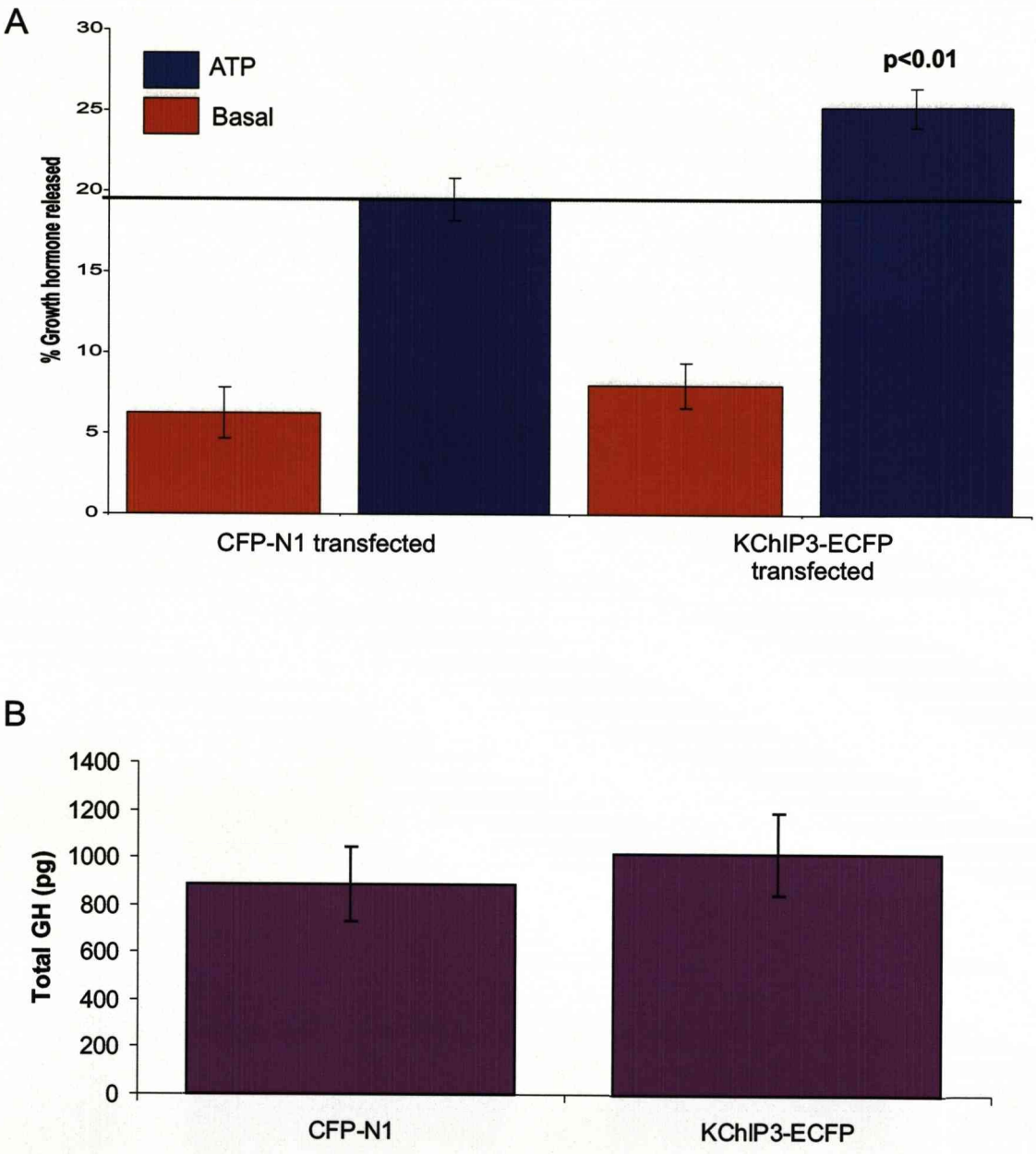

Figure 36. Overexpression of KChIP3 increases reporter growth hormone release from PC12 cells.
PC12 cells were transfected with either ECFP-N1 plasmid as a control or with KChIP3-ECFP, together with GH-encoding plasmid. After 2 days, cells were washed and incubated with no additions or with 300μM ATP for 15 minutes. **(A)** Growth hormone release was then assayed and expressed as a percentage of total cellular GH levels (n=12). Compared to the control plasmid, KChIP3 significantly enhances GH release from the cells. **(B)** Total growth hormone levels (pg).

4.2.4 Overexpression of KChIP3 has no effect on the amount of reporter growth hormone release from permeabilised PC12 cells

Previous studies on NCS-1 have demonstrated that NCS-1 results in an increase in stimulated exocytosis in intact though not in permeabilised PC12 cells where Ca^{2+} directly triggered exocytosis, implicating NCS-1 as a general but indirect regulator of neurosecretion (McFerran *et al.*, 1998). In order to investigate whether or not KChIP3 acted in a similar indirect manner, PC12 cells were transfected with either KChIP3-ECFP or ECFP-N1 as a control. Two days after transfection the cells were washed, permeabilised with digitonin and incubated in the presence of 0 or 10 μM free Ca^{2+} for 15 minutes (Graham *et al.*, 2000). Growth hormone release was assayed and expressed as a percentage of total cellular GH levels (figure 37). Cells transfected with ECFP-N1 and KChIP3-ECFP showed no significant differences in the amount of GH release in the presence of 0μM free Ca^{2+}. When the free Ca^{2+} concentration was increased to 10 μM, again no significant difference in GH release was observed, thus illustrating that KChIP3 overexpression causes an increase in stimulated exocytosis in intact but not permeabilised PC12 cells, similar to NCS-1.

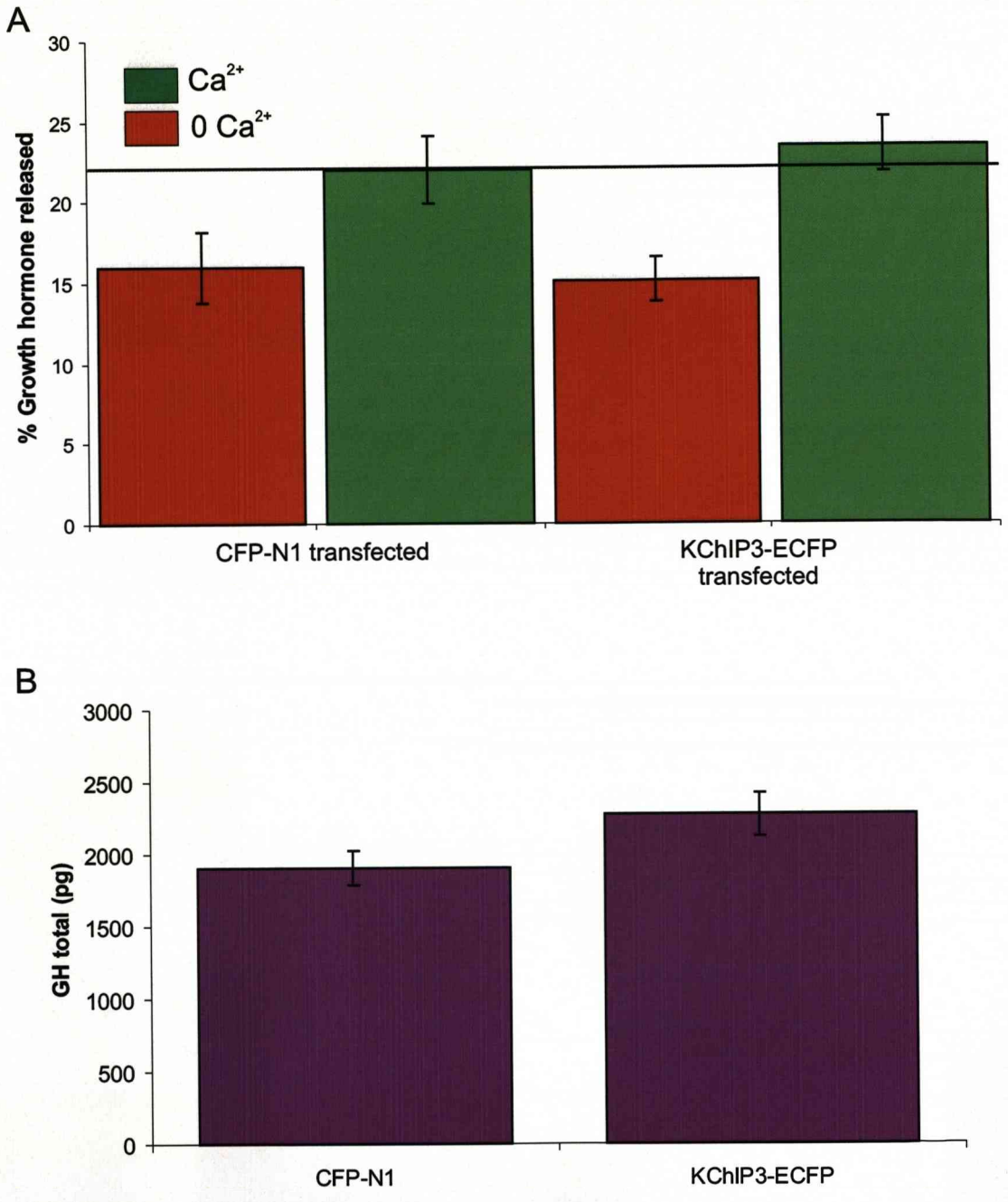

Figure 37. Lack of effect of overexpression of KChIP3 on reporter growth hormone release from permeabilised PC12 cells.
PC12 cells were transfected with either ECFP-N1 plasmid as a control or with KChIP3-ECFP, together with GH-encoding plasmid. After 2 days, cells were washed and permeabilised for 6 minutes by incubation with 20μM digitonin. **(A)** The cells were then challenged with either 0 or 10μM free Ca^{2+} and growth hormone release during a 15 minute period assayed and expressed as a percentage of total cellular GH levels (n=6). Compared to the control plasmid, KChIP3 does not exhibit an effect on GH release from permeabilised cells. **(B)** Total cellular growth hormone levels (pg).

4.2.5 Expression of presenilin 1-EGFP has no effect on stimulated exocytosis from intact PC12 cells

KChIP3 is known to interact with presenilin 1 and 2 (Buxbaum *et al.*, 1998) and the presenilins have been shown to affect ER Ca^{2+} by having a Ca^{2+} leak function (Tu *et al.*, 2006; Nelson *et al.*, 2007). Presenilins are widely expressed and, therefore, it was possible that the effect of KChIP3 secretion could have been due to an interaction with presenilin and a subsequent alteration of intracellular Ca^{2+} signalling.

To investigate this possibility further, PC12 cells were transfected to express either a control plasmid (ECFP-N1), presenilin 1-EGFP or a combination of presenilin-EGFP and KChIP3-ECFP, together with GH plasmid. Two days after transfection, cells were washed with Krebs buffer and incubated with no additions (basal) or with 300μM ATP for 15 minutes. Growth hormone release was assayed and expressed as a percentage of total cellular GH levels.

Overexpression of presenilin 1 –EGFP produced no significant effect on either basal or stimulated GH release. The results of several assays were pooled and are displayed (figure 38). Furthermore, expression of presenilin-1 in combination with KChIP3 did not modify the GH secretion enhancement observed with KChIP3 (figure 39).

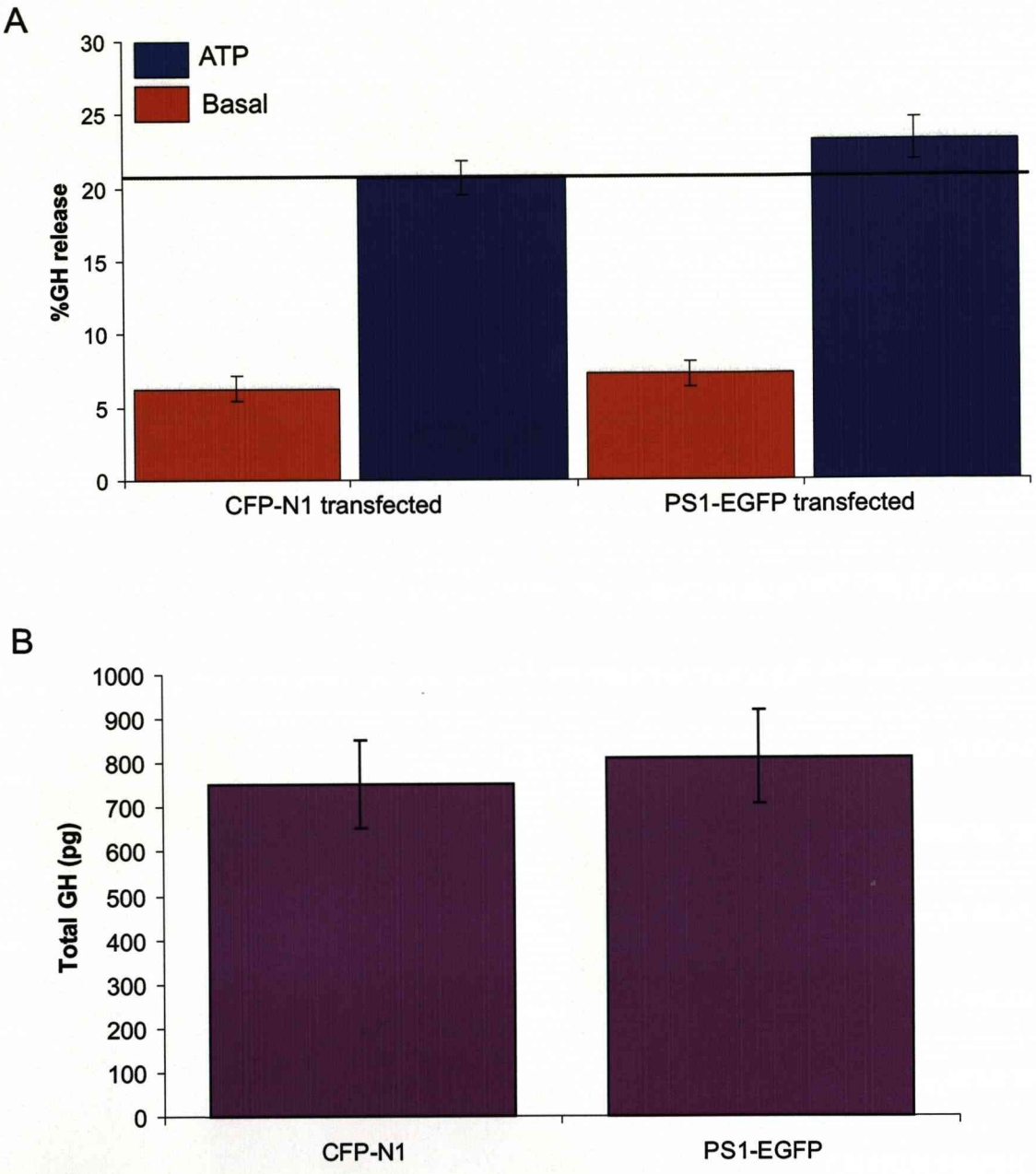

Figure 38. Overexpression of presenilin-1 has no effect on reporter growth hormone release from PC12 cells.
PC12 cells were transfected with either presenilin1-EGFP plasmid or ECFP-N1 plasmid as a control, together with GH-encoding plasmid. After 2 days, cells were washed and incubated with no additions or with 300μM ATP for 15 minutes. **(A)** Growth hormone release was then assayed and expressed as a percentage of total cellular GH levels (n=18). Compared to the control plasmid, presenilin-1 does not exhibit an effect on GH release from the cells. **(B)** Total cellular growth hormone levels (pg).

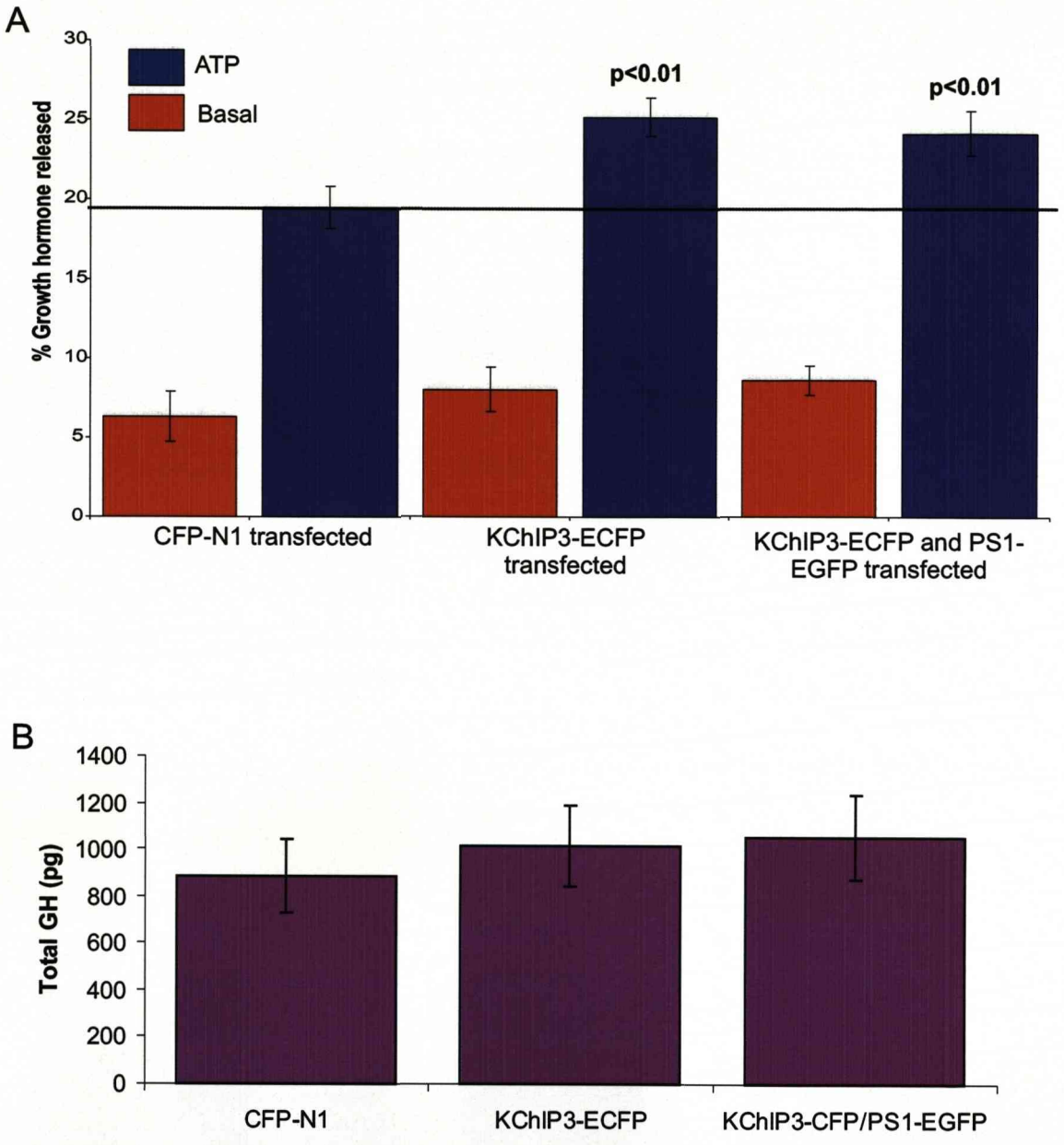

Figure 39. Overexpression of KChIP3 increases reporter growth hormone release from PC12 cells and this effect is unaltered by co-expression of presenilin-1. PC12 cells were transfected with either ECFP-N1 plasmid as a control, KChIP3-ECFP or a combination of both KChIP3-ECFP and PS1-EGFP, together with GH-encoding plasmid. After 2 days, cells were washed and incubated with no additions or with 300μM ATP for 15 minutes. **(A)** Growth hormone release was then assayed and expressed as a percentage of total cellular GH levels (n=12). Compared to the control plasmid, KChIP3 significantly enhances GH release from the cells. However, this effect is neither enhanced or diminished by co-expression of presenilin-1. **(B)** Total growth hormone levels (pg).

4.2.6 Expression of ECFP-Kv4.2 has no effect on the amount of growth hormone release from PC12 cells in response to ATP stimulation

Perhaps the best characterised of the KChIP functions is the interaction with the Kv4 channels. It was possible that the effect of KChIP3 secretion could have been due to an interaction with the Kv4 channels and a subsequent affect on cellular excitability and potentially Ca^{2+} signalling.

To investigate this possibility further, PC12 cells were transfected to express either a control plasmid (ECFP-N1), ECFP-Kv4.2 or a combination of ECFP-Kv4.2 and KChIP3-ECFP, together with GH plasmid. Two days after transfection, cells were washed with Krebs buffer and incubated with no additions (basal) or with 300μM ATP for 15 minutes. Growth hormone release was assayed and expressed as a percentage of total cellular GH levels.

Overexpression of ECFP-Kv4.2 produced no significant effect on either basal or stimulated GH release. Furthermore, expression of ECFP-Kv4.2 in combination with KChIP3 did not modify the GH secretion enhancement observed with KChIP3 (figure 40).

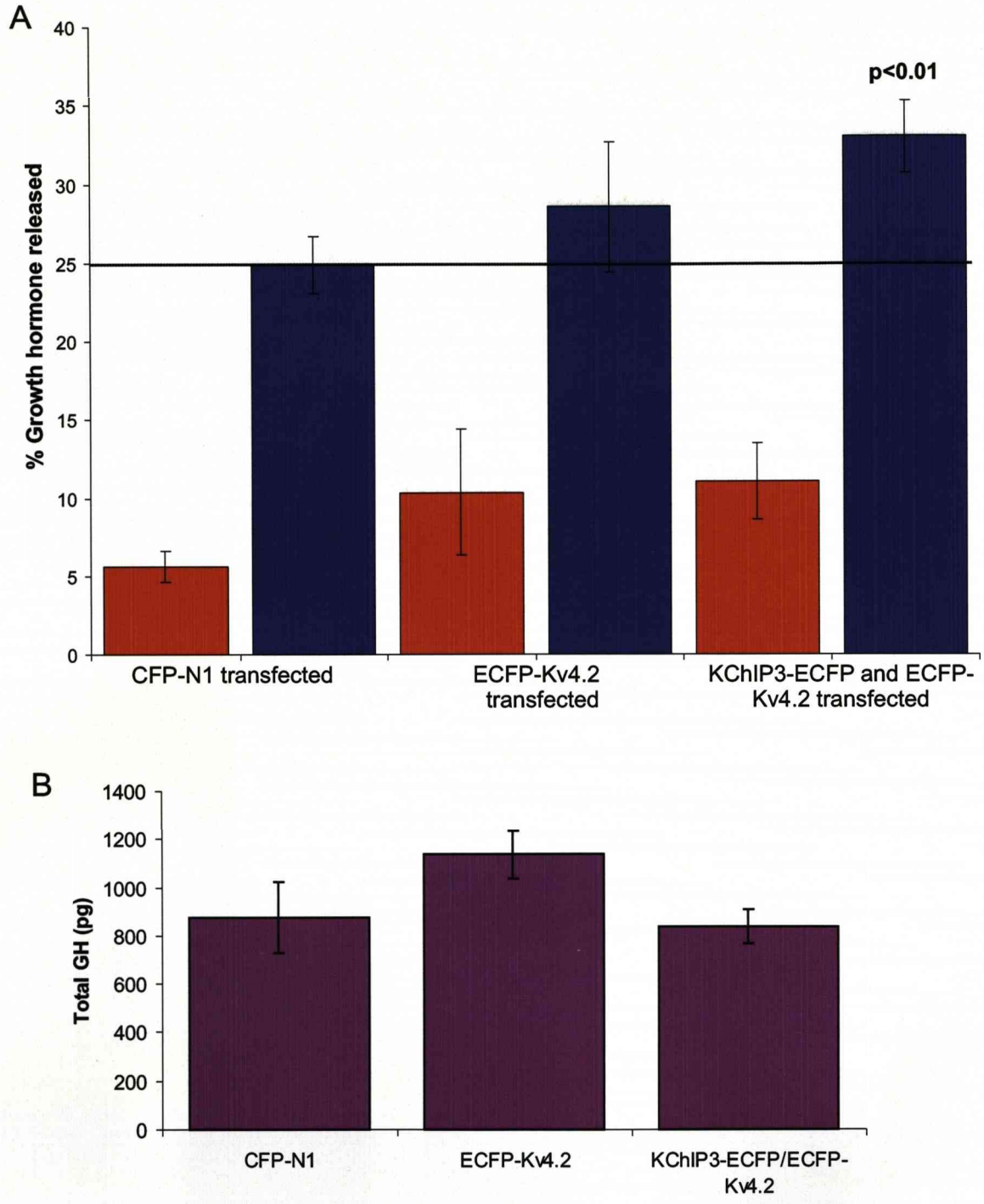

Figure 40. Overexpression of Kv4.2 has no effect on reporter growth hormone release from PC12 cells.
PC12 cells were transfected with either ECFP-Kv4.2 plasmid, ECFP-N1 plasmid as a control or a combination of ECFP-Kv4.2 and KChIP3-ECFP, together with GH-encoding plasmid. After 2 days, cells were washed and incubated with no additions or with 300μM ATP for 15 minutes. **(A)** Growth hormone release was then assayed and expressed as a percentage of total cellular GH levels (n=9). Compared to the control plasmid, Kv4.2 does not exhibit an effect on GH release from the cells. **(B)** Total cellular growth hormone levels (pg).

4.3 The effects of the KChIPs on Ca^{2+} signalling in response to purinergic stimulation

4.3.1 Overexpression of KChIP3 but none of the other KChIPs delays the decline of cytosolic Ca^{2+} concentration from the peak after stimulation with ATP

Overexpression of KChIP3 has previously been demonstrated to cause an increase in the Ca^{2+} content of the ER and to reduce the expression of the plasma membrane Na^{+}/Ca^{2+} exchanger NCX3. It is therefore possible that the effect seen on GH secretion could be due to an effect on Ca^{2+} signalling within the cell following ATP stimulation. In order to test this, cells were transfected with ECFP-tagged KChIPs, the cells loaded with the calcium indicator X-Rhod-1 and the changes in the intracellular Ca^{2+} concentration in response to ATP monitored following previously published methodology (Haynes *et al.*, 2004).

Firstly however, it was important to establish that expression of ECFP protein itself didn't have an effect on $[Ca^{2+}]_i$ responses to ATP. In cells expressing ECFP alone, robust $[Ca^{2+}]_i$ rises in response to perfusion with 300μM ATP were observed that were almost identical to responses recorded from adjacent control, nontransfected cells monitored simultaneously from the same coverslips (figure 41). This result showed that transfection and expression of ECFP did not adversely affect normal rises in $[Ca^{2+}]_i$. Averaged peak responses from a number of cells confirmed no

statistical difference in peak $[Ca^{2+}]_i$ responses to ATP between ECFP-transfected and nontransfected cells (figure 41).

In order to compare the effects of each of the KChIPs, cellular fluorescence levels of X-Rhod-1 were monitored before, during and after stimulation in high ECFP-expressing cells and adjacent nontransfected cells, and the data for expressed and nontransfected cells directly compared. Expression of KChIPs1-4 had no significant effect on the peak increase in $[Ca^{2+}]_i$ following the addition of 300μM ATP (figures 42-45). KChIPs 1, 2 and 4 had no effect on any other aspect of the Ca^{2+} signal (figures 42, 43 and 45). However, KChIP3-ECFP expression resulted in a significantly elevated $[Ca^{2+}]_i$ during the latter period of the experiment as the $[Ca^{2+}]_i$ was slowly declining (figure 44).

The data from analysis of the effect of KChIPs on changes in $[Ca^{2+}]_i$ would be consistent with the possibility that the increased GH secretion in KChIP3 expressing PC12 cells was a result of the prolongation of the increased $[Ca^{2+}]_i$ observed at later times of stimulation.

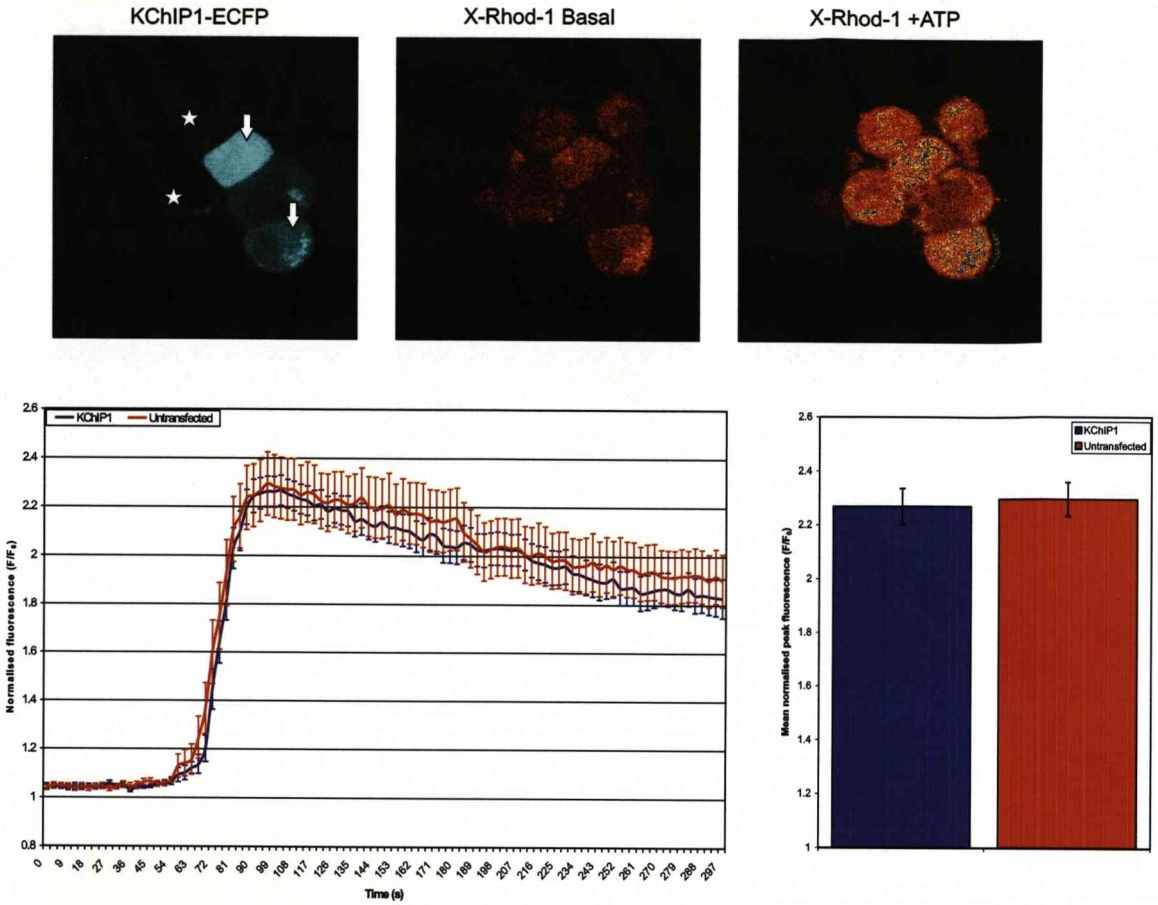

Figure 40. Expression of KChIP1 has no effect on cytosolic Ca^{2+} concentration in comparison to control cells after stimulation with ATP.

PC12 cells were transfected to express KChIP1 as an ECFP tagged construct. 48 hours after transfection cells were loaded with X-rhod-1 AM and then live cells were imaged. An ECFP image was taken to allow identification of transfected and non-transfected cells and then X-rhod-1 fluorescence was monitored before and after stimulation by perfusion with 300μM ATP. Images of ECFP and X-rhod-1 before and at the peak after stimulation are shown.After completion of the experiment average values were collected for whole cell fluorescence for transfected and adjacent control cells. Fluorescence values were normalised to the initial fluorescence for each cell and the data are shown as mean ± SEM. The numbers of cells for each condition were as follows:
KChIP1, 19 control , 17 transfected.

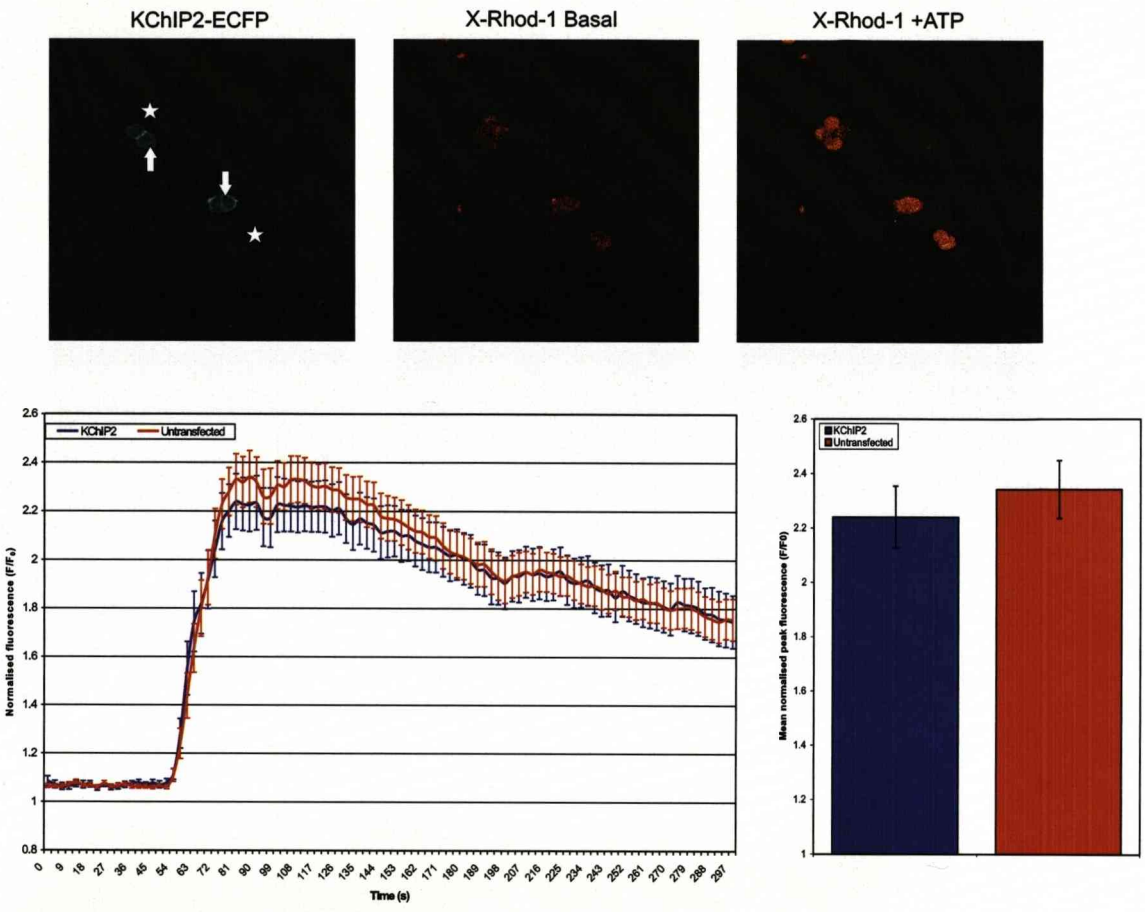

Figure 41. Expression of KChIP2 has no effect on cytosolic Ca^{2+} concentration in comparison to control cells after stimulation with ATP.

PC12 cells were transfected to express KChIP2 as an ECFP tagged construct. 48 hours after transfection cells were loaded with X-rhod-1 AM and then live cells were imaged. An ECFP image was taken to allow identification of transfected and non-transfected cells and then X-rhod-1 fluorescence was monitored before and after stimulation by perfusion with 300μM ATP. Images of ECFP and X-rhod-1 before and at the peak after stimulation are shown. After completion of the experiment average values were collected for whole cell fluorescence for transfected and adjacent control cells. Fluorescence values were normalised to the initial fluorescence for each cell and the data are shown as mean ± SEM. The numbers of cells for each condition were as follows: KChIP2, 35 control, 29 transfected.

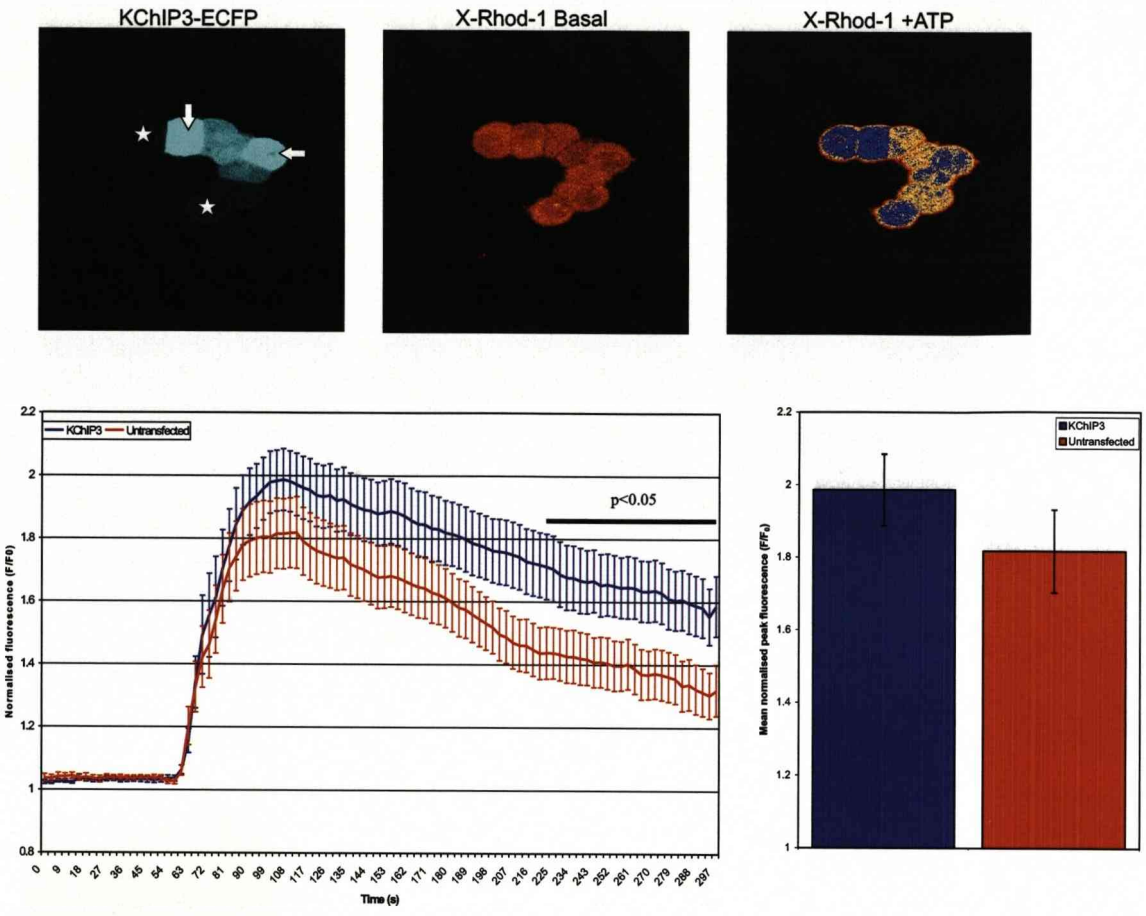

Figure 42. Expression of KChIP-3 delays the decline of cytosolic Ca^{2+} concentration from the peak after stimulation with ATP.

PC12 cells were transfected to express KChIP3 as an ECFP tagged construct. 48 hours after transfection,cells were loaded with X-rhod-1 AM and then live cells were imaged. An ECFP image was taken to allow identification of transfected and non-transfected cells and then X-rhod-1 fluorescence was monitored before and after stimulation by perfusion with 300μM ATP. Images of ECFP and X-rhod-1 before and at the peak after stimulation are shown. After completion of the experiment average values were collected for whole cell fluorescence for transfected and adjacent control cells. Fluorescence values were normalised to the initial fluorescence for each cell and the data are shown as mean ± SEM. The numbers of cells for each condition were as follows: KChIP3, 19 control, 21 transfected.

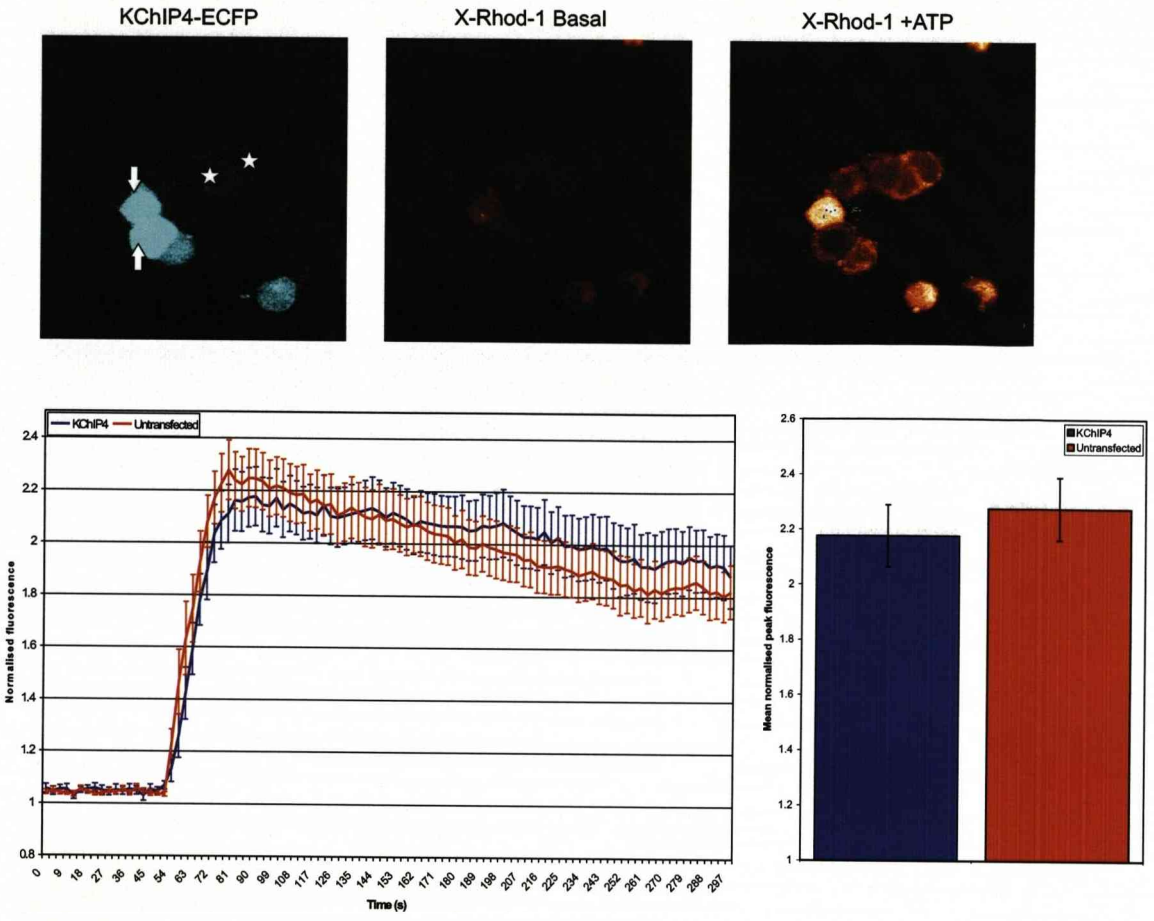

Figure 43. Expression of KChIP4 has no effect on cytosolic Ca^{2+} concentration in comparison to control cells after stimulation with ATP.

PC12 cells were transfected to express KChIP4 as an ECFP tagged construct. 48 hours after transfection cells were loaded with X-rhod-1 AM and then live cells were imaged. An ECFP image was taken to allow identification of transfected and non-transfected cells and then X-rhod-1 fluorescence was monitored before and after stimulation by perfusion with 300μM ATP. Images of ECFP and X-rhod-1 before and at the peak after stimulation are shown. After completion of the experiment average values were collected for whole cell fluorescence for transfected and adjacent control cells. Fluorescence values were normalised to the initial fluorescence for each cell and the data are shown as mean ± SEM. The numbers of cells for each condition were as follows: KChIP4, 22 control, 22 transfected.

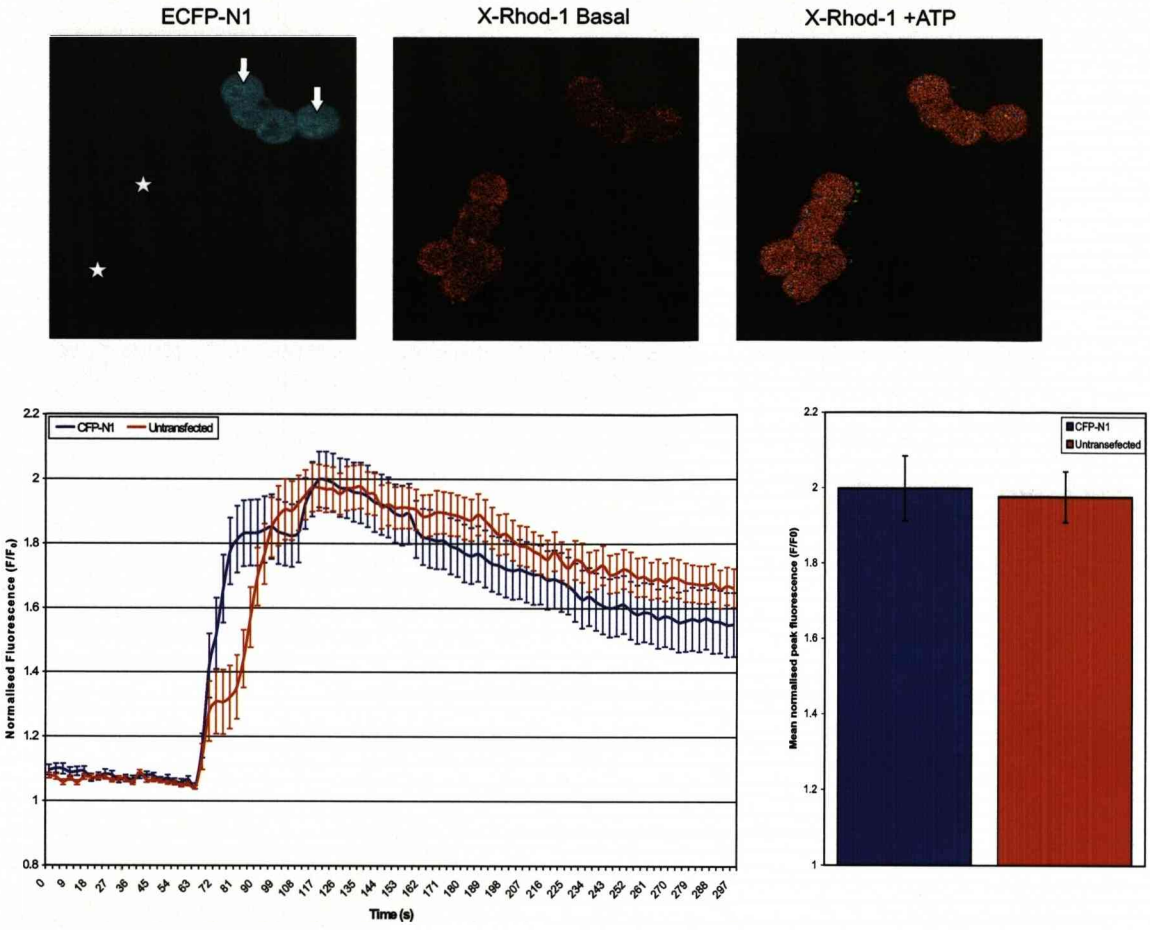

Figure 44. Expression of ECFP-N1 has no effect on Ca^{2+} concentration after stimulation with ATP in comparison to nontransfected control cells.

PC12 cells were transfected to express ECFP-N1 and 48 hours after transfection were loaded with X-rhod-1 AM and then live cells were imaged. An ECFP image was taken to allow identification of transfected and non-transfected cells and then X-rhod-1 fluorescence was monitored before and after stimulation by perfusion with 300μM ATP. Images of ECFP and X-rhod-1 before and at the peak after stimulation are shown. After completion of the experiment average values were collected for whole cell fluorescence for transfected and adjacent control cells. Fluorescence values were normalised to the initial fluorescence for each cell and the data are shown as mean ± SEM. The numbers of cells for each condition were 33 control and 28 transfected.

4.3.2 Overexpression of presenilin 1-EGFP has no effect on Ca^{2+} concentration after stimulation with ATP

To confirm that the effect of KChIP3 seen upon secretion and the intracellular Ca^{2+} concentration in PC12 cells in response to the addition of ATP related to an interaction with presenilin-1, after testing the effect of presenilin-1 overexpression on secretion (figures 38 and 39) the effect of presenilin-1-EGFP overexpression on the ATP evoked elevation of $[Ca^{2+}]_i$ was investigated.

X-rhod-1 fluorescence was recorded before, during and after stimulation of cells either strongly expressing presenilin-1-EGFP or control nontransfected cells with 300μM ATP. Expression of presenilin-1-EGFP did not significantly alter the peak $[Ca^{2+}]_i$ nor did it affect the duration of the elevation, with the rate of decline exhibited comparable to that of the control nontransfected cells (figure 45).

These data suggest that the effects seen on both secretion and upon the $[Ca^{2+}]_i$ following stimulation with ATP in cells overexpressing KChIP3 would not appear to be due to an interaction between KChIP3 and presenilin-1.

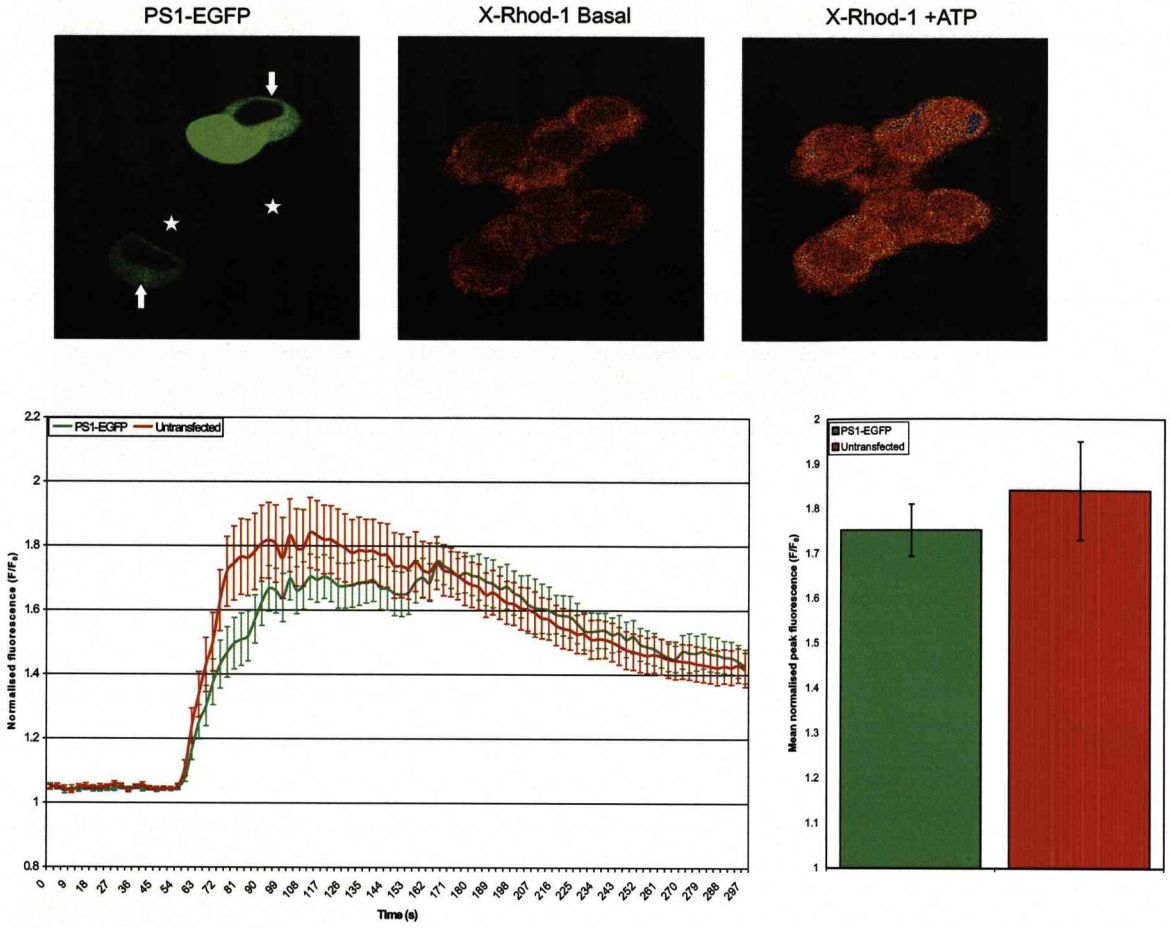

Figure 45. Expression of presenilin-1-EGFP has no effect on Ca^{2+} concentration after stimulation with ATP.

PC12 cells were transfected to express presenilin-1-EGFP (PS1-EGFP) and 48 hours after transfection were loaded with X-rhod-1 AM and then live cells were imaged. An EGFP image was taken to allow identification of transfected and non-transfected cells and then X-rhod-1 fluorescence was monitored before and after stimulation by perfusion with 300μM ATP. Images of EGFP and X-rhod-1 before and at the peak after stimulation are shown. After completion of the experiment average values were collected for whole cell fluorescence for transfected and adjacent control cells. Fluorescence values were normalised to the initial fluorescence for each cell and the data are shown as mean ± SEM. The numbers of cells for each condition were 28 control and 26 transfected.

4.4 Discussion

In this study I have demonstrated a specific effect for expression of KChIP3 compared to isoforms of KChIPs 1, 2 and 4 on Ca^{2+} responses to purinergic receptor stimulation and on secretion in PC12 cells. Although I cannot formally prove that the effects seen upon secretion are as a direct result of the modification of the Ca^{2+} signal seen in response to ATP stimulation, this does seem likely, particularly as the effect of KChIP3 is not seen in permeabilised cells in response to buffered Ca^{2+} concentration. If the increased secretion of GH observed were as a result of the prolonged increase in $[Ca^{2+}]_i$ then secretion directly triggered by an increasing Ca^{2+} to a fixed level should not be affected by KChIP3 expression, as demonstrated by these experiments.

Previous studies have highlighted that KChIP3 can have an effect on Ca^{2+} signalling; this has been attributed in one study to an increase in the filling of ER Ca^{2+} stores (Lilliehook *et al.*, 2002), whilst another investigation found that expression of a dominant active EF-hand mutant of KChIP3 resulted in the slowing of recovery from Ca^{2+} elevation in cerebellar granule cells (Gomez-Villafuertes *et al.*, 2005). The latter finding was attributed to the DREAM function of KChIP3, resulting in lower expression levels of the Na^{+}/Ca^{2+} exchanger NCX3 due to a direct repression of its transcription. This study also demonstrated that overexpression of wild-type KChIP3 could result in downregulation of NCX3.

Whilst this would apparently provide an explanation for the effects seen upon secretion and the modification of the $[Ca^{2+}]_i$ seen in response to ATP stimulation, it is important to consider that the effect on NCX3 expression is thought to be due to the DREAM transcriptional regulator function. This would in turn suggest that the changes I observed may not be as a result of a change in NCX3 expression, as all four KChIPs are known to exhibit the DREAM transcriptional regulator function and therefore I would expect all four KChIPs to exhibit similar effects on secretion and Ca^{2+} signalling if they were as a result of the DREAM function. All four KChIPs have been demonstrated to bind to DRE sites and to be equally efficient in repressing the transcription from three separate promoters (Link *et al.*, 2004). However, it would be of interest to examine whether the other KChIP isoforms do limit NCX3 expression in the same manner as KChIP3 has been shown to. This is something that is yet to be tested, and it may be that solely KChIP3 decreases NCX3 expression at the plasma membrane, whether this is through a repression of transcription or possibly an effect on the trafficking of the exchanger to the plasma membrane.

Similarly, I cannot rule out effects via Kv4 channels which are regulated by all four KChIPs, and interactions with the presenilins. The presenilins are known to interact not only with KChIP3 but also KChIP4, but the functional experiments carried out here did not implicate a presenilin interaction in the KChIP3 effect on secretion or the Ca^{2+} response in PC12 cells. Equally, functional experiments carried with the Kv4.2 channel did not implicate a channel interaction in the effects on secretion.

The increase in filling of ER Ca^{2+} stores is something that has been implied in the reported pro-apoptotic effect of KChIP3 in HeLa cells. Interestingly, my initial experiments found that KChIP3 but none of the other KChIP isoforms tested caused apoptosis in HeLa cells (data not shown), but this apoptotic effect does not appear to occur in COS-7 or PC12 cell lines. The idea that Ca^{2+} signalling could be modified by KChIP3 overexpression leading to increased filling of ER Ca2+ stores is something that could be possible. Since the $[Ca^{2+}]_i$ increase in response to ATP stimulation is due predominantly to Ca^{2+} entry rather than release from intracellular stores, increased levels of ER Ca^{2+} should not have any significant effect on peak Ca^{2+} response. However, the increase in ER Ca^{2+} could conceivably prevent further reuptake of Ca^{2+} into the ER following ATP stimulation, thus increasing the time taken for the level of $[Ca^{2+}]_i$ to decline, accounting for the observed changes here.

Obviously whilst the functional data presented here demonstrates that KChIP3 has an effect on both secretion and Ca^{2+} response to ATP stimulation, the precise mechanism by which this occurs is something that is yet to be fully elucidated. The most likely explanations would appear to be either by an increase in ER Ca^{2+} store filling or by a decrease in the expression of the plasma membrane Na^{+}/Ca^{2+} exchanger NCX3, though interactions with other proteins such as the presenilins or the Kv4 channels cannot be ruled out. Clearly more work is required to fully understand the processes by which KChIP3 is producing its effects, though the work

carried out so far would suggest that this multi-talented protein may indeed carry out another specific functional role within the cell.

CHAPTER 5:

General Discussion

A large part of this study concerned a comparative investigation into some of the known functions of the four KChIP proteins and their ability carry out multiple cellular roles. This work utilised GFP-variant fusion proteins expressed in both COS-7 and PC12 cell lines, and imaged by confocal microscopy. The key findings demonstrated that whilst the KChIPs do indeed share some functional roles, such as the stimulation of traffic of the Kv4.2 channel to the plasma membrane, they also maintain unique functions specific to each particular KChIP, such as the ability to inhibit Kv1.4 traffic to the plasma membrane (KChIP2.3), to interact with presenilin-1 (KChIP3.1), and to enhance secretion through an effect on Ca^{2+} signalling (KChIP3.1).

To date, sixteen different isoforms of the different KChIP sub-family members have been identified, with previous studies finding differences between isoforms of the same KChIP gene in terms of Kv channel modulation (Deschenes *et al.*, 2002; Holmqvist *et al.*, 2002; Van Hoorick *et al.*, 2003; Patel *et al.*, 2004). The variable effects of these different isoforms, together with the potential for the KChIPs to form homo- or hetero-oligomers (Osawa *et al.*, 2001) raise the possibility that the KChIPs may be able to subtly modify channel expression and function. It would be interesting to discover the differing expression pattern for each particular splice variant, and elucidate whether particular KChIP isoforms are limited to particular neuronal cell types along with specific Kv channel isoforms to modulate their function as desired for that individual cell type. It is quite possible that cell-type specific expression of different KChIPs and different isoforms, in conjunction with

the differential intracellular targeting displayed by different KChIP isoforms, are important in terms of regulating specific channel isoforms in specific cell types.

The extent to which KChIP functions overlap remains unclear. However, the work carried out during the course of this investigation demonstrates that whilst the KChIPs do perform some similar roles within the cell, they also maintain distinct individual properties. Only KChIP2.3 for example was found to inhibit Kv1.4 channel trafficking to the plasma membrane. However, it is KChIP3.1 that seems to possess the greatest variation in functional ability. In addition to an ability to stimulate the traffic of Kv4 channels to the plasma membrane, it has been reported to interact with the presenilins, act as a repressor of transcription through an interaction with DRE residues and possess a pro-apoptotic function. Furthermore, in this study I demonstrated a novel effect of KChIP3.1 on Ca^{2+} responses to purinergic receptor stimulation and on secretion in PC12 cells. The effect upon secretion in response to ATP stimulation is likely to be a direct result of the elongation of the Ca^{2+} signal, although the precise mechanism behind this prolonged signal remains to be determined. Previous investigations have described two potential answers to this question, with KChIP3 expression reported to result in an increased filling of the ER Ca^{2+} stores and a lower expression of the Na^{+}/Ca^{2+} exchanger NCX3 due to a direct repression of its transcription. In the latter case, the lower expression of NCX3 was attributed to the DREAM function of KChIP3, although as all four KChIPs are reported to exhibit the DREAM transcriptional regulator function, it would be interesting to investigate whether or not the other KChIPs limit NCX3 expression in

the same manner as KChIP3 has been shown to. If the expression of NCX3 is limited specifically by KChIP3, this would potentially provide an explanation for the functional data presented in this thesis. Furthermore, it would be of interest to investigate the shorter splice variant of KChIP3 (KChIP3.2) to discover whether or not this isoform shares the ability to modify the Ca^{2+} signal in a similar manner. Further work to determine the full range of target proteins for each of the KChIPs would also be of interest, potentially by completing a yeast two-hybrid screen for all four KChIPs, or by making GST-tagged versions of the proteins in order to perform a GST-pull-down assay with brain lysates, allowing subsequent identification of interacting proteins by mass spectroscopy. This could be followed up by functional studies in order to determine the significance of any potential interactions.

Clearly additional work is required in order to fully appreciate the multiple functions of this particular sub-family of the NCS proteins. It is becoming increasingly clear that differential expression of multiple isoforms of the KChIPs in neuronal cells may be important for the subtle regulation of a number of interacting partners, and that the KChIPs are much more than simply an accessory subunit of Kv4 channels. It would be of interest to discover the implications of the co-expression of multiple KChIP isoforms, based on the determination of the specific neuronal patterns of expression, and perform co-expression studies in model cell types. One example would be to investigate the co-expression of KChIP3.1 with isoforms of KChIPs 1, 2, and 4 with respect to the novel function revealed in this thesis and look for subtle modifications of secretion and Ca^{2+} signalling.

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