This electronic thesis or dissertation has been downloaded from Explore Bristol Research, http://research-information.bristol.ac.uk

Author: Charlick, James N
Title: Structural and functional properties of the novel heteromeric hSK1-hIKCa channel

General rights
Access to the thesis is subject to the Creative Commons Attribution - NonCommercial-No Derivatives 4.0 International Public License. A copy of this may be found at https://creativecommons.org/licenses/by-nc-nd/4.0/legalcode. This license sets out your rights and the restrictions that apply to your access to the thesis so it is important you read this before proceeding.

Take down policy
Some pages of this thesis may have been removed for copyright restrictions prior to having it been deposited in Explore Bristol Research. However, if you have discovered material within the thesis that you consider to be unlawful e.g. breaches of copyright (either yours or that of a third party) or any other law, including but not limited to those relating to patent, trademark, confidentiality, data protection, obscenity, defamation, libel, then please contact collections-metadata@bristol.ac.uk and include the following information in your message:

• Your contact details
• Bibliographic details for the item, including a URL
• An outline nature of the complaint

Your claim will be investigated and, where appropriate, the item in question will be removed from public view as soon as possible.
Structural and functional properties of the novel heteromeric hSK1-hIKCa channel

James Nathan Charlick

A dissertation submitted to the University of Bristol in accordance with the requirements for award of the degree of Master of Science by Research in the Faculty of Biomedical Sciences

School of Physiology, Pharmacology and Neuroscience

August 2021

Word count: 18,495
Abstract

Calcium-activated potassium (KCa) channels couple changes in calcium ion (Ca\(^{2+}\)) concentration, which commonly increases during periods of membrane or cellular excitability, to potassium ion (K\(^{+}\)) efflux that brings about a relaxation or change in cellular volume. Small-conductance KCa (SK) channels have been proposed to mediate certain KCa components of the repolarising potentials of neurons and cardiac cells. The expression of intermediate-conductance KCa (IKCa) channels has been proposed overlap with that of SK channels, and SK and IKCa channels have now been shown to preferentially co-assemble to form heteromeric hSK1-hIKCa channels. This implicates these heteromers in physiological roles that might have previously been attributed to other homomeric or heteromeric SK channels. Due to this, it is important to understand the structural and functional characteristics of heteromeric hSK1-hIKCa channels.

C-terminal coiled-coil domains (CCDs) have been proposed to mediate SK channel assembly. Site-directed mutagenesis was used to substitute the H389 residue within the second C-terminal CCD of hIKCa subunits to a glutamate residue. Outside-out patch clamp electrophysiology and pharmacological investigations revealed that this mutation disrupted but not prevent the formation of heteromeric hSK1-hIKCa channels, while having no affect upon the formation of homomeric hIKCa channels. These findings support the idea that the preferential formation of heteromeric hSK1-hIKCa channels is highly specific. Also, inside-out patch clamp was used to investigate the Ca\(^{2+}\) sensitivity of homomeric hSK1 and hIKCa as well as heteromeric hSK1-hIKCa channels. Compared to the Ca\(^{2+}\) sensitivities of both homomeric hSK1 and hIKCa channels, the Ca\(^{2+}\) sensitivity of heteromeric hSK1-hIKCa channels was found to be significantly right-shifted. These findings support the proposal that these channels are a novel member of the KCa family, with distinct functional properties that could be an indication that they play a distinct physiological role.
Acknowledgements

I must first thank Neil Marrion for being such a helpful, patient and reliable supervisor over the last two years. I have valued your mentorship and always-constructive criticism immensely.

I’d also like to thank everyone I’ve met in the lab throughout my project – somehow, you’ve managed to make doing this during a pandemic enjoyable! I am especially grateful to Andrew Butler for his incredible capacity for patience, wisdom and friendship.

Finally, thank you to my family, especially Mum, Dad, Grandad, Sam and Grace, and my partner, Caitlin, for your unwavering love, support and encouragement.

Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

SIGNED: …..JAMES NATHAN CHARLICK…… DATE:........16/08/21.............
### Table of Contents

- **Title page** i
- **Abstract** ii
- **Acknowledgements** iii
- **Author's declaration** iii
- **Table of Contents** iv
- **List of Figures** viii
- **List of Abbreviations** x

#### Chapter 1: General Introduction

1. **1.1 KCa Channel Structure and Function** 1
   - 1.1.1 KCa Channel Structure 1
   - 1.1.2 BK Channels 3
   - 1.1.3 SK Channels 4
   - 1.1.4 IKCa Channels 5
   - 1.1.5 Heteromeric KCa Channels 6

2. **1.2 The Slow Afterhyperpolarisation** 8

3. **1.3 Chapter References** 9

#### Chapter 2: General Methods

1. **2.1 Cell culture and transient transfection** 15
2. **2.2 Electrophysiology** 15
   - 2.2.1 Electrophysiology rig 16
3. **2.3 Recording & Analysis** 18
   - 2.3.1 Data collection 18
   - 2.3.2 Data analysis 19
Chapter 3: Co-assembly of hSK1 and hIKCa subunits via C-terminal CCDs

3.1 Chapter Introduction 22
   3.1.1 Preliminary co-expression of hSK3 and hIKCa subunits 22
   3.1.2 Coiled-coil domains 23
   3.1.3 The role of C-terminal CCDs in KCa channel assembly 23
   3.1.4 The H389E mutation in hIKCa subunits 27
   3.1.5 Pharmacology: Apamin 28
   3.1.6 Pharmacology: UCL1684 29

3.2 Chapter Aims 31

3.3 Chapter Methods 32
   3.3.1 Molecular biology: plasmid preparations 32
   3.3.2 Molecular biology: site-directed mutagenesis 32
   3.3.3 Voltage clamp in whole cell and outside-out patch configurations 35
   3.3.4 Electrodes 36
   3.3.5 Internal (electrode) and external (bathing) solutions 36
   3.3.6 Drugs 38

3.4 Chapter Results 39
   3.4.1 Control experiments 39
   3.4.2 Wild-type channel pharmacology: Introduction 41
   3.4.3 Homomeric hIKCa channel pharmacology 42
   3.4.4 Homomeric hSK1 and hSK3 channel pharmacology 44
   3.4.5 hSK1-hIKCa co-expression and hSK3-hIKCa co-expression 49
   3.4.6 Amplitude of hSK1-, hIKCa-, and hSK1-hIKCa-mediated currents as an indicator of plasma membrane expression 54
3.4.7 hIKCa(H389E)-mediated current amplitude and TRAM34 sensitivity  56
3.4.8 hSK1-hIKCa(H389E) co-expression  58

3.5 Chapter Discussion  67
3.5.1 Pharmacology of homomeric channels  67
3.5.2 Pharmacology of heteromeric hSK1-hIKCa channels: Apamin and TRAM34  67
3.5.3 Subunit stoichiometry of heteromeric hSK1-hIKCa channels  68
3.5.4 Pharmacology of heteromeric hSK1-hIKCa channels: UCL1684  70
3.5.5 Assembly of heteromeric hSK1-hIKCa channels  70
3.5.6 The role of the H389E mutation in the formation of homomeric and heteromeric channels  74

3.6 Chapter References  75

Chapter 4: Ca\textsuperscript{2+} sensitivity of hSK1, hIKCa and hSK1-hIKCa channels

4.1 Chapter Introduction  79
4.1.1 Homomeric SK channels  79
4.1.2 Homomeric IKCa channels  79
4.1.3 Heteromeric hSK1-hIKCa channels  80

4.2 Chapter Aims  81

4.3 Chapter Methods  82
4.3.1 Inside-out patch clamp  82
4.3.2 Electrodes  82
4.3.3 Internal (bathing) and external (electrode) solutions  82

4.4 Chapter Results  84
4.4.1 Ca\textsuperscript{2+} sensitivity of hSK1, hIKCa and hSK1-hIKCa channels  84

4.5 Chapter Discussion  87
4.5.1 Homomeric hSK1 channels sense acute changes in Ca\(^{2+}\) concentration

4.5.2 Homomeric hIKCa channels are activated by a broad range of Ca\(^{2+}\) concentrations

4.5.3 Heteromeric hSK1-hIKCa channels have a distinct Ca\(^{2+}\) sensitivity

4.5.4 Ca\(^{2+}\) sensitivity findings are limited by some experimental factors

4.6 Chapter References

Chapter 5: General Discussion & Conclusions

5.1 The H389E mutation within the C-terminal CCDs of hIKCa subunits perturbs co-assembly of hIKCa subunits with hSK1 subunits

5.2 Heteromeric hSK1-hIKCa channels are less sensitive to activation by Ca\(^{2+}\) than homomeric hSK1 and hIKCa channels

5.3 Heteromeric hSK1-hIKCa channels are strong candidates for the sAHP channel

5.4 Conclusion

5.5 Chapter References
List of Figures

1.1. KCa channel topology (adapted from Gueguinou et al., 2014) 2
1.2. AHP generated from dorsal motor nucleus of the vagus nerve in rats displaying each of the fast (f), medium (m) and slow (s) AHP (from Sah, 1996) 4
2.1. Cells were selected for electrophysiology by eliciting green fluorescence from eGFP-expressing cells 17
2.2. Example IV trace 19
3.1. Canonical $\alpha$-helical CCDs 23
3.2. Alignment of the predicted C-terminal CCDs of hSK1-3 and hIKCa subunits 24
3.3. LOGICOIL predicted C-terminal CCDs of hIKCa 26
3.4. Alignment of hIKCa(WT) and hIKCa(H389E) amino acid sequences 28
3.5. Successful point mutation of $c1166$ and $c1168$ to $g1166$ and $g1168$ in the human $KCNN4$ gene 34
3.6. tsA201 cells do not endogenously express KCa channels 40
3.7. Currents evoked from cells co-expressing either hSK1 or hSK3 and hIKCa were not affected by DMSO 41
3.8. Pharmacology of expressed hIKCa-mediated current 43
3.9. Apamin sensitivity of expressed hSK1- and hSK3-mediated currents 45
3.10. UCL1684 sensitivity of expressed hSK1- and hSK3-mediated currents 47
3.11. TRAM34 insensitivity of expressed hSK1- and hSK3-mediated currents 48
3.12. Apamin insensitivity of hSK1-hIKCa- hSK3-hIKCa-mediated currents 49
3.13. UCL1684 sensitivity of currents evoked from outside-out hSK1-hIKCa-expressing patches and hSK3-hIKCa-expressing cells 51
3.14. TRAM34 sensitivity of currents evoked from outside-out hSK1-hIKCa-expressing patches and hSK3-hIKCa-expressing cells 53
3.15. Comparison of hSK1, hIKCa and hSK1-hIKCa(WT) current amplitude

3.16. Amplitude and TRAM34 sensitivity of hIKCa(H389E)-mediated current

3.17. Apamin sensitivity of currents evoked from tsA201 cells co-expressing hSK1 and hIKCa(H389E)

3.18. UCL1684 sensitivity of currents evoked from whole tsA201 cells co-expressing hSK1 and hIKCa(H389E)

3.19. TRAM34 sensitivity of currents evoked from whole tsA201 cells co-expressing hSK1 and hIKCa(H389E)

4.1. The Ca²⁺ sensitivity of hSK1-, hIKCa, and hSK1-hIKCa-mediated currents

4.2. ‘Ca²⁺-dependence of hippocampal SK channel activity’ (adapted from Hirschberg et al., 1999 (‘Figure 1’))

5.1. hIKCa subunits contain two predicted C-terminal CCDs, whereas hSK1 subunits contain one predicted C-terminal CCD
List of Abbreviations

KCa: Ca^{2+}-activated K^{+} channel/conductance/current
Ca^{2+}: calcium ion
K^{+}: potassium ion
pS: picosiemens (10^{-12} siemens)
AHP: afterhyperpolarisation
sAHP: slow AHP
mAHP: medium AHP
fAHP: fast AHP
CA1: cornu ammonis area 1
BK: big/large-conductance KCa channel
IKCa: intermediate-conductance KCa channel
SK: small-conductance KCa channel
KCNMA1: potassium Ca^{2+}-activated channel subfamily M alpha 1 gene
Slo1: slowpoke 1 gene
KCNN: potassium intermediate/small conductance Ca^{2+}-activated channel subfamily
N
TMD: transmembrane domain
S1-6: KCa subunit TMD segments 1-6
RCK domain: regulator of K^{+} conductance domain
Cav: voltage-activated Ca^{2+} channels/conductance/current
TEA: tetraethylammonium
CTX: charybdotoxin
CCD: coiled-coil domain
CaM: calmodulin
CaMBD: CaM-binding domain

h(KCa): human KCa channel

r(KCa): rat KCa channel

tsA201: Transformed temperature-sensitive T antigen-expressing HEK293 cell line

DMEM: Dulbecco’s Modified Eagle Medium

FBS: Foetal bovine serum

cDNA: complementary DNA

eGFP: enhanced green fluorescent protein

PEI: polyethylenimine

HCl: hydrochloric acid

KOH: potassium hydroxide

kHz: kilohertz

Kv: voltage-gated K+ channels/conductance/current

IV: current (I)-voltage(V)

EC50: effective concentration 50; or, throughout this project, the concentration of Ca2+ that activates a KCa channel by 50% its maximal activity

IC50: inhibitory concentration 50; or, throughout this project, the concentration of drug that blocks 50% of KCa-mediated current

nH: Hill coefficient/slope

SEM: standard error of the mean

Imax: maximal current amplitude measured at -60 mV

I: current amplitude measured at -60 mV under a given condition (e.g. Ca2+ concentration); or, current amplitude measured at -60 mV in the presence of drug

Icontrol: current amplitude measured at -60 mV in the absence of drug

Amin: Imin/Icontrol
$A_{\text{max}} = \frac{I_{\text{max}}}{I_{\text{control}}}$

$A_{\text{frac}}$: Current amplitude at the maximum of the high-sensitivity component $\frac{I_{\text{frac}}}{I_{\text{cont}}}$

$IC_{50,a}$: the IC$_{50}$ of the high-sensitivity component of a two-component Hill equation

$nH_{a}$: the Hill coefficient of the high-sensitivity component of a two-component Hill equation

$IC_{50,b}$: the IC$_{50}$ of the low-sensitivity component of a two-component Hill equation

$nH_{b}$: the Hill coefficient of the low-sensitivity component of a two-component Hill equation

H389: histidine residue at position 389 in the second predicted C-terminal CCD of the hIKCa amino acid sequence

H358: histidine residue at position 358 in the first predicted C-terminal CCD of the hIKCa amino acid sequence

H389E: point mutation, where the H389 residue of hIKCa is substituted for a glutamate (E) residue

H475: histidine residue at position 475 in the predicted C-terminal CCD of the hSK1 amino acid sequence

H475E: point mutation, where the H475 residue of hSK1 is substituted for a glutamate (E) residue

NDPK-B: nucleoside diphosphate kinase B

WT: wild-type

NAM: negative allosteric modulator

UCL1684: 6,10-Diaza-3(1,3),8(1,4)-dibenzena-1,5(1,4)-diquinolinacyclodecaphane

TRAM34: 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole

$I^{-60 \text{ mV}}$: current amplitude measured at -60 mV

PCR: polymerase chain reaction
dsDNA: double-stranded DNA

dNTP: deoxyribonucleotide triphosphate

bp: base pairs

OD: outside density

ID: inside density

mOhm: megaohm

M: concentration in molar (mol.L⁻¹)

mM: concentration in millimolar (10⁻³ M)

µM: concentration in micromolar (10⁻⁶ M)

nM: concentration in nanomolar (10⁻⁹ M)

pM: concentration in picomolar (10⁻¹² M)

Osm: osmolarity in osmole

mOsm: osmolarity in milliosmole (10⁻³ Osm)

DMSO: dimethyl sulfoxide

dH₂O: deionised H₂O

Vₘ: membrane potential

mV: millivolt

HEK293: human embryonic kidney cell line 203

[X]: concentration of X (drug/Ca²⁺)

LS: low-sensitivity component

HS: high-sensitivity component

STORM: Stochastic Optical Resonance Microscopy

FRET: Fluorescence Resonance Energy Transfer

CHO: Chinese Hamster Ovary cell line

COS-7: African green monkey kidney fibroblast cell line
CaMKII: CaM kinase II
Chapter 1: General Introduction

1.1 KCa Channel Structure and Function

The presence of a calcium (Ca\textsuperscript{2+})-activated potassium (K\textsuperscript{+}) (KCa) conductance was first described in erythrocytes (Gardos, 1958) and was later shown to be important in regulating the firing frequency of both invertebrate and vertebrate nervous tissue (Meech, 1978). KCa conductances of 220 pS and 19 pS were eventually shown to contribute to the afterhyperpolarisation (AHP) phase of action potentials in CA1 pyramidal neurones of the hippocampus (Lancaster and Adams, 1986, Lancaster \textit{et al}., 1991), with the family of KCa ion channels underlying these currents cloned soon after. These KCa channel types are big- (BK, KCa1), intermediate- (IKCa, SK4, KCa3.1) and small- (SK1-3, KCa2.1-KCa2.3) conductance, named for their respective single-channel conductances of 100-250 pS, 30-50 pS, and 2-10 pS, respectively (Adelman \textit{et al}., 1992, Kohler \textit{et al}., 1996, Ishii \textit{et al}., 1997, Joiner \textit{et al}., 1997).

1.1.1 KCa Channel Structure

The pore-forming components of human BK, SK and IKCa channels are expressed at the plasma membrane as tetramers of \(\alpha\)-subunits encoded by \textit{KCNMA1}/\textit{Slo1} and \textit{KCNN1}-4 genes, respectively. These \(\alpha\)-subunits share a similar topology, made up of 6 (SK and IKCa subunits) or 7 (BK subunits) transmembrane domains (TMDs), as well as key molecular properties, including a TMD segment (S) 5-6 pore-forming domain that contains the K\textsuperscript{+} selectivity motif, GYGD (Shen \textit{et al}., 1994, Tseng-Crank \textit{et al}., 1994, Kohler \textit{et al}., 1996, Joiner \textit{et al}., 1997) (Fig. 1.1)
**Figure 1.1. KCa channel topology (adapted from Gueguinou et al., 2014).** **Top.** SK and IKCa α-subunit. Key properties shown are the 6 TMD topology, S5-S6 pore-forming domain containing the S6 GYGD motif, intracellular N- and C-terminal domains, tethered calmodulin and predicted coiled-coil domains. Four SK and IKCa α-subunits assemble to form one tetrameric channel. **Bottom.** BK α-subunit. BK subunits are visibly similar to SK and IKCa channels in topology and also assemble as tetramers. Key differences between BK and SK/IKCa channels shown are the 7 TMD topology, large extracellular N-terminus, voltage-sensitive S4 domain, long intracellular C-terminal tail region containing the Ca²⁺ bowl, and β/γ subunits. **Right.** Representation of a K⁺-conducting KCa channel.
**1.1.2 BK Channels**

BK channels are coincidence detectors for membrane depolarisation and intracellular Ca\(^{2+}\) (Marty *et al.*, 1981, Latorre *et al.*, 1982). The S4 domain contains four cationic amino acid residues that are able to promote conformational changes that open BK channels in response to depolarisation (Diaz *et al.*, 1998). Subunits also have a long intracellular C-terminus containing two regulator of K+ conductance (RCK) domains that provide Ca\(^{2+}\) sensitivity (Jiang *et al.*, 2002). A string of five aspartate residues within RCK2 constitutes the BK 'Ca\(^{2+}\) bowl', which promotes BK activation at Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_{i}\)) below 10 \(\mu\)M (Schreiber & Salkoff, 1997, Jiang *et al.*, 2002). In neurons, the Ca\(^{2+}\)-dependent component of BK activity relies upon Ca\(^{2+}\) entry through voltage-gated Ca\(^{2+}\) (Ca\(_{v}\)) channels that are in physical contact with BK channels (Marrion & Tavalin, 1998, Fakler & Adelman, 2008).

Alternative splicing of auxiliary \(\beta\) and \(\gamma\) subunits confers tissue-specific biophysical properties and pharmacology upon BK channels (Knaus *et al.*, 1994b, Knaus *et al.*, 1994c, Brenner *et al.*, 2000, Xia *et al.*, 2000, Zhang & Yan, 2014) (Fig. 1.1).

BK channels underlie the fast (f) component of the AHP (fAHP) (Fig 1.2), making them crucial in the repolarisation of neurons and in regulating the intervals between action potential spikes. Both BK channels and the fAHP are sensitive to block by tetraethylammonium (TEA) and charybdotoxin (CTX), but insensitive to block by the honeybee venom peptide toxin, apamin, a highly-selective SK blocker (Sah, 1996).
Figure 1.2. AHP generated from dorsal motor nucleus of the vagus nerve in rats displaying each of the fast (f), medium (m) and slow (s) AHP (from Sah, 1996). Left (‘E’). The fAHP can be seen as a downward deflection activating and decaying within milliseconds, while the mAHP activates within tens of milliseconds and decays more slowly, and the sAHP activates after a few hundred milliseconds and decays over several seconds. Right (‘F’). Outward currents underlying the fAHP and mAHP (I_{AHF}) and sAHP (I_{sAHF}) (Sah, 1996).

1.1.3 SK Channels

Despite sharing some structural features with BK channels (Fig. 1.1), SK channels have a number of distinct structural properties that allow them to carry out different functional roles. Functional SK channels are made up of four SK α-subunits, each containing six TMD segments (S1-S6) (Kohler et al., 1996) and intracellular N- and C-terminal domains. These subunits can assemble as either homomeric or heteromeric channels (Church et al., 2015), and assembly is proposed to occur via coiled-coil domains (CCDs) within subunit C-terminal tail domains (Tuteja et al., 2010). SK channels contain two cationic residues within S4, but unlike BK channels, they are insensitive to changes in membrane potential (Kohler et al., 1996). In further contrast to BK channels, the Ca^{2+} gating of SK channels is not mediated by
a Ca\textsuperscript{2+} bowl region. Instead, a calmodulin (CaM) is constitutively tethered to each SK subunit by a CaM-binding domain (CaMBD) within each subunit C-terminus. Each tetrameric channel contains four CaM domains, conferring cooperativity of SK activation by Ca\textsuperscript{2+} (Xia et al., 1998). The Ca\textsuperscript{2+} sensitivity of SK channels will be investigated and discussed in Chapter 4.

1.1.4 IKCa Channels

The $\alpha$-subunit of IKCa channels, like that of SK channels, is composed of six TMD segments (S1-S6) with intracellular N- and C-terminal domains (Joiner et al., 1997) (Fig. 1.1). The amino acid sequences of SK and IKCa $\alpha$-subunits share 40% sequence similarity, resulting in IKCa initially being named ‘SK4‘ (Joiner et al., 1997, Ishii et al., 1997). These channels are now mostly referred to as IKCa, since properties such as their larger single-channel conductance of 30-50 pS, unique pharmacology, and differing range of physiological roles distinguish them from SK1-3 channels (Joiner et al., 1997, Ishii et al., 1997). IKCa channels are also tetrameric in composition. The C-terminus of IKCa subunits contain predicted CCDs, through which runs a leucine zipper motif that is involved in IKCa trafficking (Syme et al., 2003), though the importance of this region in tetrameric assembly specifically has yet to be determined. Like SK subunits, each IKCa subunit comprising the tetrameric channel possesses a constitutively tethered CaM, conferring cooperativity of activation by Ca\textsuperscript{2+} upon IKCa channels (Joiner et al., 1997, Khanna et al., 1999, Lee & MacKinnon, 2018). The assembly of IKCa subunits has also been proposed to be mediated by these tethered CaM domains (Joiner et al., 2001). IKCa channels sense cytosolic changes in Ca\textsuperscript{2+} concentration in non-neuronal tissues to contribute to processes such as erythrocyte volume control (Gardos et al., 1958), endothelial hyperpolarisation (Quilley et al., 1997), and cancer cell proliferation (Lallet-Daher et al., 2009, Wulff & Castle, 2010). However, the true Ca\textsuperscript{2+} sensitivity of IKCa channels, after more than 20 years since being cloned, is still vague, as will be discussed in Chapter 4.
The initial belief that the roles of IKCa and SK channels shared little overlap has been challenged in recent years by immunocytochemistry, electrophysiology and pharmacology that maps IKCa subunit expression to SK-expressing neurons (Stocker & Pedarzani, 2000, Sailer et al., 2002, Turner et al., 2015, King et al., 2015, Turner et al., 2016) and cardiac tissue (Tuteja et al., 2005, Weisbrod, 2013). With such widespread expression in the body and potentially important involvement in processes such as neuronal and cardiac excitability, it is clear that the IKCa channel has been a chronically under-investigated member of the KCa family.

1.1.5 Heteromeric KCa Channels

Heteromeric assembly of human (h) SK1-hSK2 channels has been shown to be preferential to the formation of either homomeric channel when both subunits are co-expressed (Church et al., 2015). Indeed, Western blot analysis showed that co-expression of hSK1 with rat (r) SK2 subunits increased plasma membrane expression of Myc-tagged rSK2, whereas co-expression of rSK1 with rSK2 held more rSK2 subunits within cytosolic compartments (Autuori et al., 2019). This suggests that SK1 subunits might act as chaperones of other SK subtypes, either reducing or enhancing plasma membrane expression. hSK1 subunits and hIKCa subunits also preferentially co-assemble when co-expressed in a heterologous expression system (Higham et al., 2019), suggesting that hSK1 subunits might act as chaperones for IKCa subunits as well as other SK subunits. Further evidence that might be relevant to this theory comes from the fact that, although IKCa subunits have been proposed to be involved in the sAHP of CA1 neurons, the pharmacology of the sAHP differs slightly from that of homomeric IKCa channels (King et al., 2015) and resembles more closely the pharmacology of the novel heteromeric hSK1-hIKCa channel (Higham et al., 2019). Obviously, the existence of hSK1-hIKCa heteromers does not confirm that SK1 subunits chaperone IKCa subunits to the plasma membrane and further experiments would be
needed to determine whether the expression levels of heteromeric channels differs from that of homomeric channels.

There is a real possibility that heteromeric KCa channels are important in a number of physiological processes, not only in the brain. For example, pharmacological and immunocytochemistry evidence indicates that the repolarisation of action potentials in atrial myocytes is mediated in part by heteromeric SK2-SK3 channels (Hancock et al., 2015). Moreover, since SK1-SK2, SK2-SK3 and SK1-IKCa heteromers all display either subtly or markedly different pharmacology when compared with each respective homomeric channel, uncovering the differential distribution of SK and IKCa subunits, and mapping heteromer expression, could become an important step in beginning to develop subtype-specific therapies for diseases involving SK and IKCa channels.
1.2 The Slow Afterhyperpolarisation

The sAHP is a hyperpolarising potential that peaks 2 seconds after a train of action potentials and decays after 5 seconds (Sah et al., 1996). The sAHP results from the activation of a Ca\(^{2+}\)-activated current (Lancaster & Adams, 1986) that is not sensitive to the highly-selective homomeric SK blocker, apamin (Lancaster et al., 1991, Sah & McLachlan, 1991, Sah et al., 1996) but is sensitive to the IKCa and SK1-IKCa channel blocker, TRAM34 (King et al., 2015, Higham et al., 2019), and can be modulated by a variety of neurotransmitters (Nicoll, 1988, Church et al., 2019). Noradrenaline, for example, suppresses the sAHP via activation of \(\beta\)-adrenoceptors (Madison & Nicoll, 1982, Sah & Isaacson, 1995), reducing spike frequency adaptation and in turn increasing intrinsic neuronal excitability, which can entrain learning in CA1 pyramidal cells; the opposite – reduced intrinsic excitability and greater spike frequency adaptation due, in part, to an increase in sAHP amplitude – occurs in aging neurons (Disterhoft & Oh, 2006). Also, in CA1 neurons in Alzheimer’s disease (AD) brains, the upregulation of L-type Ca\(_V\) channel expression and subsequent impairment of cellular Ca\(^{2+}\) homeostasis has been linked to significant neuron loss (Coon et al., 1999). Since Ca\(^{2+}\) entry through L-type Ca\(_V\) channels creates Ca\(^{2+}\) microdomains that activates co-localised SK-type channels (Marrion & Tavalin, 1998), it is possible that L-type Ca\(_V\) channel upregulation increases sAHP amplitude by potentiating the activity of the KCa channel underlying the sAHP. If the KCa channel(s) underlying the sAHP in CA1 pyramidal neurons can be identified, sAHP channel-specific therapeutics could be developed to modify diseases in which neuronal excitability is involved.
1.3 Chapter References


Chapter 2: General Methods

2.1 Cell culture and transient transfection

tsA 201 cells were incubated in culture at 37°C and 5% CO₂. Cells were maintained in culture flasks with Dulbecco’s Modified Eagle Medium (DMEM) supplemented with foetal bovine serum (FBS) (10%) and penicillin and streptomycin (1%) in a ratio of 1:8 to 1:32. Cells were passaged at around 80% confluence.

The cDNA of all KCa channel subunits used, as well as enhanced green fluorescent protein (eGFP), were contained within pcDNA3.1 plasmid vectors, which can be efficiently transfected into tsA201 cells by polyethylenimine (PEI) (Merck/Sigma, UK). To prepare the PEI solution at a concentration of 0.2 µg/µL, 10 mg PEI salt was first dissolved in dH₂O by reducing the pH to 2 using dropwise additions of 12 M hydrochloric acid (HCl). The pH of the PEI solution was then raised to 7.4 by dropwise additions of 7 M potassium hydroxide (KOH) solution. The PEI solution was then made up to 50 mL using dH₂O, divided into 1 mL aliquots and frozen at -20°C. The transfection mixture was prepared under a sterile cell culture hood by adding channel plasmid and eGFP to a sterile eppendorf tube in a 4:1 ratio alongside 80-120 µL of PEI. This mixture was incubated for 40 minutes in pure DMEM and then added dropwise to 35 mm cell culture plates containing tsA201 cells, which were incubated for 24-48 hours before being used for electrophysiology.

2.2 Electrophysiology

Electrophysiology is a technique, invented and pioneered in the late 1930s by Hodgkin and Huxley, that uses electrodes to record the electrical changes that occur due to the movement of ions across cell membranes. Since then, the technique has become more sophisticated, and the term electrophysiology encompasses numerous specialised and distinct techniques. Throughout this project, voltage clamp was used in whole cell, outside-
and inside-out patch configurations to record the movement of K⁺ ions across tsA201 cell membranes and membrane patches, revealed, for the most part, by voltage ramp protocols.

2.2.1 Electrophysiology rig

A 35 mm tissue culture dish (Falcon) containing tsA201 cells was placed into a chamber within an adjustable platform that was mounted onto an inverted light microscope (Zeiss Axiovert 100, Germany). To resolve the amplitude, shape and sensitivity of currents it was important to minimize the noise picked up from the rig during recording. Therefore, this platform was mounted on a nitrogen-inflated air table, which isolated the set-up from surrounding vibrations. The rig was also grounded by metal clips to various metal parts of the rig. Patch electrodes were inserted into a CV201A head-stage (Axon Instruments, CA), which was mounted onto the adjustable platform and had inputs from and outputs to an Axopatch 200A amplifier (Axon Instruments, CA). The head-stage was also connected to a PatchStar micromanipulator (Scientifica), which adjusted the orientation of the head-stage and therefore the position of the patch electrode.

Cells were chosen for electrophysiology by first using visible light from the inverted light microscope to identify a viable cell. Ultraviolet (UV) light from a mercury lamp was then reflected into the path of the cell plate using a dichroic mirror, which would excite expressed eGFP in successfully transfected cells, emitting green fluorescence (Figure 2.1). Viable fluorescent cells were then used for electrophysiology.
Figure 2.1. Cells were selected for electrophysiology by eliciting green fluorescence from eGFP-expressing cells. A. tsA201 cell exposed to visible light through an inverted light microscope. B. The same tsA201 cell under UV light reflected by a dichroic mirror emitting green fluorescence.

tsA201 cells and excised patches were superfused with a bathing solution of around 1 mL in 35 mm tissue culture dishes. This solution was applied via an in-flow pipe connected to 60 mL reservoir syringes, which were filled with 50 mL bathing solution at any time during recording. Bathing solution flowed into the dish via gravity at a rate of 10-12 mL/min that was determined by the tightness of an adjustable clamp attached to the in-flow tube. Bathing solution was sucked from the plate, through an out-flow pipe, by a Watson Marlow peristaltic pump (Falmouth, Cornwall, England). Flow pipes were diametrically opposed in relation to one another to ensure that the stream of bathing solution flowed through the centre of the plate, so that patches were continually exposed to flowing solution.

Pulse 8.80 software (HEKA Elektronik) was used to apply voltage protocols and command the membrane potential at which the Axopatch 200A amplifier would hold either excised patches or whole cell membranes. During recording, data was low-pass filtered (Bessel, Frequency Devices) at a rate of 1 kHz and then acquired by Pulse 8.80 at a rate of 10 kHz. This created a safety factor of 10 between the A/D rate (10 kHz) and filter bandwidth (1 kHz), minimising the distortion of data that can be produced when filtering and acquisition rates are too close together. All recordings were made at room temperature (around 22°C).
2.3 Recording & Analysis

2.3.1 Data collection

tsA201 cells endogenously express voltage-gated K⁺ (Kᵥ) channels that activate around +30 mV. It was important to measure current amplitude at a voltage within the -100 mV to +100 mV voltage ramp where no endogenous ion channels were active, as they could contaminate the expressed KCa component of evoked current traces. In addition, expressed currents should have been bigger than endogenous currents. As shown in Figure 2.2, under isotonic K⁺ conditions the current trace at -60 mV is mostly linear, larger than and uncontaminated by endogenous K⁺ channels. Current amplitudes were always measured at -60 mV (I₋₆₀ mV) throughout this project as a result. To maximise the linear region of current traces, all electrophysiology that was performed on cells expressing KCa channel subunits was carried out under isotonic K⁺ conditions, which forced the K⁺ reversal potential to be 0 mV (Figure 2.2). Origin 9.0 software (Microcal Software Inc., MA) was used to generate current-voltage (IV) graphs.
Figure 2.2. Example IV trace. This trace was recorded by applying a voltage ramp from -100 mV to +100 mV to an outside-out patch excised from an eGFP-positive tsA201 cell transiently expressing hSK1 subunits under isotonic K⁺ conditions. The leftmost red arrow indicates the point on the current trace at which amplitude would be measured when reading from -60 mV. The rightmost red arrow indicates the onset of inward rectification as the membrane potential becomes more positive.

2.3.2 Data analysis

The statistical significance of the differences between groups was determined throughout this project using the Student's unpaired t-test within Graphpad Prism 7 software. The p values quoted correspond to the following certainty that the difference between the means occurred by chance: p > 0.05 indicates less than 95% certainty that the difference was significant; p < 0.05* indicates 95% certainty that the difference was significant; p < 0.01** indicates 99% certainty that the difference was significant; and p < 0.001*** indicates 99.9% certainty that the difference was significant. Graphpad Prism 7 was also used to construct all concentration-response and concentration-inhibition curves. Curves were fitted to data using
either one or two variable four-parameter Hill equations, which are provided below. Values for the concentration of drug required to inhibit 50% of total current (IC50), concentration of Ca2+ required to evoke 50% of the total current response (EC50), maximum response/inhibition (%), and Hill coefficient (nH) were quoted as a mean value ± standard error of the mean (SEM). To generate SEM values, first curves were fitted to each replicate to produce values for each of the parameters listed above. These values were then grouped and analysed for descriptive statistics to produce mean ± SEM values for each parameter.

The following equations were used throughout:

i) Normalised current:

\[
\text{Normalised current} = \frac{I_{\text{max}} - I}{I_{\text{max}}}
\]

where \( I_{\text{max}} \) is the maximal current amplitude measured at -60 mV, and \( I \) is the current amplitude produced by a given concentration of Ca2+ ;

ii) Percentage inhibition:

\[
\text{Inhibition \( (%) \)} = \frac{I - I_{\text{control}}}{I_{\text{control}}} \times 100
\]

where \( \text{inhibition \( (%) \)} \) is the percentage reduction in current amplitude produced by addition of drug, \( I_{\text{control}} \) is current amplitude measured at -60 mV in the absence of drug, and \( I \) is current amplitude in the presence of drug;

iii) Hill equation

\[
\frac{I}{I_{\text{control}}} = A_{\text{min}} + \left( \frac{A_{\text{max}} - A_{\text{min}}}{1 + 10^{(\log EC_{50} - X) \times nH}} \right)
\]
where $I_{\text{control}}$ is current amplitude in the absence of drug \((X)\), expressed in logarithmic units, $I$ is current amplitude in the presence of drug, $A_{\text{min}}$ is $I_{\text{min}} / I_{\text{control}}$, $A_{\text{max}}$ is $I_{\text{max}} / I_{\text{control}}$, $IC_{50}$ is the concentration of drug that blocks 50% of KCa-mediated current, and $n_H$ is the Hill coefficient.

iv) Two-component Hill equation, used to fit biphasic concentration-inhibition curves

$$\frac{I}{I_{\text{control}}} = A_{\text{min}} + \left(\frac{A_{\text{frac}} - A_{\text{min}}}{1 + 10^{(LogIC_{50,a} - X) \times n_{H,a}}}\right)$$

$$+ \left(\frac{A_{\text{max}} - A_{\text{min}}}{1 + 10^{(LogIC_{50,b} - X) \times n_{H,b}}}\right)$$

where $A_{\text{frac}}$ is current amplitude at the maximum of the high-sensitivity component $I_{\text{frac}} / I_{\text{control}}$, $IC_{50,a}$ is the IC$_{50}$ of the high-sensitivity component, $n_{H,a}$ is the Hill coefficient of the high-sensitivity component, $IC_{50,b}$ is the IC$_{50}$ of the low-sensitivity component, and $n_{H,b}$ is the Hill coefficient of the low-sensitivity component.
Chapter 3: Co-assembly of hSK1 and hIKCa subunits via C-terminal CCDs

3.1 Chapter Introduction

3.1.1 Preliminary co-expression of hSK3 and hIKCa subunits

In this chapter, the primary aim was to investigate the preferential assembly of heteromeric hSK1-hIKCa channels. First, the primary sequences of SK2 and SK3 subtypes were assessed for motifs that might preclude their preferential co-assembly with hIKCa subunits. Unpublished data from the Marrion lab indicates that hSK2 subunits do indeed form heteromers when co-expressed with hIKCa subunits, so the sequence of hSK3 was considered instead. The amino acid sequences of hSK1 and hSK3 subunits were aligned using EMBOSS Needle (EMBL EBI) (Madeira et al., 2019) software, revealing a 57% similarity (49.2% sequence identity). This suggested that there could be structural differences in hSK3 that make co-assembly with hIKCa subunits less favourable. However, the results of the pharmacological experiments presented below suggested that hSK3 and hIKCa subunits co-assemble to form heteromeric channels. Therefore, sequence differences between hSK1 and hSK3 would not be useful for investigating the molecular determinants of heteromeric hSK1-hIKCa channel formation. C-terminal CCDs have been proposed to mediate SK subunit assembly (Tuteja et al., 2010). Therefore, the predicted C-terminal CCDs within hIKCa and hSK1 subunits were considered as sites that could influence preferential subunit co-assembly, rather than differences between the amino acid sequences of hSK1 and hSK3 subunits.
3.1.2 Coiled-coil domains

α-Helical CCDs are secondary protein structures that can mediate protein-protein interactions and folding, or form as larger structural features (Woolfson et al., 2012). They assemble from two or more supercoiled amino acid α-helices (Crick, 1953, O’Shea et al., 1991) and as shown in Figure 3.1, consist of repeating heptad motifs denoted \( abcd\rightarrow\), where residues at \( a \) and \( d \) positions are hydrophobic and residues at \( bcefg \) positions are polar (Woolfson et al., 2012). However, many proteins contain variations of this motif, which can be structurally or functionally significant (Woolfson et al., 2012).

![Figure 3.1. Canonical α-helical CCDs (from Woolfson et al., 2012) Top (‘a’). Right-handed coiled-coil of tropomyosin. Bottom. The helical-wheel diagram on the left (‘b’) shows the orientation of residues within the typical CCD heptad repeat, which is also apparent in the yeast transcriptional activator GCN4 on the right (‘c’)](image)

3.1.3 The role of C-terminal CCDs in KCa channel assembly

CCDs are predicted to occur distal to the CaMBD in the C-terminus of human and rat SK1-3 as well as IKCa subunits (Fig. 3.2). Crystal structures of rSK2 and hIKCa CCDs have now
been resolved (Kim et al., 2007, Ji et al., 2018), confirming the presence of predicted C-terminal CCDs in these channels and increasing the degree to which CCD prediction programmes can be trusted for KCa channels of which we do not have crystal structures.

**Figure 3.2 Alignment of the predicted C-terminal CCDs of hSK1-3 and hIKCa subunits.**

Primary sequences of hSK1-3 and hIKCa were entered into Clustal Omega (EMBL EBI) (Madeira et al., 2019) for multiple sequence alignment. Above is the C-terminal tail region of each channel subunit directly after the CaMBD. Predicted CCDs of each sequence are outlined in black, the H389 residue of hIKCa is outlined in red, and the first two predicted heptad repeats of hIKCa’s C-terminal CCD are indicated by the heptad letter coding as a superscript.

C-terminal CCDs have been implicated in SK subunit assembly. One study synthesised inhibitory peptides based upon SK subunit C-terminal CCDs and intracellularly infused them during whole cell recordings from tsA201 cells and atrial myocytes. They found that SK current was inhibited following infusion of these inhibitory peptides (Tuteja et al., 2010). This would suggest that CCDs maintain subunit assembly at the plasma membrane. Another study reported that when co-expressed in HEK293 cells, hSK1 and rSK2 subunits did not co-assemble to form heteromeric hSK1-rSK2 channels, but hSK1 and hSK2 subunits did. The reason for this species-specific assembly was isolated to a pair of differing amino acids within the C-terminal CCDs of rSK2 (T543/N547) and hSK2 (A542/S546). Substitution of the
T543/N547 residues present in rSK2 with A542/S546 in hSK2 resulted in co-assembly of hSK1 with mutant rSK2 subunits (Church et al., 2015). This showed that SK subunit assembly was reliant upon two amino acids present within C-terminal CCDs, suggesting that assembly is mediated by this region and in a way that is highly specific.

Coiled-coil predication programmes suggest that hIKCa subunits contain two separate C-terminal CCDs (Fig. 3.3). The CCD that is N-terminal to the CaMBD contains a histidine at position 358 (H358) (Fig. 3.3, B). It has been suggested that H358-Cu²⁺ interactions within the IKCa channel four helix bundle prevents the conformational changes involved in channel opening (Srivastava et al., 2016) and that phosphorylation of H358 by nucleoside diphosphate kinase B (NDPK-B) is required to disrupt the H358-Cu²⁺ interactions and permit CaM-mediated activation (Srivastava et al., 2006). However, a crystal structure of IKCa’s C-terminus containing H358 has never been resolved in support of this. Another histidine residue is contained within IKCa’s second C-terminal CCD that is C-terminal to the CaMBD, at position 389 (H389) (Fig. 3.3, B), of which a crystal structure has been resolved (Ji et al., 2018). This crystal structure suggested that the H389 residue also bound Cu²⁺ (Ji et al., 2018), though it did not shed any light upon whether this occurs natively, or whether H389-Cu²⁺ interactions are involved in IKCa channel activation. Nevertheless, these crystal structures contribute to the pool of evidence signalling that histidine residues might be particularly important in protein function and assembly. For example, the phosphorylation state of H358 in hIKCa regulates channel activity (Srivastava et al., 2006), and protonation of the imidazole side chain of histidine at lower intracellular pH can be important in protein-protein interactions and assembly (Schonichen et al., 2013). It is therefore reasonable to believe that H389 could play a role in one of the folding, assembly, trafficking, or function of hIKCa or heteromeric hSK1-hIKCa channels. Since H389 occurs within C-terminal CCDs, which have been proposed to mediate SK channel assembly, the experiments in this chapter primarily investigated whether mutating H389 in hIKCa subunits affected the assembly of homomeric hIKCa, and heteromeric hSK1-hIKCa channels.
Figure 3.3. LOGICOIL predicted C-terminal CCDs of hIKCa. **A** LOGICOIL (Vincent et al., 2013) output of the likelihood (%) that CCDs are present (P(coiled-coil)) across amino acid positions from the N- to C-terminus of the primary sequence of hIKCa. As indicated by the red peaks (Marcoil confidence level), from the start of the C-terminus of hIKCa (right side of x-axis) the likelihood that CCDs are present increases from around 30% to almost 100%. **B** Primary sequence of the C-terminal region of hIKCa, containing H358 and H389 residues outlined in red (top), and the predicted heptad repeat position of each residue (bottom). Black outlined boxes indicate inconsistencies between the LOGICOIL output and the residue denotation proposed by the IKCa CCD crystal structure in Ji et al. For example, the ‘ISKM’ sequence is predicted to be at defg positions within a heptad repeat by LOGICOIL, whereas the crystal structure suggests a break in the CCDs here, producing aefg instead. This results in the H358 residue assuming an inward-facing a position, like H389 (Ji et al., 2018).
3.1.4 The H389E mutation in hIKCa subunits

In wild-type hIKCa (hIKCa(WT)) subunits, H389 was substituted with a glutamate/E residue to produce the mutant hIKCa(H389E) (Fig. 3.4). Glutamate was chosen, firstly, because its carboxylic acid side chain has a pKa of 4.3, making it negatively charged at the pH of the cytosol (around 7.2 in tsA201 cells) and intracellular organelles proposed to be involved in KCa channel assembly, such as the Golgi. This would eliminate the potential for protonation at this residue and remove the influence this protonation might have on subunit assembly. Moreover, the intra- and inter-chain interactions made by a negatively charged E389 might differ from those made by H389, which might disrupt CCD interactions. Finally, because glutamate is a similar sized amino acid compared with histidine, any impact of this mutation should be limited to either the lack of E389 protonation, or the interactions removed or added. It should be noted that mutagenesis has not been used before to disrupt KCa C-terminal CCDs. Rather, a previous study infused inhibitory peptides during whole cell recordings to target and impair CCD assembly at the plasma membrane (Tuteja et al., 2010). Although there was no template for disrupting the C-terminal CCDs of hIKCa subunits using mutagenesis, this method would capture the involvement of this region in channel assembly from translation through to plasma membrane expression.
Figure 3.4. Alignment of hIKCa(WT) and hIKCa(H389E) amino acid sequences. Clustal Omega (EMBL EBI) (Madeira et al., 2019) was used to align hIKCa(WT) (‘KCNN4_HUMAN_WT’) and hIKCa(H389E) (‘KCNN4_HUMAN_H389E’) primary sequences. The only difference between the two sequences is the substitution at position 389 of a histidine (H) for a glutamate (E), which is outlined in black. Asterisks indicate identical residues; black points indicate different residues.

3.1.5 Pharmacology: Apamin

The presence of homomeric SK channels in cells can be revealed using the bee venom peptide toxin, apamin. The only SK channel that cannot be identified using apamin is rSK1, which accumulates within the Golgi rather than being expressed at the plasma membrane (Church et al., 2015). Apamin is a highly negative selective allosteric modulator (NAM) of SK

3.1.6 Pharmacology: UCL1684

Bis-quinolinium cyclophane SK blockers with nanomolar potency have also been designed, such as UCL1684 (Campos Rosa et al., 1998, Hancock et al., 2015). UCL1684 is also a NAM of SK channels, but uses a different mechanism than apamin: though block does require that the S3-S4 extracellular loop be in a certain conformation, it does not bind directly to the S3-S4 loop (Weatherall et al., 2011). Although the novel, apamin-insensitive heteromeric hSK1-hIKCa channel was reported to be insensitive to UCL1684 at the concentration used (100 nM) to initially characterise its pharmacology, some of this data indicated that block might occur at higher concentrations (Higham et al., 2019). If a full concentration-inhibition relationship for UCL1684 on heteromeric hSK1-hIKCa-mediated current could be produced, it might make UCL1684 useful in identifying hSK1-hIKCa channels in native tissues. Like apamin, UCL1684 has no effect upon IKCa-mediated current (Higham et al., 2019).

3.1.7 Pharmacology: TRAM34

Using the antifungal clotrimazole as a template, TRAM34 was designed to be a highly-selective IKCa blocker (IC50 = 20-40 nM) (Wulff et al., 2000, Wulff et al., 2001, Higham et al., 2019). Indeed, TRAM34 does not affect SK-mediated current (Higham et al., 2019). However, heteromeric hSK1-hIKCa channels are sensitive to TRAM34, with a right-shifted
IC$_{50}$ and reduced maximum block (IC$_{50}$ = 557 nM), compared to its effect on hIKCa-mediated current (Higham et al., 2019).
3.2 Chapter Aims

The experiments in this chapter aimed to:

I. use outside-out patch clamp to determine the apamin, UCL1684 and TRAM34 pharmacology of homomeric hSK1-, hSK3- and hIKCa-mediated currents;

II. use outside-out patch clamp to determine the apamin, UCL1684 and TRAM34 pharmacology of heteromeric hSK1-hIKCa-mediated currents;

III. use whole cell voltage clamp to determine the apamin, UCL1684 and TRAM34 pharmacology of currents evoked from cells co-expressing hSK3 and hIKCa;

IV. carry out site-directed mutagenesis of the H389 residue in hIKCa subunit cDNA to E389;

V. use outside-out patch clamp to determine the effect of the H389E mutation on the amplitude and TRAM34 pharmacology of hIKCa(H389E)-mediated current;

VI. use whole cell voltage clamp to investigate the apamin, UCL1684 and TRAM34 pharmacology of currents evoked from cells co-expressing hSK1 and hIKCa(H389E) subunits, using this pharmacology as an indication of the population(s) of channels expressed at the plasma membrane of these cells and therefore of the effect of the H389E mutation on the co-assembly of hSK1 and hIKCa(H389E) subunits;

VII. generate concentration-inhibition curves and derive IC50, Hill coefficient (nH), and maximum inhibition (%) values for the above aims where possible.
3.3 Chapter Methods

3.3.1 Molecular biology: plasmid preparations

i) Transformation of DH5α competent *E. coli* cells

Each of hSK1, hSK3, hIKCa and hIKCa(H389E) subunit cDNAs were contained within pcDNA3.1 plasmid expression vectors. Qiagen plasmid maxi-prep kits were used to make up a large volume of stock plasmids. DH5α competent *E. coli* cells were transformed by mixing with plasmid DNA in 1:10,000 (DNA:DH5α) ratio and imposing a regime whereby cells were heat shocked at 42°C, incubated, and centrifuged to permeate plasma membranes and allow DNA entry. Streaks of transformed cells were made on ampicillin selective plates and incubated overnight to produce bacterial colonies that were then selected for amplification.

ii) Bacterial colony amplification and DNA purification

A single colony of transformed *E. coli* cells was selected and amplified using ampicillin-treated LB broth, then put through several centrifugation and incubation steps to produce a pellet of transformed cells. This pellet was then resuspended and centrifuged to produce a supernatant, from which DNA was then eluted using gravity flow and precipitated using isopropanol. After a final centrifugation step, the DNA pellet was washed with 70% ethanol, redissolved in TE buffer, and applied to a nanodrop. Maxi-preps yielded a final DNA concentration of around 3000 ng.µL⁻¹.

3.3.2 Molecular biology: site-directed mutagenesis

i) Amplification of mutant DNA using PCR

A polymerase chain reaction (PCR) mixture was made using the Agilent QuikChange II XL kit that included the hIKCa double-stranded (ds) DNA template, forward and reverse
oligonucleotide primers (Fig. 3.5, A), a deoxyribonucleotide triphosphate (dNTP) mix, QuikSolution reagent, ddH₂O and PfuUltra HF DNA polymerase. This mixture underwent eight PCR cycles that consisted of temperature changes which separated the dshIKCa DNA template, so that the heat-resistant PfuUltra DNA polymerase could synthesise mutant DNA strands from the forward and reverse primers. The final step of each cycle lasted one minute for every 1000 base pairs (bp) within the pcDNA3.1 hIKCa plasmid. Since the KCNN4 gene is 1,500 bp long and pcDNA3.1 is 5,500 bp long, together they consist of 7,000 bp. Therefore, the final step of the PCR lasted for seven minutes. After the PCR was finished, the mixture was cooled to below 37°C and the Dpn-1 restriction enzyme was added to digest supercoiled parental DNA.

ii) Transformation of XL10-Gold Ultracompetent cells

Dpn-1-treated DNA was added to XL10-Gold ultracompetent E.coli cells, which were then subjected to heat shock at 42°C and added to pre-warmed SOC broth, incubated at 225 rpm and then spread onto ampicillin selective plates, which were incubated overnight.

iii) Bacterial colony amplification and purification of hIKCa(H389E) DNA

A single colony was selected and amplified in ampicillin-treated LB broth for 12-16 hours at 37°C with vigorous shaking. A series of centrifugation, resuspension and washing steps were then followed, as specified in the QuikChange II XL kit instructions, to purify mutant DNA from transformed bacterial cells. Mutant DNA was then eluted and nanodropped before diluting to a concentration of 100 ng.µL⁻¹ and being sent to Eurofins Scientific for sequencing. The nucleotide sequence received was aligned with that of wild-type KCNN4 using the BLAST programme, revealing successful mutation of the histidine 389 codon, cac (1166-1168), to a glutamate codon, gag (Fig. 3.5, B). This mini-prep of hIKCa(H389E) was then maxi-prepped using the Qiagen kit and protocol outlined above.
Figure 3.5. Successful point mutation of c1166 and c1168 to g1166 and g1168 in the human KCNN4 gene. A. Forward and reverse oligonucleotide primer sequences used by PfuUltra HF DNA polymerase to synthesise mutant DNA strands. B. Two cytosines either side of an adenine were substituted with two guanines to change the histidine 389 codon,
cac (1166-1168), to a glutamate, cac, codon (outlined in black). Nucleotide sequence alignment was performed using BLAST. The presence of solid black lines connecting single-letter codes of nucleotides indicates that nucleotides of each sequence are identical, whereas the absence of black lines indicates nucleotide differences.

### 3.3.3 Voltage clamp in whole cell and outside-out patch configurations

By using appropriate pharmacological agents, it is possible to dissect a total macroscopic current of which there might be more than one component ion channel population. With whole cell voltage or outside-out patch clamp, the pharmacological dissection of macroscopic currents can be done with a high resolution during a single recording.

**i) Outside-out patch clamp**

By excising a small patch of plasma membrane from a cell, outside-out patch clamp isolates regions of membrane from the electrical activity of the rest of the cell. Outside-out patches expose the outside face of the plasma membrane to the extracellular solution and the inside face to the electrode solution. If cells are known to express only one population of ion channels at the plasma membrane, outside-out patches allow the properties of the current mediated by these channels to be investigated pharmacologically with higher resolution than whole cell configuration does. Outside-out patches were used to investigate homomeric hSK1, hSK3, hIKCa(WT), hIKCa(H389E), and heteromeric hSK1-hIKCa(WT) currents.

**ii) Whole cell voltage clamp**

Whole cell voltage clamp was chosen to investigate the pharmacology of currents evoked from cells co-expressing hSK3 and hIKCa subunits, as well as cells co-expressing hSK1 and hIKCa(H389E) subunits. If there was more than one population of channel present in co-
expressing cells, excised outside-out patches might not include them all. Currents evoked from whole cells on the other hand are more likely to be composed of each expressed ion channel population.

### iii) Voltage ramp protocol

Currents were revealed from whole cells and outside-out patches using a voltage ramp protocol. From a holding potential of 0 mV, continuous voltage ramps from -100 mV to +100 mV of 1 second duration, were applied across patches every second. This voltage range pushed the membrane potential positive enough to reveal the presence and extent of any current rectification, which can be both an indication of the type of channel mediating the current and the amount of contaminating leak current.

### 3.3.4 Electrodes

For whole cell voltage clamp and outside-out macropatch recordings, micropipettes were fabricated from thick-walled (1.5 mm outside density (OD) x 0.86 inside density (ID)) borosilicate glass capillaries (Harvard Apparatus) using a Narishige model PP-830 puller, fire polished using a model MF-830 Narishige micro-forge, and filled with internal solution, giving pipettes a resistance of 2-4 mOhm for whole cell and 4-6 mOhm for outside-out recordings.

### 3.3.5 Internal (electrode) and external (bathing) solutions

**i) Solutions used to measure endogenous currents from non-transfected tsA201 cells**

The profile of currents endogenous to the tsA201 cell line was investigated using a voltage step protocol in whole cell voltage clamp. During these experiments, cells were perfused with an internal solution containing, in mM, 97 K+ Gluconate, 20 KCl, 1.5 Na\textsubscript{2}ATP, 10 EGTA,
9.65 or 6.2 CaCl$_2$ (1 $\mu$M or 60 nM free Ca$^{2+}$), 10 HEPES Na$^+$, 2.5 or 0 MgCl$_2$ (1 $\mu$M or 0 free Mg$^{2+}$), of pH 7.4 and with an osmolarity between 280-310 mOsm. The pH was adjusted initially to 7 using KOH pellets, followed by dropwise additions of 1 M KOH solution until a reading of pH 7.4 was given by the pH meter (Fisherbrand, USA). The osmolarity was determined to be between 280-310 mOsm using an osmometer (Wescor, ELITech Group, USA), calibrated with standard solutions of 100, 290 and 1000 mOsm (Reagecon, R.O.I) respectively. Cells were bathed in high Na$^+$, low K$^+$ external solution containing, in mM, 150 NaCl, 2.5 KCl, 10 HEPES Na, 10 D-glucose, 1.2 MgCl$_2$, 2.5 CaCl$_2$; pH 7.4, 280-310 mOsm. The pH and osmolarity of external solution was achieved and measured as above for internal solution.

ii) Solutions used to measure expressed currents in whole cell and outside-out patches

The same internal solution used to record endogenous currents from non-transfected tsA201 cells, described above, was used to record expressed currents evoked from tsA201 cells transfected with KCa subunit cDNA in both whole cell and outside-out patch configurations. Transfected cells were bathed in high K$^+$ external solution, containing, in mM, 97 K$^+$ Aspartate, 30 KCl, 10 HEPES Na$^+$, 6.19 CaCl$_2$ (1 $\mu$M free Ca$^{2+}$), 1.44 MgCl$_2$ (1 $\mu$M free Mg$^{2+}$); pH 7.4, 280-310 mOsm. This combination of internal and external solutions created isotonic K$^+$ conditions (around 150 mM intracellularly and extracellularly), resulting in a reversal potential for K$^+$ of 0 mV during voltage ramps.
3.3.6 Drugs

i) TRAM34 and UCL1684

TRAM34 and UCL1684 solids (both Tocris Bioscience, Biotechne, UK) were dissolved in dimethyl sulfoxide (DMSO) (Sigma/Merck, UK) with gentle warming and divided into 100 µL aliquots at 10 mM (TRAM34), 100 µM and 1 mM (UCL1684), which were immediately frozen at -20°C. When required, TRAM34 and UCL1684 stock aliquots were defrosted, then serial dilutions were made using DMSO and dH2O, with aliquots maintained on ice until used. The ratio of dH2O to DMSO was increased with each dilution to limit the concentration of DMSO to which tsA201 cells, and patches excised from them, were exposed.

ii) Apamin

Apamin solid (Tocris Bioscience, Biotechne, UK) was dissolved in dH2O and divided into 50 µL aliquots at 100 µM and immediately frozen at -20°C. When required, apamin stock aliquots were defrosted and serial dilutions were made using dH2O, which were then maintained on ice until they were used.

iii) Cumulative concentration-inhibition regiment

Throughout this chapter, the concentration-inhibition relationships were generated using a cumulative drug addition protocol; this removed the need for drug wash cycles, which can be time-consuming and risk the integrity of excised patches.
3.4 Chapter Results

3.4.1 Control experiments

i) Endogenous tsA201 currents in the presence and absence of intracellular free Ca^{2+}

Endogenous KCa currents have been shown to be absent from HEK293 cells (Yu & Kerchner, 1998), suggesting that they are absent from the transformed HEK293 line, tsA201 as well. However, it was important to determine this first-hand. tsA201 cells were held at -60 mV in whole cell voltage clamp, from which the membrane potential (V_m) was stepped in 10 mV increments negative to -100 mV, and then positive to +50 mV. This voltage step protocol was applied to tsA201 cells perfused with an internal (electrode) solution containing either a low (60 nM) or high (1 µM) concentration of free Ca^{2+}. The IV relationships for each condition would be indistinguishable if tsA201 cells expressed no endogenous Ca^{2+}-activated channels. Indeed, there was no significant difference (p >0.05) between the amplitude of endogenous tsA201 cell currents revealed by this voltage step protocol when cells were perfused with either 60 nM free Ca^{2+} (n = 3) or 1 µM free Ca^{2+} (n = 3) (Fig. 3.6). This confirmed that the tsA201 cell line used throughout this project did not endogenously express hIKCa, hSK1 or hSK3 channels, or any contaminating Ca^{2+}-activated currents.
Fig 3.6. tsA201 cells do not endogenously express KCa channels. A. Step currents evoked from cells perfused with 60 nM (left) or 1 µM (right) free Ca\(^{2+}\). B. IV relationship showing the effect of 10 mV step depolarisations from -100 mV to +50 mV on whole cell I\(^{60}\) mV. To allow comparison between cells, the peak current amplitude (pA) was divided by the whole cell capacitance (pF) of each cell to obtain a normalised amplitude (pA/pF). Recordings that exhibited more than 1 nA of leak current were rejected.

ii) Insensitivity of expressed currents to DMSO

At the end of one cumulative TRAM34 or UCL1684 concentration-inhibition protocol, tsA201 cells or outside-out patches would have been exposed to 0.326% DMSO. To produce a
concentration of 0.326% DMSO in the bathing solution, 163 \( \mu \)L DMSO was added to the reservoir of 50 mL external solution. Current evoked from whole cells co-expressing either hSK1 or hSK3 and hIKCa were unaffected by 0.326% DMSO (Fig. 3.7). This suggested that using DMSO as a solvent for TRAM34 and UCL1684 did not confound the validity of experiments involving these drugs.

**Fig. 3.7** Currents evoked from cells co-expressing either hSK1 or hSK3 and hIKCa were not affected by DMSO. A. Ramp currents evoked from tsA201 cells co-expressing hSK1 and hIKCa (left), or hSK3 and hIKCa (right) did not decrease in amplitude following application of 0.326% DMSO (purple).

### 3.4.2 Wild-type channel pharmacology: Introduction

Expressed SK currents rectify strongly at positive membrane potentials under isotonic K\(^+\) conditions. In the presence of drug, a loss of this rectification, which results in a virtually linear current trace, can be taken to represent 100% inhibition of expressed current. Therefore, throughout this section, a loss of rectification of hSK1 and hSK3-mediated currents following application of apamin or UCL1684 was assumed to be equal to 100% inhibition of expressed current. Weak or no rectification of currents evoked from patches
excised from tsA201 cells expressing SK channels indicated that the patch had a significant amount of leak current. These leaky patches were discarded from analysis because they did not accurately represent high-resolution SK-mediated current. Complete loss of rectification of hIKCa-mediated current in the presence of drug was not taken as 100% block, since the rectification observed for hIKCa-mediated currents was in general very mild and therefore could not serve as an indicator of remaining expressed current.

3.4.3 Homomeric hIKCa channel pharmacology

Voltage ramps applied to outside-out patches excised from tsA201 cells expressing hIKCa subunits revealed a current exhibiting, in general, little rectification and TRAM34 sensitivity, with 10 \( \mu \text{M} \) TRAM34 inhibiting current by 93.9 ± 3.0%. The concentration-inhibition relationship was best fit by a single Hill equation with an \( n_H \) of 0.970 ± 0.2 and an IC\(_{50} \) of 51.1 ± 12.3 nM (n = 4) (Fig. 3.8, B). Neither apamin (100 nM, n = 3) nor UCL1684 (100 nM, n = 3) inhibited hIKCa-mediated (Fig. 3.8, A).
3.4.4 Homomeric hSK1 and hSK3 pharmacology

i) Apamin

Voltage ramps applied to outside-out patches excised from tsA201 cells expressing either hSK1 or hSK3 subunits revealed strongly rectifying currents. Apamin (100 nM) addition caused a flattening of the current trace with complete loss of inward rectification, an indication that apamin inhibited 100% of both hSK1- and hSK3-mediated current (Fig. 3.9, A). The concentration-inhibition relationship for inhibition of hSK1 and hSK3 channels were best fit by single Hill equations with $n_H$ values of $1.39 \pm 0.6$ and $1.12 \pm 0.07$ that were not significantly different ($p = 0.550$), and IC$_{50}$s of $1.02 \pm 0.3$ nM ($n = 4$) and $1.58 \pm 0.5$ nM ($n = 7$), respectively, which were not significantly different ($p = 0.440$) (Fig. 3.9, B, C).
Figure 3.9. Apamin sensitivity of expressed hSK1- and hSK3-mediated currents. A. hSK1- (left) and hSK3- (right) mediated ramp currents and the concentration-dependent inhibitory effect of apamin (red) on these currents. B. Single variable four-parameter Hill equations were used to produce monophasic concentration-inhibition curves comparing the inhibition of hSK1- (black circles) and hSK3- (white circles) mediated current by increasing log [apamin] (M). C. Bar charts comparing the $n_H$ and $IC_{50}$ (nM) values of apamin on each current.

ii) UCL1684

UCL1684 (100 nM) inhibited both hSK1- and hSK3-mediated current by 100% (Fig. 3.10, A). The concentration-inhibition relationships for inhibition of hSK1 and hSK3 by UCL1684 were best fit by single Hill equations with $n_H$ values of 1.75 ± 0.8 and 1.24 ± 0.2 that were not significantly different ($p = 0.565$) and with $IC_{50}$s of 783 ± 377 pM ($n = 3$) and 4.23 ± 0.4 nM ($n = 3$), respectively, which were significantly different ($p = 0.0033^{**}$) (Figure 3.10, B, C).
Figure 3.10. UCL1684 sensitivity of expressed hSK1- and hSK3-mediated currents. A: hSK1- (left) and hSK3- (right) mediated ramp currents and the concentration-dependent inhibition of UCL1684 on hSK1 and hSK3 currents.

B: Logarithmic plot showing the concentration-response curve for UCL1684 inhibition of hSK1 and hSK3 currents.

C: Comparison of nH and IC50 values between hSK1 and hSK3.
inhibitory effect of UCL1684 (blue) on these currents. B. Single variable four-parameter Hill equations were used to produce monophasic concentration-inhibition curves comparing the inhibition hSK1- (black circles) and hSK3- (white circles) mediated current by increasing log [UCL1684] (M). C. Bar charts comparing the \( n_H \) and IC\( _{50} \) (nM) of UCL1684 on these currents.

### iii) TRAM34

The TRAM34 sensitivity of hSK1- and hSK3-mediated currents was investigated by applying a single 10 \( \mu \)M addition of TRAM34; this would inhibit over 90% of hIKCa-mediated current (Figure 3.8). Neither hSK1- nor hSK3-mediated currents were found to be sensitive to 10 \( \mu \)M TRAM34 (\( n = 3 \)) (Figure 3.11).

**Figure 3.11. TRAM34 insensitivity of expressed hSK1- and hSK3-mediated currents.**

Ramp currents evoked from outside-out patches excised from tsA201 cells expressing either hSK1 (left) or hSK3 (right) subunits were not inhibited by a single addition of 10 \( \mu \)M TRAM34 (green).
3.4.5 hSK1-hIKCa co-expression and hSK3-hIKCa co-expression

i) Apamin

Heteromeric hSK1-hIKCa current has been shown to be insensitive to apamin (Higham et al., 2019). Ramp currents evoked from outside-out patches excised from tsA201 cells co-expressing hSK1 and hIKCa were found to be completely insensitive to a single 100 nM addition of apamin (n = 4) (Fig. 3.12). Ramp currents evoked from whole cells co-expressing hSK3 and hIKCa were also found to be completely insensitive to apamin (n = 3) (Fig. 3.12).

![Figure 3.12 Apamin insensitivity of hSK1-hIKCa- hSK3-hIKCa-mediated currents.](image)

Ramp currents evoked from outside-out patches excised from tsA201 cells co-expressing hSK1 and hIKCa subunits (left) were not inhibited by 100 nM apamin (red). Ramp currents evoked from whole tsA201 cells co-expressing hSK3 and hIKCa were also not inhibited by 100 nM apamin (red).

ii) UCL1684

Heteromeric hSK1-hIKCa-mediated current has been reported to be relatively insensitive to 100 nM UCL1684 (Higham et al., 2019). Here, a concentration-inhibition relationship was generated that was best fit by a single Hill equation with a similar n_H of 1.65 ± 0.7 (p =
0.928), greater IC_{50} of 236 ± 82.3 nM (p = 0.0460*), and a reduced maximum block of 44.9 ± 2.5% (p <0.0001****), (n = 3), compared to that of UCL1684 on homomeric hSK1 (Fig. 3.13).

The UCL1684 sensitivity of current evoked from cells co-expressing hSK3 and hIKCa has not been investigated before. A concentration-inhibition relationship was generated that was best fit by a single Hill equation with a similar n_{H} of 0.876 ± 0.06 (p = 0.0972), greater IC_{50} of 609 ± 86.5 nM (p = 0.0022**), and a reduced maximum block of 53.8 ± 4.2% (p = 0.0004****) (n = 3), compared to that of UCL1684 on homomeric hSK3 (Fig. 3.13).

These results show that the UCL1684 sensitivity of current evoked from hSK1-hIKCa-expressing patches and hSK3-hIKCa-expressing cells was similar. Indeed, neither the n_{H} (p = 0.310) nor maximum inhibition (p = 0.141) of either curve was significantly different. The IC_{50} of UCL1684 on current evoked from hSK3-hIKCa co-expressing cells was however significantly right-shifted (p = 0.0354*) compared with its IC_{50} on hSK1-hIKCa-mediated current (Fig. 3.13).
Figure 3.13 UCL1684 sensitivity of currents evoked from outside-out hSK1-hIKCa-expressing patches and hSK3-hIKCa-expressing cells. A. Concentration-dependent
UCL1684 (blue) sensitivity of hSK1-hIKCa- (left) and hSK3-hIKCa- (right) mediated ramp currents evoked from outside-out patches and whole tsA201 cells, respectively. **B.** Single variable four-parameter Hill equations were used to produce monophasic concentration-inhibition curves comparing the effect of increasing log [UCL1684] (M) on hSK1- (black circles), hSK3- (white circles), hSK1-hIKCa- (black triangles) and hSK3-hIKCa- (white squares) mediated ramp currents. **C.** Bar charts comparing the maximum inhibition (%), nH, and IC_{50} (nM) of UCL1684 on each current.

### iii) TRAM34

Voltage ramps applied to outside-out patches excised from tsA201 cells co-expressing hSK1 and hIKCa subunits evoked current that displayed moderate rectification and concentration-dependent TRAM34 block (Fig. 3.14, A). The concentration-inhibition relationship for this current was best fit by a single Hill equation with a similar nH of 0.664 ± 0.05 (p = 0.0810), greater IC_{50} of 597 ± 82 nM (p = 0.0024**), and a reduced maximum block of 74.5 ± 2.0% (p = 0.0006***; n = 4), compared with that of homomeric hIKCa (Fig. 3.14, B, C). Whole cell currents evoked from cells co-expressing hSK3 and hIKCa also displayed moderate rectification and concentration-dependent TRAM34 block (Fig. 3.14, A). The concentration-inhibition relationship for this current was fit by a monophasic nonlinear regression with a similar nH of 0.705 ± 0.06 (p = 0.235), greater IC_{50} of 402 ± 111 nM (p = 0.0135*), and similar maximum block of 87.1 ± 2.1 (p = 0.142) (n = 3), compared with that of TRAM34 on homomeric hIKCa (Fig. 3.14, B, C).

The TRAM34 sensitivity of heteromeric hSK1-hIKCa and hSK3-hIKCa currents overlap considerably, with neither the IC_{50}s (p = 0.202) nor the nH values (p = 0.591) being significantly different. The only significant difference was between the maximum block of each current by TRAM34 (p = 0.0067**).
Figure 3.14 TRAM34 sensitivity of currents evoked from outside-out hSK1-hIKCa-expressing patches and hSK3-hIKCa-expressing cells. **A.** Concentration-dependent TRAM34 (green) sensitivity of hSK1-hIKCa- (left) and hSK3-hIKCa- (right) mediated ramp currents evoked from outside-out patches and whole tsA201 cells, respectively. **B.** Single variable four-parameter Hill equations were used to produce monophasic concentration-inhibition curves comparing the effect of increasing log [TRAM34] (M) on hIKCa- (black squares) hSK1-hIKCa- (black triangles) and hSK3-hIKCa- (white squares) mediated currents. **C.** Bar charts comparing the maximum inhibition (%), nH, and IC50 (nM) of TRAM34 on each current.

### 3.4.6 Amplitude of hSK1-, hIKCa-, and hSK1-hIKCa-mediated currents as an indicator of plasma membrane expression

The I_{-60 mV} evoked from outside-out patches can be used as an indicator of plasma membrane expression of expressed ion channels. In outside-out patches, the I_{-60 mV} of hIKCa-mediated current was 150 ± 24.1 pA (n = 11), which was significantly smaller than that of hSK1-mediated current (300 ± 38.9 pA) (n = 15) (p = 0.0063**) and hSK1-hIKCa-mediated current (450 ± 57.5) (n = 23) (p = 0.0014**) (Fig. 3.15).
Figure 3.15. Comparison of hSK1, hIKCa and hSK1-hIKCa(WT) current amplitude. A. Representative hSK1- (left), hIKCa- (middle), and hSK1-hIKCa(WT)- (right) ramp currents evoked from outside-out patches excised from tsA201 cells. Replicates were only included if they were produced from cells that were used for electrophysiology between 24 and 48 hours after transfection.
3.4.7 hIKCa(H389E)-mediated current amplitude and TRAM34 sensitivity

First, hIKCa(H389E) cDNA alone was transiently expressed in tsA201 cells. Ramp currents evoked from outside-out patches excised from these cells revealed a mildly rectifying current with a mean $I_{-60\,mV}$ of $0.168 \pm 0.03\, \text{pA}$ ($n = 3$), which was not significantly different ($p = 0.713$) from the mean $I_{-60\,mV}$ of hIKCa(WT)-mediated current of $0.153 \pm 0.02\, \text{pA}$ ($n = 8$) (Fig. 3.16, A). A single 10 \,\mu M addition of TRAM34 produced $83.7 \pm 2.01\%$ ($n = 3$) block of hIKCa(H389E)-mediated current, which was not significantly different ($p = 0.437$) from the block of hIKCa(WT)-mediated current produced by the same concentration of TRAM34 ($87.0 \pm 2.6\%$) ($n = 6$) (Fig. 3.16, B).
Figure 3.16. Amplitude and TRAM34 sensitivity of hIKCa(H389E)-mediated current. A.

Left: ramp currents evoked from outside-out patches excised from tsA201 cells expressing hIKCa(H389E).
either hIKCa(WT) (top) or hIKCa(H389E) subunits (bottom). Right: Bar chart comparing the mean $I_{-60\text{mV}}$ (nA) of hIKCa(WT)- and hIKCa(H389E)-mediated currents. B. Left: inhibition of hIKCa(WT)- (top) and hIKCa(H389E)- (bottom) mediated ramp currents by TRAM34 (10 µM) (green). Right: Bar chart comparing the inhibition (%) of these currents produced by 10 µM TRAM34.

3.4.8 hSK1-hIKCa(H389E) co-expression

i) Apamin

First, apamin was applied to whole tsA201 cells co-expressing hSK1 and hIKCa(H389E) to determine whether they contained homomeric hSK1 channels. Application of 100 nM apamin to outside-out patches excised from these cells blocked current by 13.0 ± 8.2% ($n = 4$), though this was not significantly different from the absence of apamin block on hSK1-hIKCa(WT)-mediated current ($n = 4$) ($p = 0.162$) (Fig. 3.17). The degree of apamin block varied greatly between outside-out patches, ranging from the absence of block in some to almost 40% in others. If there was more than one population of expressed channels, the populations of channels present within excised patches could have been inconsistent, which might produce variability in the pharmacology of currents. Therefore, whole cell voltage clamp was used from this point to be able to evoke currents that included all possible populations of expressed channels. The application of 1 nM apamin to cells co-expressing hSK1 and hIKCa(H389E) subunits blocked whole cell ramp currents by 3.95 ± 0.7% ($n = 3$), which was significantly greater than the absence of block by 100 nM apamin on hSK1-hIKCa(WT)-mediated current ($p = 0.0051^{**}$). Furthermore, when 100 nM apamin was applied to cells co-expressing hSK1 and hIKCa(H389E) subunits, current was blocked by 17.3 ± 1.8% ($n = 6$), which was also significantly greater than the absence of block by 100 nM apamin on hSK1-hIKCa(WT)-mediated current ($p = 0.0003^{***}$) (Fig. 3.17).
A

hSK1-hIKCa(WT) outside-out

hSK1-hIKCa(H389E) outside-out

hSK1-hIKCa(WT) whole cell

hSK1-hIKCa(H389E) whole cell

B

Inhibition (%)

ns

***

**

hSK1-hIKCa(WT) 100 nM (outside-out)
hSK1-hIKCa(H389E) 100 nM (outside-out)
hSK1-hIKCa(WT) 100 nM (whole cell)
hSK1-hIKCa(H389E) 100 nM (whole cell)
Figure 3.17. Apamin sensitivity of currents evoked from tsA201 cells co-expressing hSK1 and hIKCa(H389E) subunits. A. *Left:* ramp currents showing that hSK1-hIKCa(WT)-mediated current was not blocked by apamin (red) in either outside-out patch or whole cell configuration (100% of these patches and cells were not blocked by apamin). In the bottom left trace, 10 µM TRAM34 (green) was applied first, inhibiting current, followed by 100 nM apamin (red), which did not further inhibit current. *Right:* ramp currents showing that hSK1-hIKCa(H389E)-mediated current was blocked by apamin. The top trace shows apamin block of outside-out current, which occurred in 75% of patches. The bottom trace shows apamin block of whole cell current, which occurred in 100% of cells. B. Bar chart comparing the level of inhibition (%) by apamin of current evoked from outside-out patches and whole cells co-expressing either hSK1 and hIKCa(WT) or hSK1 and hIKCa(H389E) subunits.

ii) UCL1684

Compared to hSK1-mediated current, the sensitivity of hSK1-hIKCa(WT)-mediated current to UCL1684 was shown to be right shifted 200-fold (Fig. 3.13). The considerable separation between heteromeric and homomeric sensitivity meant that UCL1684 could be used, in addition to apamin, to investigate the population(s) of channels present within cells co-expressing hSK1 and hIKCa(H389E) subunits. Whole cell currents evoked from cells co-expressing hSK1 and hIKCa(H389E) subunits displayed sensitivity to UCL1684 that was best fit by a two-component Hill equation, producing a biphasic concentration-inhibition curve with maximal inhibition of 50.9 ± 12.5% that was significantly reduced compared to that of hSK1- (p = 0.0171*) but not hSK1-hIKCa(WT)-mediated currents (p = 0.535). The IC$_{50,a}$ of 511 ± 0.2 pM and n$_{H,a}$ of 0.910 ± 0.02 (n = 3) of the high-sensitivity component was not significantly different from the IC$_{50}$ (p = 0.545) and n$_{H}$ (p = 0.354) of UCL1684 on homomeric hSK1-mediated current. Also, the IC$_{50,b}$ of 301 ± 84.6 nM and n$_{H,b}$ of 0.930 ± 0.1 (n = 3) of the low-sensitivity component was not significantly different from the IC$_{50}$ (p = 0.609) and n$_{H}$
(p = 0.350) of UCL1684 on heteromeric hSK1-hIKCa(WT)-mediated current (Fig. 3.18).
**A** hSK1

<table>
<thead>
<tr>
<th>V (mV)</th>
<th>I (nA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>V (mV)</th>
<th>I (nA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**hSK1-hIKCa(WT)**

<table>
<thead>
<tr>
<th>V (mV)</th>
<th>I (nA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>V (mV)</th>
<th>I (nA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

**Inhibition (%)**

- hSK1
- hSK1-hIKCa(H389E)
- hSK1-hIKCa(WT)

**log [UCL1684] (M)**

- Max inhibition (%)
- $h_n$
- $IC_{50}$ (nM)

<table>
<thead>
<tr>
<th>log [UCL1684] (M)</th>
<th>Max inhibition (%)</th>
<th>$h_n$</th>
<th>$IC_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* ns
iii) TRAM34

Compared to its effect on hIKCa-mediated current, the TRAM34 concentration-inhibition curve for hSK1-hIKCa(WT)-mediated current was right-shifted 10-fold, with a reduced maximum block (Fig. 3.19). The concentration-inhibition relationship obtained from current evoked from cells co-expressing hSK1 and hIKCa(H389E) subunits displayed sensitivity to TRAM34 that was best fit by a single Hill equation, with a greater IC$_{50}$ of 250 ± 53 nM (p = 0.008*), similar n$_H$ of 0.836 ± 0.3 (p = 0.682), and reduced maximum block of 68.2 ± 6.9% (p = 0.0267*) (n = 3), compared to its effect on homomeric hIKCa-mediated current (Fig. 3.19). The IC$_{50}$ of current evoked from hSK1-hIKCa(H389E)-expressing cells was also significantly reduced compared to that of heteromeric hSK1-hIKCa(WT)-mediated current (p = 0.0247*), while the n$_H$ (p = 0.462) and maximum block (p = 0.250) values were not significantly different. This placed the IC$_{50}$ for TRAM34 on current evoked from cells co-expressing hSK1
and hIKCa(H389E) subunits intermediate between its IC$_{50}$ on homomeric hIKCa(WT)- and heteromer hSK1-hIKCa(WT)-mediated currents.
Figure 3.19. TRAM34 sensitivity of currents evoked from whole tsA201 cells co-expressing hSK1 and hIKCa(H389E). A. Concentration-dependent TRAM34 (green) sensitivity of hIKCa-, hSK1-hIKCa(WT)-, and hSK1-hIKCa(H389E)-mediated ramp currents evoked from outside-out patches (hSK1 and hSK1-hIKCa(WT)) and whole tsA201 cells (hSK1-hIKCa(H389E)), respectively. HS: high-sensitivity component; LS: low-sensitivity component. B. CI curve comparing the effect of increasing log [TRAM34] (M) on hIKCa-(black squares), hSK1-hIKCa(WT)-(black triangles), and hSK1-hIKCa(H389E) (black diamonds)-mediated currents. C. Bar charts comparing the maximum inhibition, nH, and IC50 (nM) of TRAM34 on each current.
3.5 Chapter Discussion

3.5.1 Pharmacology of homomeric channels

The pharmacology of homomeric channels presented above reflects published findings. Homomeric hSK1 channels have been reported to be sensitive to apamin with an IC$_{50}$ of 3-8 nM and $n_H$ of approximately 1 (Strobaek et al., 2000, Shah & Haylett, 2000), and to UCL1684 with an IC$_{50}$ of 0.8-3 nM (Campos Rosa et al., 1998, Strobaek et al., 2000). Homomeric hSK3 channels have been reported to be sensitive to apamin with an IC$_{50}$ of 6 nM and $n_H$ of 1.4 (Lamy et al., 2010), which is similar to its effect on hSK1 channels. This lack of selectivity between hSK1 and hSK3 channels by apamin is reproduced here. The IC$_{50}$ of UCL1684 on hSK3 compared to hSK1 was found to be around 3-fold right-shifted however, which reflects the difference between UCL1684’s published IC$_{50}$ of 760 pM on hSK1 and 2.5 nM on hSK3 (Stroebeck et al., 2000, Hancock et al., 2015). SK channels are known to be insensitive to the hIKCa blocker TRAM34 (Wulff et al., 2001, Higham et al., 2019), and neither apamin nor UCL1684 have been reported to block hIKCa channels (de-Allie et al., 1996, Wulff et al., 2001, Higham et al., 2019); these are all findings that have been reproduced in this chapter. TRAM34 is a highly selective hIKCa blocker, with a reported IC$_{50}$ of 20-40 nM and $n_H$ of 1 (Wulff et al., 2001, Higham et al., 2019). The IC$_{50}$ (50 nM) and $n_H$ (0.97) found here are in line with these previously published values.

3.5.2 Pharmacology of heteromeric hSK1-hIKCa channels: Apamin and TRAM34

The only study of heteromeric hSK1-hIKCa channels was carried out by Higham et al. in 2019, who used pharmacology, single channel recordings and Stochastic Optical Resonance Microscopy (STORM) to determine that when hSK1 and hIKCa subunits were transiently co-expressed in tsA201 cells they co-assembled in a preferential manner to form a single population of heteromeric hSK1-hIKCa channels. In line with Higham et al., this project found that currents evoked from outside-out patches excised from co-expressing
cells to be completely insensitive to apamin, which confirms the absence of homomeric hSK1 channels. The rightward shift in the TRAM34 sensitivity of this current could mean that both homomeric hIKCa and heteromeric hSK1-hIKCa channels were present. If this were the case, the TRAM34 concentration-inhibition relationship of this current would be biphasic, with a HS component representing homomeric hIKCa-mediated current and LS component representing heteromeric hSK1-hIKCa-mediated current. Alternatively, a monophasic relationship might be produced with a shallow $n_H$. However, here a monophasic concentration-inhibition relationship was produced with a $n_H$ that was not significantly different from the concentration-inhibition relationship of TRAM34 on homomeric hIKCa-mediated current. This TRAM34 pharmacology confirms the absence of homomeric hIKCa channels.

Together, the apamin and TRAM34 pharmacology of current evoked from patches excised from cells co-expressing hSK1 and hIKCa subunits strongly suggests that only heteromeric hSK1-hIKCa channels were present.

### 3.5.3 Subunit stoichiometry of heteromeric hSK1-hIKCa channels

Apamin binding to SK channels has been proposed to display positive cooperativity, requiring donation of the S3-S4 loop of one SK subunit to the outer pore of an adjacent SK subunit (Weatherall et al., 2011). Since hSK1-hIKCa-mediated current was insensitive to apamin, it is likely that hSK1 subunits within the hSK1-hIKCa heteromer were not adjacent. TRAM34 binding to IKCa channels occurs within the conduction pore (Wulff et al., 2001), with high-sensitivity TRAM34 block requiring the presence of all four pyrazole ring V275 side chains, each donated by an individual IKCa subunit (Nguyen et al., 2017). The reduced TRAM34 sensitivity of the hSK1-hIKCa heteromer reported here suggests that its conduction pore contains less than four pyrazole rings. From these proposed binding sites, the present findings can be used to deduce an hSK1-hIKCa subunit stoichiometry, which could be either...
of: one hSK1 subunit and three hIKCa subunits; or two of each subunit, with subunits of each type diametrically opposed. Both of these configurations would render heteromers unable to bind apamin and less suited to binding TRAM34. Although it cannot be confirmed by data presented here, it might be more likely that a heteromer containing two of each subunit dominates. If, for example, heteromers were comprised of three hIKCa subunits and one hSK1 subunit, it would be expected that a subpopulation of homomeric hSK1 channels would form from leftover hSK1 subunits; this cannot be the case due to the aforementioned apamin insensitivity of current evoked from all patches excised from co-expressing cells. Likewise, if heteromers were made up of three hSK1 subunits and one hIKCa subunit, heteromers would contain adjacent hSK1 subunits, allowing apamin to bind and inhibit current. In this scenario, a subpopulation of homomeric hIKCa channels would also be left over, conferring a high sensitivity TRAM34 component upon this current. The pharmacology presented here and in Higham et al. does not match either of these scenarios but does match that in which two of each hIKCa and hSK1 subunits, diametrically opposed, make up the heteromer. A further conclusion that can be drawn from these results is that the stoichiometry of hSK1 and hIKCa subunits in heteromeric channels is fixed. This is likely because the pharmacology of current evoked from patches excised from cells co-expressing hSK1 and hIKCa subunits does not vary. Variation between patches would indicate that different channel populations were present at the plasma membrane of different cells, which would be observed for heteromers with random subunit stoichiometries. This is significant, as if the stoichiometry of heteromeric hSK1-hIKCa channels is fixed, there must be structural elements of either hSK1 or hIKCa subunits that consistently mediate the formation of a particular configuration of subunits within the heteromer. This in turn strengthens the argument that heteromeric hSK1-hIKCa channels form natively where the expression of hSK1 and hIKCa subunits overlaps.
3.5.4 Pharmacology of heteromeric hSK1-hIKCa channels: UCL1684

Higham et al. reported that hSK1-hIKCa-mediated current was insensitive to UCL1684 because the highest concentration of UCL1684 applied (100 nM) inhibited a very small percentage of the total current, which was indistinguishable from the absence of block. By adding higher concentrations of UCL1684 it was shown here that heteromeric hSK1-hIKCa-mediated current was in fact moderately sensitive to UCL1684, with a 204-fold right-shifted IC\textsubscript{50} and around 50% maximum block, compared with its effect on hSK1-mediated current. This is a unique pharmacology amongst KCa channels.

The reduced sensitivity of heteromeric current suggests that the extent of UCL1684 block is dependent upon the number of SK subunits comprising the tetramer. However, the fact that some UCL1684 block was retained suggests that UCL1684 binding is not completely reliant upon cooperation between SK subunits. This difference between the mechanism of binding of apamin and UCL1684 has been previously reported (Weatherall et al., 2011) and the findings presented here support this theory.

3.5.5 Assembly of heteromeric hSK1-hIKCa channels

i) hSK3 and hIKCa subunits co-assemble

First, hSK3 subunits were co-expressed in tsA201 cells with hIKCa subunits. As described in the introduction to this chapter, alignment of hSK1 and hSK3 amino acid sequences revealed around 50% sequence similarity, suggesting that there could be structural differences in hSK3 that make co-assembly with hIKCa subunits less favourable. However, the pharmacology of whole cell currents evoked from cells co-expressing hSK3 and hIKCa was very similar to the pharmacology of heteromeric hSK1-hIKCa channels, suggesting that only heteromeric hSK3-hIKCa channels were present. Further experiments would be needed to confirm this; nonetheless, these results meant that amino acid differences between hSK1
and hSK3 would not be the starting point for investigating the molecular determinants of heteromeric hSK1-hIKCa channel assembly.

ii) The H389E mutation in hIKCa did not affect the amplitude or TRAM34 sensitivity of hIKCa(H389E)-mediated currents

The H389 residue in hIKCa was chosen to be substituted with a glutamate residue, as discussed in the introduction to this chapter. When expressed alone, hIKCa(H389E)-mediated current appeared to have an amplitude and pharmacology in outside-out patches that was not different from that of hIKCa(WT)-mediated current. This suggests that the H389E mutation did not affect the formation of tetrameric hIKCa channels. With more time, it would be desirable to use whole cell voltage clamp with cell capacitance compensation to compare normalised current amplitude of cells expressing hIKCa(WT) with cells expressing hIKCa(H389E) to confirm this.

iii) Apamin caused a 20% reduction in the amplitude of currents evoked from tsA201 cells co-expressing hSK1 and hIKCa(H389E) subunits

hIKCa(H389E) subunits were subsequently co-expressed with hSK1 subunits to determine whether the H389E mutation affected heteromeric co-assembly. The pharmacological markers of current mediated by only heteromeric hSK1-hIKCa channels, uncovered in Higham et al., are total insensitivity to apamin, lack of UCL1684 sensitivity, and a right-shifted sensitivity to TRAM34. Interestingly, current evoked from cells co-expressing hSK1 and hIKCa(H389E) subunits was partially but clearly inhibited by apamin. It is unlikely that the H389E mutation conferred apamin sensitivity upon heteromeric hSK1-hIKCa(H389E)-mediated current as the binding sites for apamin have been proposed to be within the outer pore and S3-S4 loop of SK subunits, not C-terminus (Weatherall et al., 2011). Therefore, apamin block strongly suggests the presence of homomeric hSK1 channels at the plasma
membrane of these cells. Application of 100 nM apamin inhibited 100% of homomeric hSK1-mediated current (Fig. 3.9), so the proportion of apamin-sensitive current evoked from cells co-expressing hSK1 and hIKCa(H389E) subunits can be used as a measure of homomeric hSK1 channel plasma membrane expression. Apamin blocked around 20% of the total current evoked from these cells, suggesting that homomeric hSK1 channels comprised 20% of expressed channels. The remaining population of channels could either be comprised of hIKCa or hSK1-hIKCa(H389E) channels only, or a combination of the two.

iv) Current evoked from tsA201 cells co-expressing hSK1 and hIKCa(H389E) subunits was inhibited with both high and low sensitivity by UCL1684

Current evoked from cells co-expressing hSK1 and hIKCa(H389E) subunits was blocked with a biphasic sensitivity by UCL1684. The IC_{50,a} of this relationship was not significantly different from the IC_{50} of UCL1684 on hSK1-mediated current. Homomeric hSK1-mediated current was 100% blocked by 100 nM UCL1684 (Fig. 3.10). The HS component of the biphasic relationship plateaus at around 20%, supporting the idea that the proportion of homomeric hSK1 channels expressed at the plasma membrane of these cells was around 20%. The LS component of this relationship had an IC_{50,b} that was not significantly different from the IC_{50} of UCL1684 on hSK1-hIKCa(WT)-mediated current. This showed that heteromeric hSK1-hIKCa(H389E) channels were present as well as homomeric hSK1 channels.

v) TRAM34 blocked current evoked from tsA201 cells co-expressing hSK1 and hIKCa(H389E) subunits with an intermediate IC_{50}

The above representative trace (Fig. 3.19, A) shows that currents evoked from cells co-expressing hSK1 and hIKCa(H389E) subunits, that were sensitive to apamin, were blocked by concentrations of TRAM34 at which hSK1-hIKCa(WT)-mediated currents were not (3
nM). This trace also shows that currents evoked from cells co-expressing hSK1 and hIKCa(H389E) subunits were more sensitive to concentrations of TRAM34 that have a small effect upon hSK1-hIKCa(WT)-mediated current (300 nM). This resembles the TRAM34 pharmacology of hIKCa channels more closely than hSK1-hIKCa(WT) channels. However, the IC50 of this relationship was right-shifted compared to that of TRAM34 on hIKCa-mediated currents, and left-shifted compared to that of TRAM34 on hSK1-hIKCa(WT)-mediated currents. On one hand, this could suggest that current evoked from cells co-expressing hSK1 and hIKCa(H389E) subunits was mediated by a population of heteromeric channels with an augmented TRAM34 sensitivity. This is unlikely, since TRAM34 is proposed to block within the conduction pore of hIKCa channels, away from the C-terminus (Wulff et al., 2001). Alternatively, this intermediate IC50 value could represent the high and low TRAM34 sensitivities of separate populations of homomeric hIKCa and heteromeric hSK1-hIKCa(H389E) channels. Attempts to generate a biphasic concentration-inhibition curve using a two-component Hill equation produced fits with very wide confidence intervals for each of the curve’s four parameters and IC50 values that were not plausible. This was probably due to the small number of replicates gathered; without obtaining a substantial number of replicates, the comparatively small (10-fold) separation between the TRAM34 sensitivity of homomeric hIKCa and heteromeric hSK1-hIKCa(WT) channels would make reliably fitting two Hill equations to this data difficult. Moreover, the I-60mV of hIKCa-mediated current in outside-out patches was around half that of hSK1-mediated current (Fig. 3.15), which suggests that homomeric hIKCa channels were expressed at the plasma membrane of tsA201 cells around half as much as homomeric hSK1 channels. In cells co-expressing hSK1 and hIKCa(H389E) subunits, this would result in a plasma membrane expression of homomeric hIKCa channels of only 10% of the total population of expressed channels. It would be difficult to define a component of a biphasic pharmacology that was made up of only 10% of the total expressed current, even with a large number of replicates. Therefore, although there is evidence here that suggests homomeric hIKCa channels could be expressed at the plasma membrane of these cells, alongside homomeric hSK1 and
heteromeric hSK1-hIKCa channels, further experiments will be required to illuminate this problem.

3.5.6 The role of the H389E mutation in the formation of homomeric and heteromeric channels

Mutating the H389 residue in hIKCa subunits to E389 disrupted the formation of heteromeric hSK1-hIKCa channels but did not affect the formation of homomeric hIKCa channels. A previous study proposed that hIKCa channel assembly and trafficking is mediated by a leucine zipper motif within subunit C-terminal CCDs (Syme et al., 2003). Although H389 is situated within hIKCa’s C-terminal leucine zipper, it is possible that the H389E mutation does not perturb this leucine zipper. This could be because glutamate residues are similar to histidine residues in size and shape, reducing the impact of the H389E mutation on the shape of this motif. Another study proposed that constitutively tethered CaM domains mediate the interaction and assembly of IKCa subunits, as well as channel gating (Joiner et al., 2001). H389 is around 50 residues C-terminal to the 62 amino acid run within the first C-terminal region proposed to be involved in IKCa CaM binding (Fanger et al., 1999), so it is unlikely that the H389E mutation would disrupt any CaM-mediated interactions between IKCa subunits. Further possible explanations of how the H389E mutation disrupts the formation of heteromeric hSK1-hIKCa but not homomeric hIKCa channels are discussed in Chapter 5.

Although it is not possible to pinpoint reason that the H389E mutation specifically disrupts the formation of heteromeric channels, it is possible to conclude that the C-terminal CCDs of hIKCa subunits are involved in the preferential formation of heteromeric hSK1-hIKCa channels.
3.6 Chapter References

Campos Rosa J, Galanakis D, Ganellin CR, Dunn PM, Jenkinson DH (1998). Bis-Quinolinium Cyclophanes: 6,10-Diaza-3(1,3),8(1,4)-dibenzena-1,5(1,4)-diquinolinacyclodecaphane (UCL 1684), the first nanomolar, non-peptidic blocker of apamin-sensitive Ca\textsuperscript{2+}-activated K\textsuperscript{+} channel. J Med Chem 41: 2-5.


Chapter 4: Ca\(^{2+}\) sensitivity of hSK1, hIKCa and hSK1-hIKCa channels

4.1 Chapter Introduction

4.1.1 Homomeric SK channels

Expressed SK1 channels have been reported to be activated by Ca\(^{2+}\) with an EC\(_{50}\) between 300-500 nM and an n\(_H\) of 4-5 (Kohler et al., 1996, Xia et al., 1998). In hippocampal neurons, KCa conductances of around 20 pS have been proposed to be activated by Ca\(^{2+}\) entering through L-type Ca\(_V\) channels that are co-localised at a 50-150 nm distance within the plasma membrane (Marrion & Tavalin, 1998). These KCa currents were proposed to be mediated by SK channels.

4.1.2 Homomeric IKCa channels

No co-localisation between IKCa channels and Ca\(_V\) channels has been reported. This would mean that IKCa channels must be able to detect small fluctuations in cytosolic Ca\(^{2+}\) concentration via its constitutively tethered CaM domains. This property would align with the proposed physiological roles of IKCa channels, such as erythrocyte volume control (Gardos et al., 1958). To detect small fluctuations in cytosolic Ca\(^{2+}\) concentration, IKCa channels would have to have a higher sensitivity to activation by Ca\(^{2+}\) than SK channels. When IKCa channels were initially cloned, the EC\(_{50}\) for activation by Ca\(^{2+}\) was reported to be as low as 95 nM, with a steep n\(_H\) of 3.2 (Joiner et al., 1997). However, a separate group simultaneously published a higher EC\(_{50}\) of 300 nM and a shallower n\(_H\) of 1.7 (Ishii et al., 1997). These findings appear divergent, though this could be due to differences in the methods used in each study, which will be discussed later.
4.1.3 Heteromeric hSK1-hIKCa channels

When hSK1 subunits were co-expressed with hIKCa subunits in tsA201 cells, heteromeric hSK1-hIKCa channels preferentially formed (Higham et al., 2019). Heteromeric channels could be present in CA1 pyramidal neurons and cardiac cells, where the expression of SK1 and IKCa subunits are proposed to overlap (Stocker & Pedarzani, 2000, Sailer et al., 2002, Weisbrod et al., 2013, Turner et al., 2015, 2016, King et al., 2015). In this chapter, the Ca$^{2+}$ sensitivity of heteromeric hSK1-hIKCa channels will be investigated for the first time. Importantly, the Ca$^{2+}$ sensitivity of heteromeric hSK1-hIKCa channels might help determine whether or not these channels are expressed in native cells. The Ca$^{2+}$ sensitivity of hSK1 and hIKCa channels will also be investigated so that each channel can be compared directly.
4.2 Chapter Aims

The experiments in this chapter aimed to:

I. generate concentration-response relationships for the activation by Ca$^{2+}$ of currents evoked from macroscopic inside-out patches excised from tsA201 cells transiently expressing either hSK1 subunits, hIKCa subunits, or co-expressing both hSK1 and hIKCa subunits, deriving values for the EC$_{50}$ and n$_H$ of each channel.
4.3 Chapter Methods

4.3.1 Inside-out patch clamp

Previous studies of KCa channel Ca\(^{2+}\) sensitivity have used either whole cell voltage clamp (Joiner et al., 1997) or macroscopic inside-out patch clamp (Kohler et al., 1996, Ishii et al., 1997, Hirschberg et al., 1998, Xia et al., 1998). Throughout the experiments in this chapter, macroscopic inside-out patch clamp was used. This allowed internal solutions with increasing concentrations of free Ca\(^{2+}\) to be exchanged underneath the inside-out patch rapidly and in real-time. The same voltage ramp protocol as the one used in Chapter 3 was again used here to evoke currents from inside-out patches: from a holding potential of 0 mV, continuous depolarising voltage ramps from -100 mV to +100 mV of 1 second duration, were applied across patches every second.

4.3.2 Electrodes

For inside-out macropatch recordings, micropipettes were fabricated from thin-walled (1.5 mm OD x 1.17 mm ID) borosilicate glass capillaries (Harvard Apparatus) using a Narishige model PP-83 puller. After being fire-polished and filled with external solution, micropipette resistance was between 3-4 mOhm. This produced large micropipette tips, meaning the size of excised patches could be larger and so the number of expressed channels within each patch could be greater.

4.3.3 Internal (bathing) and external (electrode) solutions

For inside-out patch recordings, cells were perfused with the same high K\(^+\) solution used for outside-out patch recordings in Chapter 3. When making each batch of seven internal Ca\(^{2+}\) solutions, a base solution containing, in mM, 97 K Gluconate, 30 KCl, 10 HEPES(Na) and 10 EGTA was made first. This base was then separated into seven aliquots of equal volume,
which were then separately supplemented with different concentrations of CaCl₂ and MgCl₂ to achieve the desired free Ca²⁺ concentration in each.

Free Ca²⁺ concentrations were calculated using the REACT programme (University of Strathclyde) which takes into account the binding constants of EGTA for Ca²⁺, Mg²⁺ and H⁺ at room temperature (22°C). The range of free Ca²⁺ concentrations used, and the CaCl₂ and MgCl₂ concentrations added to produce them, was as follows: 30 nM (3.31 mM CaCl₂ and 1.56 mM MgCl₂ in 1 L), 100 nM (6.22 mM CaCl₂ and 1.32 mM MgCl₂ in 1 L), 300 nM (8.32 mM CaCl₂ and 1.14 mM MgCl₂ in 1 L), 500 nM (8.92 mM CaCl₂ and 1.09 mM MgCl₂ in 1 L), 750 nM (9.25 mM CaCl₂ and 1.06 mM MgCl₂ in 1 L), 1 μM (9.43 mM CaCl₂ and 1.05 mM MgCl₂ in 1 L), and 3 μM (9.81 mM CaCl₂ and 1.02 mM MgCl₂ in 1 L). The pH of each solution was then increased to 7.4 by adding KOH pellets at first, then 1 M KOH solution, dropwise. The osmolarity of each solution was determined to be between 280 and 310 mOsm using the method described in Chapter 3.
4.4 Chapter Results

4.4.1 Ca$^{2+}$ sensitivity of hSK1, hIKCa and hSK1-hIKCa channels

Ramp currents evoked from inside-out patches excised from tsA201 cells expressing hSK1 subunits showed marked inward rectification and Ca$^{2+}$ dependence. Homomeric hSK1-mediated current was activated by Ca$^{2+}$ with an EC$_{50}$ of 498 ± 40 nM and a steep $n_H$ of 3.66 ± 0.9, with 3 µM Ca$^{2+}$ evoking a maximal response ($n = 4$). Homomeric hIKCa-mediated ramp currents displayed very mild rectification and were also Ca$^{2+}$-dependent, with an EC$_{50}$ of 419 ± 35 nM and a less steep $n_H$ of 2.62 ± 0.5 ($n = 5$). Finally, heteromeric hSK1-hIKCa-mediated ramp currents displayed similar rectification as hSK1-mediated currents, and again showed Ca$^{2+}$-dependence, with a Ca$^{2+}$ EC$_{50}$ of 600 ± 24 nM, $n_H$ of 3.47 ± 0.4, and maximal response at 3 µM Ca$^{2+}$ ($n = 7$) (Fig. 4.1, B).

Statistical analysis revealed that the EC$_{50}$ values for activation of homomeric hSK1 and hIKCa channels by Ca$^{2+}$ were not significantly different ($p = 0.180$). However, the EC$_{50}$s for activation by Ca$^{2+}$ were significantly different between homomeric hSK1 channels and heteromeric hSK1-hIKCa channels ($p = 0.0438^*$) as well as homomeric hIKCa channels and heteromeric hSK1-hIKCa channels ($p = 0.0013^{**}$). There was no significant difference when comparing the $n_H$ values of hSK1 and hIKCa channels ($p = 0.321$), hSK1 and hSK1-hIKCa channels ($p = 0.817$), or hIKCa and hSK1-hIKCa channels ($p = 0.180$) (Fig 4.1, C).
A

hSK1

hIKCa

hSK1-hIKCa

B

Normalised current

[Free Ca$^{2+}$] (µM)

C

$P_i$

$EC_{50}$ (nM)
Figure 4.1. The Ca$^{2+}$ sensitivity of hSK1-, hIKCa, and hSK1-hIKCa-mediated currents. 

A. Representative ramp currents evoked from inside-out patches excised from tsA201 cells expressing either hSK1 (left), hIKCa (middle), or both hSK1 and hIKCa subunits (right). Ramp shade darkens from light grey to black to indicate sweeps elicited by increasing concentrations of Ca$^{2+}$. B. A single variable four-parameter Hill equation was used to produce monophasic concentration-inhibition curves. These curves show the effect of increasing concentrations of free Ca$^{2+}$ (µM) on currents evoked from inside-out patches excised from tsA201 cells expressing either hSK1 (black points), hIKCa (black squares), or co-expressing hSK1 and hIKCa (black triangles) subunits. The maximum current amplitude recorded during each concentration-response regime was normalised to 1, which was then used to calculate normalised levels of current elicited by each concentration of Ca$^{2+}$. C. Bar charts comparing the $n_H$ (left) and EC$_{50}$ (nM) (right) for activation by Ca$^{2+}$ of hSK1-, hIKCa- and hSK1-hIKCa-mediated current.
4.5 Chapter Discussion

4.5.1 Homomeric hSK1 channels sense acute changes in Ca\textsuperscript{2+} concentration

When hSK1 channels were first cloned and expressed, they were reported to be activated by Ca\textsuperscript{2+} with an EC\textsubscript{50} of 710 nM and n\textsubscript{H} of 3.9 (Kohler et al., 1996). Subsequent studies showed that SK channels were more sensitive to Ca\textsuperscript{2+} than this, with an EC\textsubscript{50} of 300-500 nM and n\textsubscript{H} of 4-5 (Xia et al., 1998, Hirschberg et al., 1998). Each of these values were obtained using currents evoked from inside-out patches excised from Xenopus Oocytes transfected with hSK1 subunits, the caveats of which are discussed below. SK channels native to CA1 hippocampal neurons were then proposed to have an EC\textsubscript{50} of 560 nM and n\textsubscript{H} of 4.6 (Hirschberg et al., 1999). The Ca\textsuperscript{2+} sensitivity of hSK1 reported here (EC\textsubscript{50} = 498 nM, n\textsubscript{H} = 3.66), using the mammalian tsA201 expression system, is in line with these previously reported values.

4.5.2 Homomeric hIKCa channels are activated by a broad range of Ca\textsuperscript{2+} concentrations

Here, homomeric hIKCa channels were shown to have a significantly higher Ca\textsuperscript{2+} sensitivity (reduced EC\textsubscript{50}) compared to heteromeric hSK1-hIKCa (p <0.01**), but not homomeric hSK1 channels (p >0.05). hIKCa channels were also around 10% active when exposed to 100 nM Ca\textsuperscript{2+}, which is approximately the resting Ca\textsuperscript{2+} concentration in native cells. In contrast, when exposed to 100 nM Ca\textsuperscript{2+}, hSK1 (p = 0.0147*) and hSK1-hIKCa channels (p = 0.0018**) were totally inactive. These findings correspond to the known physiological role of IKCa channels in regulating permeability of nonconducting cells, such as red blood cells and lymphocytes, by detecting fluctuations in cytosolic Ca\textsuperscript{2+} (Gardos, 1958, Wulff & Castle, 2010).

The EC\textsubscript{50} and n\textsubscript{H} reported here reflect aspects of both previous studies of hIKCa channel Ca\textsuperscript{2+} sensitivity (Joiner et al., 1997, Ishii et al., 1997). Joiner et al. reported a highly sensitive channel, with an EC\textsubscript{50} for activation by Ca\textsuperscript{2+} of 95 nM, and an n\textsubscript{H} of 3.2. Ishii et al. found that
the EC₅₀s for hIKCa and rSK2 channels were the same (300 nM), but that the nₜₜ for hIKCa activation was almost half as steep as rSK2 activation. Ishii et al. also found that hIKCa channels were around 10% active when exposed to 100 nM Ca²⁺. Here, the concentration-response relationship for hIKCa channels was leftward of both homomeric hSK1 and heteromeric hSK1-hIKCa channel relationships (only the latter significantly), aligning more with Joiner et al. Despite this, the EC₅₀ for Ca²⁺-mediated activation of hIKCa channels was more than 4-fold greater than the one reported by Joiner et al., resembling the value of 300 nM reported by Ishii et al. more closely. This makes the findings in Joiner et al. difficult to reconcile with those presented here. One similarity shared by the present findings and those of both Joiner et al. and Ishii et al. was that, at what would be a resting concentration of Ca²⁺ inside native cells, hIKCa channels were active, whereas SK channels were not.

4.5.3 Heteromeric hSK1-hIKCa channels have a distinct Ca²⁺ sensitivity

Here, heteromeric hSK1-hIKCa channels were found to have a significantly right-shifted EC₅₀ (600 nM) for activation by Ca²⁺ compared with homomeric hSK1 (498 nM) (p <0.05*) and hIKCa channels (419 nM) (p <0.01**). Native SK channels, which were thought might underlie the sAHP, have an EC₅₀ of 560 nM and nₜₜ of 4.6, as measured using inside-out patches excised from acutely isolated hippocampal CA1 neurons. The same study showed that native SK channels were silent at 100 nM Ca²⁺ (Fig. 4.2) (Hirschberg et al., 1999). Therefore, heteromeric hSK1-hIKCa channels have a similar Ca²⁺ sensitivity (EC₅₀ = 600 nM, inactive at 100 nM Ca²⁺) compared with neuronal SK-type channels, but not expressed SK channels (EC₅₀ = 498 nM, inactive at 100 nM Ca²⁺), nor homomeric hIKCa channels (EC₅₀ = 419 nM, 10% active at 100 nM Ca²⁺).
**Figure 4.2.** ‘Ca$^{2+}$-dependence of hippocampal SK channel activity’ (adapted from Hirschberg *et al.*, 1999 (*Figure 1*)). **A.** Single-channel recordings of current evoked from inside-out patches excised from CA1 neurons held at -60 mV and exposed to increasing concentrations of Ca$^{2+}$, with downward deflections indicating channel openings (‘- c’ annotations indicate SK channels in the closed state). Note that no channel openings are observed when Ca$^{2+}$ concentration is 100 nM. **B.** Concentration-response curve showing the effect of increasing concentrations of Ca$^{2+}$ (µM) on the open probability ($P_o$) hippocampal SK
channels. These SK channels have a maximal $P_o$ of 0.74 from 1 µM Ca$^{2+}$, an $EC_{50}$ of 560 nM and an $n_H$ of 4.6.

The association between hSK1 and hIKCa subunits co-expressed in tsA201 cells has been shown to be close enough to produce a signal in a fluorescence resonance energy transfer (FRET) assay, and to show overlapping clusters of tagged subunits in STORM. Current evoked from co-expressing cells also exhibited a unique pharmacology and single-channel conductance, which together with imaging assays strongly suggested that a single population of heteromeric hSK1-hIKCa channels formed preferentially to either homomer (Higham et al., 2019). The findings presented in this chapter demonstrate that currents evoked from tsA201 cells co-expressing hSK1 and hIKCa subunits are activated by Ca$^{2+}$ in a way that is best fit by a monophasic nonlinear regression, with a significantly right-shifted $EC_{50}$ compared with that of both hSK1 and hIKCa. If hIKCa homomers were present in co-expressing cells, the concentration-response relationship for activation by Ca$^{2+}$ would be biphasic, displaying a high-sensitivity component representing hIKCa homomers. No such high sensitivity component was resolved, meaning that hIKCa homomers were not present. If hSK1 homomers were present in co-expressing cells, the biphasic relationship would have a low-sensitivity component with an $EC_{50}$ overlapping with that of hSK1 homomers. The $EC_{50}$ of current evoked from co-expressing cells was significantly higher than that of hSK1-mediated current, making it unlikely that hSK1 homomers were present, either. Therefore, these findings bolster the argument made in Higham et al. that a single population of heteromeric hSK1-hIKCa channels is expressed at the plasma membrane of tsA201 cells co-expressing hSK1 and hIKCa subunits. Also, the distinct Ca$^{2+}$ sensitivity of heteromeric channels represents another functional property that is unique, in addition to its single-channel conductance. These results, together with those from Higham et al., make a strong case that heteromeric hSK1-hIKCa channels are a functionally distinct member of the KCa family, which, if present in native cells known to express both SK1 and IKCa subunits, could carry out a specific physiological role.
4.5.4 Ca\textsuperscript{2+} sensitivity findings are limited by some experimental factors

When interpreting the findings in this chapter, as well as those from similar studies in the past, it is important to recognise the different advantages and disadvantages of the methods used to record KCa channel Ca\textsuperscript{2+} sensitivity. Kohler et al., Ishii et al., Xia et al. and Hirschberg et al. all used macroscopic inside-out patches excised from Xenopus Oocytes to characterise hIKCa and SK channel Ca\textsuperscript{2+} sensitivity. In their favour, inside-out macropatches allow the Ca\textsuperscript{2+} concentration to which channels are exposed to be changed in real time, reflecting native conditions. However, excising patches into a large reservoir of bathing solution removes the internal elements of KCa channels, including the constitutive CaM domain, from any intracellular components that might be involved in function. Excision into a bathing solution also exposes the channel to approximately homogenous Ca\textsuperscript{2+} conditions. This does not reflect cellular conditions, where the dynamic roles of Ca\textsuperscript{2+} ions means that Ca\textsuperscript{2+} concentration is in a constant state of flux. In contrast, Joiner et al. used whole cell voltage clamp to record the current amplitude evoked from a single IKCa-expressing Chinese Hamster Ovary (CHO) cell at each Ca\textsuperscript{2+} concentration, changing internal solutions and re-recording from the same CHO cell to create a concentration-response relationship. Although this would keep the intracellular components involved in IKCa channel function intact, the quality of the individual CHO cell used would likely diminish with each recording, which could impair the function of membrane proteins, including IKCa channels, and could contaminate recordings with noise from increased leak through a damaged plasma membrane. Macroscopic inside-out patch clamp was used in all experiments presented in this chapter because full concentration-response relationships could be obtained relatively quickly with a single patch. In addition, using inside-out macropatches would permit easier comparison between the findings of this project and those from the majority of studies conducted on KCa channels or sAHP Ca\textsuperscript{2+} sensitivity, as these generally also use inside-out macropatches.
The species of expression system might also have an impact upon observed Ca\(^{2+}\) sensitivity. When SK channels were initially cloned from rat brain and expressed in *Xenopus* Oocytes, rSK2 channels were found to be apamin-sensitive, with an EC\(_{50}\) of 63 pM, whereas hSK1 channels were 30-fold less sensitive to apamin, with an EC\(_{50}\) of 76.2 µM (Kohler *et al*., 1996). Subsequent studies showed that hSK1 channels were indeed sensitive to apamin when expressed in mammalian systems, such as HEK293 or COS-7 cells (Shah & Haylett, 2000), which makes species an important factor to consider when making conclusions about KCa channel properties, such as their pharmacology. Although the observed Ca\(^{2+}\) sensitivity of SK channels has increased over the years from around 700 nM when initially reported in 1996 by Kohler *et al*., to around 300 nM in 1998 by Xia *et al*., both of these values were based upon recordings from inside-out macropatches excised from *Xenopus* Oocytes. Since the Ca\(^{2+}\) sensitivity of native SK channels has been reported to lie between these values (Hirschberg *et al*., 1999), it is difficult to conclude the importance of species in KCa channel Ca\(^{2+}\) sensitivity.

A limitation of the results reported here, and from other Ca\(^{2+}\) sensitivity experiments is that, although the Ca\(^{2+}\) concentrations of all internal solutions used were internally consistent, they were not measured first-hand. So, even though the position of each of the curves in Fig. 1, B are accurate in relation to each other, the actual values quoted might not reflect physiological sensitivity. Time and resource permitting, the real Ca\(^{2+}\) concentration in each internal solution could have been measured using a Ca\(^{2+}\) electrode, which detects the molar Ca\(^{2+}\) concentration within an aqueous solution.

Despite these limitations, the Ca\(^{2+}\) sensitivity of SK and IKCa channels have been notoriously difficult to record and various factors make comparing different studies challenging. It is also worth noting that previous studies gave no indication that the free Ca\(^{2+}\) concentration of solutions used was measured first-hand, so how well reported values capture the true sensitivity of these channels is dependent upon the reliability of the programme used in each case to calculate desired free Ca\(^{2+}\) concentrations. Therefore, the
Ca\textsuperscript{2+} sensitivity of hSK1 and hIKCa channels reported here can be viewed as being generally consistent with previously reported values. As a result of this, the findings presented in this chapter can propose a distinct Ca\textsuperscript{2+} sensitivity for the novel heteromeric hSK1-hIKCa channel, which could be used, alongside the findings in Higham et al., to determine whether this heteromeric channel is present in native cells and which physiological role it might perform.
4.6 Chapter References


Shah M, Haylett DG (2000). The pharmacology of hSK1 Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels expressed in mammalian cell lines. Br J Pharmacol. 129: 627-630.


Weisbrod D, Peretz A, Ziskind A, Menaker N, Oz S, Barad L et al. (2013). SK4 Ca\textsuperscript{2+} activated K\textsuperscript{+} channel is a critical player in cardiac pacemaker derived from human embryonic stem cells. Proc Natl Acad Sci USA 110(8): E1685-94.


Chapter 5: General Discussion & Conclusions

The aims of this project were to use different patch clamp configurations and pharmacology to study heteromerisation of hSK1 and hIKCa subunits to form functional channels. The project investigated how that might be achieved and determined the Ca$^{2+}$ sensitivity of heteromeric hSK1-hIKCa channels.

5.1 The H389E mutation within the C-terminal CCDs of hIKCa subunits perturbs co-assembly of hIKCa subunits with hSK1 subunits

The pharmacology of hSK1-hIKCa channels was previously shown to be distinct from the pharmacology of homomeric hSK1 or hIKCa channels (Higham et al., 2019), and this pharmacology was confirmed by the results presented in Chapter 3. Total apamin insensitivity of currents evoked from cells co-expressing hSK1 and hIKCa subunits confirmed that homomeric hSK1 channels were not expressed at the plasma membrane. A right-shifted, monophasic concentration-inhibition curve for TRAM34 on these currents confirmed the absence of homomeric hIKCa channels. As this occurred in every patch excised from co-expressing cells, it can be concluded that a single population of heteromeric channels formed in every cell. The formation of homomeric hIKCa(H389E) channels was not affected by the H389E mutation. However, currents evoked from cells co-expressing hSK1 and hIKCa(H389E) subunits were sensitive to apamin and showed a biphasic sensitivity to UCL1684, confirming the presence of homomeric hSK1 channels and in turn indicating that the formation of heteromeric hSK1-hIKCa(H389E) channels had been disrupted. As the mutated H389 residue occurs within the C-terminal CCDs of hIKCa subunits, these findings propose that the preferential formation of heteromeric hSK1-hIKCa channels is at least in part mediated by this region of the subunit.
One explanation of why the H389E mutation appears to specifically disrupt the co-assembly of hSK1 and hIKCa subunits is that replacing the cationic imidazole side chain of histidine with the anionic carboxylic acid side chain of glutamate at position 389 perturbs the matrix of intra- and inter-chain interactions between the C-terminal CCDs of hSK1 and hIKCa subunits. The imidazole side chain of H389 has a pKa of approximately 6.04. In the more acidic environment of the Golgi and endoplasmic reticulum (Wu et al., 2000), where KCa channels have been proposed to form (Church et al., 2015), H389’s imidazole ring would be protonated to a high degree. This would allow ionic interactions to form between H389 and other charged residues in neighbouring coiled-coils. Since H389 would lose most of its protonation in the cytosol, these ionic interactions might be lost when channels are expressed at the plasma membrane. However, whilst moving through the more acidic intracellular organelles, they could provide a scaffolding that favours the co-assembly of hSK1 and hIKCa subunits to form functional heteromeric channels. It is possible that, because of this scaffolding, interactions between hSK1 and hIKCa subunits are more energetically favourable than interactions between hSK1 and hSK1 or hIKCa and hIKCa subunits. In this theory, the H389E mutation reduces the preferential formation of heteromeric channels enough to produce three subpopulations of channels, with the heteromeric population dominating.

Preliminary experiments were conducted to investigate the effect of introducing the equivalent mutation into hSK1 (H475E) subunits. Although these data were incomplete, initial findings indicated that the H475E mutation disrupted the formation of homomeric hSK1 channels, since the amplitude of hSK1(H475E)-mediated current was reduced compared to the amplitude of hSK1(WT)-mediated current. Also, currents evoked from cells co-expressing hSK1(H475E) and hIKCa(WT) subunits were blocked by TRAM34 in a biphasic manner, suggesting that homomeric hIKCa and heteromeric hSK1(H475E)-hIKCa(WT) channels were present at the plasma membrane and in turn indicating that the preferential formation of heteromeric channels had been perturbed. These findings indicate that the
H475 residue in hSK1 subunits is involved in the assembly of both homomeric hSK1 and heteromeric hSK1-hIKCa channels. This is revealing, since the H389E mutation in hIKCa subunits only disrupted the formation of heteromeric channels, not homomeric hIKCa channels. Why could this be? The output of the coiled-coil prediction program, COILS (Lupas et al., 1991) predicts that hSK1 subunits have only one C-terminal CCD, whereas hIKCa subunits have two C-terminal CCDs (Fig. 5.1). The C-terminal CCD of hSK1 has been proposed to mediate the assembly of functional SK channels (Tuteja et al., 2010). As this CCD is the only one predicted to occur in hSK1 subunits, it might be involved in the assembly of both homomeric hSK1 channels and heteromeric hSK1-hIKCa channels. Therefore, by disrupting this CCD, the H475E mutation disrupted the assembly of homomeric hSK1 channels and co-assembly of heteromeric hSK1-hIKCa channels. In contrast, the H389E mutation would only disrupt the second C-terminal CCD of hIKCa subunits, but not the first. Since hIKCa(H389E) subunits form functional channels, it is possible that hIKCa(H389E) subunit assembly was mediated by the first C-terminal CCD and that heteromeric hSK1-hIKCa channel co-assembly was mediated by the second C-terminal CCD.

It would be interesting to determine the effect of introducing different mutations within the C-terminal CCDs of hSK1 and hIKCa subunits. For example, substituting H389 in hIKCa subunits to an arginine residue. At cytosolic pH, the guanidinium side chain of arginine is virtually always protonated, whereas the proportion of histidine residues containing a protonated imidazole side chain at cytosolic pH is low. This substitution would reveal the importance of having a cationic residue at this position within the C-terminal CCD of hIKCa.
subunits, which would in turn reveal whether or not the protonation of H389’s imidazole side chain is in fact important in the co-assembly of hIKCa and hSK1 subunits.

Figure 5.1. hIKCa subunits contain two predicted C-terminal CCDs, whereas hSK1 subunits contain one predicted C-terminal CCD. The COILS (Lupas et al., 1991) output compares primary amino acid sequences (y-axis) to a database of known CCDs to predict the likelihood that the sequence will form a CCD (x-axis). When using a 28 amino acid reading frame (red bar), only one CCD was predicted to occur in the primary sequence of hSK1 (left), whereas two CCDs were predicted to occur within the primary sequence of hIKCa (right). These CCDs are contained within the C-terminal tail of both channel subunits.

5.2 Heteromeric hSK1-hIKCa channels are less sensitive to activation by Ca$^{2+}$ than homomeric hSK1 and hIKCa channels

Ca$^{2+}$ gating is arguably the most important functional and physiological property of SK and IKCa channels; channel activation relies upon it. Now that heteromeric hSK1-hIKCa channels have been proposed as a novel functional KCa channel subtype, it was important to determine its Ca$^{2+}$ gating sensitivity. Knowing this property might help identify the channel in native cells.
and shed some light on what its physiological roles could be. The findings presented in Chapter 4 show that heteromeric hSK1-hIKCa channels have a Ca\(^{2+}\) gating sensitivity that is significantly right-shifted compared with that of either homomeric hSK1 or hIKCa channels. This lower sensitivity to Ca\(^{2+}\), alongside the pharmacology of heteromeric channels, aligns with the proposed role of these heteromers in the sAHP of CA1 hippocampal neurons, as will be discussed below.

The sAHP is a Ca\(^{2+}\)-activated potential with a component that is thought to be mediated by KCa channels, with both SK1 and IKCa subunits proposed, in different studies, to be involved (Pedarzani et al., 2001, King et al., 2015). The sAHP has a slow time course, peaking and decaying over several seconds, which was proposed to be due to the diffusion of Ca\(^{2+}\) from a spatially distant source to a KCa channel with remarkably high Ca\(^{2+}\) sensitivity (EC\(_{50}\) = 150 nM) but markedly slow activation kinetics (Lancaster & Adams, 1986, Sah & Clements, 1999). The first issue with this theory is that the open probability of the sAHP peaks at 0.4 – 0.6 when exposed to around 1 \(\mu\)M Ca\(^{2+}\) (Sah & Issacson, 1995, Hirschberg et al., 1999), which would mean that a large bulk of Ca\(^{2+}\) would have to diffuse from the Ca\(^{2+}\) source to the sAHP channel. Since cells work to prevent bulk increases in Ca\(^{2+}\), this seems unlikely. Indeed, the highest bulk concentration of Ca\(^{2+}\) reported to diffuse through hippocampal neurons is around 0.1 \(\mu\)M (Knopfel et al., 1990). In addition, there is strong evidence to show that the channels proposed to underlie the sAHP are co-localised with a Ca\(^{2+}\) source, rather than being spatially distant. Immunohistochemistry has demonstrated that Cav1.3 channels and SK1 subunits co-localise in the soma of rat hippocampal neurons (Bowden et al., 2001). In addition, single-channel recordings from patches excised from hippocampal CA1 neurons revealed co-localisation of L-type Cav conductances and SK-type outward conductances at a fixed distance of 100-150 nm (Marrion & Tavalin, 1998). Another issue with the theory that Ca\(^{2+}\) diffuses from a spatially distant source to activate sAHP channels is that the Ca\(^{2+}\) sensitivity of these channels would have to be much higher than the Ca\(^{2+}\) sensitivity of homomeric SK channels reported here and elsewhere, as well as the Ca\(^{2+}\) sensitivity of heteromeric hSK1-hIKCa channels reported here.
An EC\textsubscript{50} for activation of the sAHP channel by Ca\textsuperscript{2+} of 150 nM (Sah & Clements, 1999) does align with the EC\textsubscript{50} of cloned IKCa channels reported by Joiner \textit{et al.} in 1997 of 95 nM. However, both the experimental problems in Joiner \textit{et al.} discussed in Chapter 4 and EC\textsubscript{50} values reported previously, and in this project, for the activation of IKCa channels by Ca\textsuperscript{2+} suggest that the EC\textsubscript{50} of 95 nM was a considerable underestimation by Joiner \textit{et al.} In addition, an EC\textsubscript{50} of 150 nM would mean that a significant proportion of sAHP channels were active at resting concentrations of Ca\textsuperscript{2+} (around 100 nM), for which there is no evidence. Taking these factors into account, an EC\textsubscript{50} of 150 nM for activation of the sAHP channel by Ca\textsuperscript{2+} does not fit with any published KCa channel Ca\textsuperscript{2+} sensitivity data and does not reflect the observed characteristics of the sAHP.

How then can the time-course of the sAHP be as slow as it is, peaking 1 second after a train of action potentials and decaying over 3-5 seconds, if the Ca\textsuperscript{2+} source is co-localised? Abundant evidence from both past and recent studies proposes that sAHP channels are activated by Ca\textsuperscript{2+} that accumulates within submembrane microdomains following delayed facilitation of co-localised Ca\textsubscript{V} channels. In one study, the rising and decaying phases of the delayed facilitation of L-type Ca\textsuperscript{2+} channels that occur following a train of action potentials overlapped with the time-course of the rise and decay phases of the sAHP (Bowden \textit{et al.}, 2001). Another study found that external ionophoresis of the Ca\textsuperscript{2+}-chelator BAPTA onto the soma of CA1 hippocampal neurons inhibited the sAHP, which would only be possible if the Ca\textsuperscript{2+} source was generated by the continual entry of Ca\textsuperscript{2+} throughout the sAHP – not a bolus of intracellular Ca\textsuperscript{2+} occurring during the train of action potentials, which then diffuses to the sAHP channel (Lima & Marrion, 2007). A more recent study proposed that, following a 50 Hz train of action potentials, prolonged facilitation of Ca\textsubscript{V}1.3 channel activity was produced when Ca\textsubscript{V}1.3 subunits were co-expressed with both the scaffolding protein, densin, and CaM kinase II (CaMKII) in tsA201 cells. The subsequent co-expression of IKCa subunits revealed that IKCa channel-mediated conductance was enhanced following stimulation of this delayed facilitation (Sahu \textit{et al.}, 2017). IKCa channel subunits have been proposed to underlie the
sAHP (King et al., 2015), and indeed the sAHP of rat hippocampal pyramidal neurons was inhibited by concomitant application of CaV1.3 and CaMKII blockers and knockdown of densin (Sahu et al., 2017). However, as discussed previously, it is unlikely that homomeric hIKCa channels underlie the sAHP; their significant level of activity at resting concentrations of Ca\textsuperscript{2+} would mean that the sAHP contributes to the resting membrane potential, hyperpolarising it and in turn reducing intrinsic neuronal excitability.

If delayed facilitation of CaV channels does underlie the activation of the sAHP, it would allow submembrane Ca\textsuperscript{2+} to accumulate within discrete, co-localised microdomains throughout the duration of the sAHP. This would accommodate the delayed activation of the sAHP. Also, as the maximal open probability of the sAHP channel is reached at around 1 µM Ca\textsuperscript{2+} (Sah and Issacson, 1995), this channel would require a relatively high EC\textsubscript{50} for activation by Ca\textsuperscript{2+}, around 500 nM. This channel would also be maximally active when exposed to 1 µM Ca\textsuperscript{2+}. These values reflect the Ca\textsuperscript{2+} sensitivity of homomeric hSK1 and heteromeric hSK1-hIKCa channels reported in this project.

5.3 Heteromeric hSK1-hIKCa channels are strong candidates for the sAHP channel

It is unlikely that the sAHP in hippocampal pyramidal neurons is mediated by SK1 channels because the sAHP in these cells is not blocked by apamin, whereas expressed hSK1 channels are (Stocker et al., 1999, Bowden et al., 2001). Moreover, the deletion of SK subunits from cultured cortical neurons was reported to inhibit the mAHP, but not the sAHP (Villalobos et al., 2004), and in transgenic SK knock-out mice the sAHP was reported to be intact (Bond et al., 2004). The fact that the sAHP of CA1 neurons is sensitive to TRAM34 (King et al., 2015), whereas SK channels are not, also makes it unlikely that SK channels are involved. However, the lack of homomeric SK involvement in the sAHP is less clear than might be represented in current literature. For example, the pharmacological evidence supporting this proposal was
gathered in rat CA1 neurons (Stocker et al., 1999, Bowden et al., 2001) and cloned rSK1 channels are known to be insensitive to apamin (Kohler et al., 1996). It will be important to determine the apamin sensitivity of the sAHP of human CA1 hippocampal neurons to resolve the involvement of SK channels.

Re-appraising findings from previous studies reveals that hippocampal conductances initially thought to be mediated by SK channels were actually too large for this to be the case. For example, cell attached patch recordings from rat hippocampal neurons reported a sAHP conductance of around 19 pS. However, the IV relationship for this conductance exhibited non-linearity due to Goldman-Hodgkin-Katz rectification, which can lead to underestimation of unitary conductance (Lima & Marrion, 2007). A conductance that is larger than 20 pS resembles more closely the unitary conductance of heteromeric hSK1-hIKCa channels (36 pS) (Higham et al., 2019) than that of SK channels (2-10 pS) (Kohler et al., 1996). Since both SK1 and IKCa subunits have now been proposed to be expressed in CA1 hippocampal pyramidal neurons (Stocker & Pedarzani, 2000, Sailer et al., 2002, King et al., 2015, Turner et al., 2015, 2016), it is therefore possible that Lima & Marrion were recording heteromeric hSK1-hIKCa channels and underestimating their single-channel conductance. Another indication that heteromeric hSK1-hIKCa channels could underlie the sAHP can be derived from the finding, in this project, that the Ca$^{2+}$ sensitivity of these heteromers is right-shifted, compared with that of either homomeric hSK1 or hIKCa channel. Both hSK1 and hIKCa channels contain four constitutively bound CaMs (Xia et al., 1998, Khanna et al., 1999), so it might be assumed that a heteromer of these subunits should also possess four CaMs, and that these CaMs assemble such that heteromeric Ca$^{2+}$ sensitivity is intermediate between that of the two homomeric channels. However, if heteromeric channels were not simply a by-product of co-expressing both homomeric subunits, but physiologically distinct and purposeful channels in their own right, this assumption of intermediate Ca$^{2+}$ sensitivity would prove unscrupulous, ignoring the fact that these channels might be finely tuned for a specific role.
The fact that heteromeric hSK1-hIKCa channels have a right-shifted Ca\(^{2+}\) sensitivity strongly suggests that their functional properties are specific and meaningful.

The formation of heteromeric hSK1-hIKCa channels occurs preferentially when subunits are expressed together (Higham et al., 2019) in a manner that is likely to be mediated by C-terminal CCDs with a high degree of specificity. These structural features are indicative of a channel that is assembled within cells to carry out a distinct role. In addition, heteromeric channels display insensitivity to apamin and sensitivity to TRAM34, possess a single-channel conductance of around 36 pS (Higham et al., 2019) and a Ca\(^{2+}\) activation EC\(_{50}\) of 600 nM. These functional properties overlap with the properties of the KCa channel underlying the sAHP with more accuracy than any other known member of the KCa channel family. The novel insights into the structural and functional properties of heteromeric hSK1-hIKCa channels uncovered in this project strengthen the case for investigating whether or not these channels are present in neurons. These findings also add further evidence in support of the theory that these heteromeric channels underlie the sAHP.

### 5.4 Conclusion

This project asserts that the formation of heteromeric hSK1-hIKCa channels is made preferential, over the formation of homomeric channels, in part, by the H389 residue within the second C-terminal CCD of hIKCa subunits. This project also asserts that heteromeric hSK1-hIKCa channels are less sensitive to Ca\(^{2+}\) than homomeric hSK1 or hIKCa channels, and that the Ca\(^{2+}\) sensitivity of hSK1-hIKCa channels aligns with the Ca\(^{2+}\) activation properties of the sAHP.
5.5 Chapter References


Bowden SHE, Fletcher S, Loane DJ, Marrion NV (2001). Somatic Colocalization of Rat SK1 and D Class (CaV 1.2) L-type Calcium Channels in Rat CA1 Hippocampal Pyramidal Neurons. J Neurosci 21: RC175 1-6.


