2015-01-12

Novel Expression and Roles for IKCa in Hippocampal Pyramidal Neurons

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Novel Expression and Roles for IKCa in Hippocampal Pyramidal Neurons

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN NEUROSCIENCE

CALGARY, ALBERTA

November, 2014

Abstract

This study reports novel roles of the calcium-activated potassium channel of intermediate conductance, called IKCa, in pyramidal neurons of the CA1 region of the rat and mouse hippocampus. In addition to pharmacological profiling of the IKCa channel in these neurons, conducted using potent blockers of IKCa such as TRAM-34 and ChTx, single channels were found that fit the profile of IKCa including a potassium-selective single channel of ~30 pS conductance activated by calcium influx. In addition, the role of IKCa in generating the calcium-activated slow afterhyperpolarization (sAHP) was investigated. The molecular identity of the sAHP has eluded discovery for over 30 years despite it representing one of the strongest inhibitory responses in the brain. Here it is reported that IKCa channels generate a long-lasting, calcium-activated sAHP in response to bursts of synaptic activity and neuron firing, which is inhibited by TRAM-34. This work is important in identifying a novel ion channel in the brain and one of the primary contributors to the sAHP. These data will help to further our understanding of hippocampus-related phenomena, including behaviours such as spatial memory, with potential implications in other brain regions expressing IKCa channels.

Acknowledgements

Thank you to the members of the Turner laboratory for fruitful discussions and educating me in experimental procedures, science, and life. I would especially like to thank my supervisor Dr. Ray Turner for all of his guidance, knowledge, motivation, and support. I would like to acknowledge Arsalan Rizwan for his extensive assistance in all single channel recordings, as well as Ted Bartoletti, Colin Heath, Jordan Engbers, Steven Dykstra, Hadhimulya Asmara, and Alex Kim for their assistance throughout the project. I would also like to thank Shahid Hameed for providing and transfecting HEK cells and Mirna Kruskic for her help in the laboratory. Lastly, I would like to thank all sources of funding that have made this thesis possible including, but not limited to, CIHR, the Hotchkiss Brain Institute, the University of Calgary, and the Government of Alberta.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
4-AP	4-aminopyridine
aCSF	Artificial cerebrospinal fluid
ACTH	Adrenocorticotropic hormone
AHP	Afterhyperpolarization
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BAPTA	1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid
BK	Big conductance potassium channel
BSA	Bovine serum albumin
CA	Cornu ammonis
CaCC	Calcium-activated chloride channel
CaM	Calmodulin
cAMP	3'-5'-cyclic adenosine monophosphate
Cav	Voltage-activated calcium channel
Cd^{2+}	Cadmium
ChTx	Charybdotoxin
CICR	Calcium-induced calcium release
CNS	Central nervous system
CsCl	Cesium chloride
DC-EBIO	5,6-Dichloro-1-ethyl-1,3-dihydro-2*H*-benzimidazol-2-one
DG	Dentate gyrus
DL-AP5	DL-2-amino-5-phosphonopentanoic acid
DMSO	Dimethylsulfoxide
DNQX	6,7-dinitroquinoxaline-2,3-dione
DRG	Dorsal root ganglion
EC	Entorhinal cortex
EGTA	Ethylene glycol tetraacetic acid
EPSC	Excitatory post-synaptic current
EPSP	Excitatory post-synaptic potential
fAHP	Fast afterhyperpolarization
GABA	γ-aminobutyric acid
GFP	Green fluorescent protein
HCN	Hyperpolarization-activated cyclic nucleotide-gated
HEK	Human embryonic kidney
HEL	Human erythroleukemia
HPA axis	Hypothalamus-pituitary-adrenal axis
HVA	High-voltage activated
IbTx	Iberiotoxin
IKCa	Intermediate conductance potassium channel
I_M	M-current

I*sAHP*	Slow afterhyperpolarizing current
KCa	Calcium-activated potassium channel
KO	Knockout
Kv	Voltage-gated potassium channel
LVA	Low-voltage activated
mAHP	Medium afterhyperpolarization
MgTx	Margatoxin
mRNA	Messenger RNA
NFA	Niflumic acid
NMDA	N-methyl-D-aspartate
NPPB	5-nitro-2-[(3-phenylpropyl)amino]benzoic acid
PKA	cAMP-dependent protein kinase
PKA*Cat*	Catalytic subunit of PKA
RCK	Regulator of potassium conductance domain
RT-PCR	Reverse transcriptase polymerase chain reaction
sAHP	Slow afterhyperpolarization
SK	Small conductance potassium channel
SR	Stratum radiatum
TEA	Tetraethylammonium
TM	Transmembrane
TTX	Tetrodotoxin

Chapter One: INTRODUCTION

1.1 Membrane excitability

The regulation of central neuron action potential generation and a neuron's response to synaptic input from other neurons is the basis for neural coding and information processing. The ability of a neuron to regulate its level of excitability or rate of firing, as well as its level of excitation from an input, is therefore crucial to the normal functioning of neuronal circuitry. This regulation can come from multiple sources in the form of ion channels and chemical messengers of differing properties and roles. In the central nervous system (CNS), the most powerful postsynaptic contributors to the reduction of excitability derive from voltage- and calcium-gated potassium channels. These channels are capable of opening under various conditions of excitation to allow for the efflux of potassium and the consequent generation of a repolarizing or hyperpolarizing current. For example, many different voltage-gated potassium (Kv) channels are highly expressed in neurons to assist in immediate repolarization of the membrane following the upstroke of an action potential (for reviews see (Coetzee et al., 1999; Pongs, 1999)). While there are only two classes of calcium-activated potassium (KCa) channels widely recognized in central neurons, they are critically important in that they tie changes in the concentration of intracellular calcium to neuron excitability, generating afterhyperpolarizations (AHPs) following action potentials that regulate firing rates over the longer time frames required for synaptic integration.

1.2 Biophysical and pharmacological properties of the KCa family in the CNS

There are three main groups of KCa channels, classified initially on the magnitudes of their single channel potassium conductance: the big conductance KCa channel (BK, *slo*, Maxi-K, KCa1.1, KCNMA1) has the largest conductance at 100-250 pS (Berkefeld et al., 2010), whereas a family of small conductance KCa channels (SK1-3, KCa2.1-2.3, KCNN1-3) have the smallest conductance at around 10-20 pS (Blatz and Magleby, 1986; Hirschberg et al., 1999; Adelman et al., 2012). These two types of KCa channels are recognized to be widely expressed in the CNS to control neuronal excitability, and contribute to different aspects of AHPs (discussed below). The intermediate conductance KCa channel (IKCa, KCa3.1, KCNN4), on the other hand, was reportedly absent from central neurons (Ishii et al., 1997; Joiner et al., 1997; Logsdon et al.,

1997; Jensen et al., 1998; Jensen et al., 2001; Andrade et al., 2012). As its name suggests, IKCa channels have an intermediate conductance of between 20-90 pS (Ishii et al., 1997; Logsdon et al., 1997). The biophysical and pharmacological properties of these three KCa channel classes are compared in **Table 1** and **Fig. 1**. While KCa channels in each of the respective families have major similarities in their biophysical properties, there are important differences that allow each variant to contribute to neuron excitability in different ways.

1.2.1 BK channels

In addition to their conductances, BK channels are different from SK and IKCa channels in that they are gated by voltage as well as internal calcium (Cui and Aldrich, 2000; Fakler and Adelman, 2008). These two BK channel gating mechanisms occur independently yet interactively, in which higher levels of internal calcium lowers the voltage range for activation of the channel (Cox et al., 1997; Cui et al., 1997). Whereas SK and IKCa channels have a similar structural topography to the Kv family (6 transmembrane domains, S1-S6, plus a conserved pore domain), BK channel α subunits have 7 transmembrane domains, with an external N-terminus and a longer cytoplasmic C-terminus than SK or IKCa channels (Latorre et al., 2010). BK channels are also distinct in the nature of calcium sensing in that the long C-terminus of each of its 4 subunits contain two "regulator of potassium conductance" (RCK) domains, one of which that binds calcium at a structurally determined calcium bowl motif (**Fig. 1**). While this relieves the requirement for calcium-sensing accessory subunits, it also confers the lowest sensitivity of KCa channels to internal calcium, with a threshold for activation in the range of 1-10 μM (Womack and Khodakhah, 2002; Berkefeld et al., 2010; van Welie and du Lac, 2011). However, BK channels also interact with any of several regulatory β-subunits. For example, the neuronal β4 subunit slows BK gating kinetics, modulates its calcium sensitivity, and reduces its sensitivity to iberiotoxin (IbTx), a BK channel blocker (Brenner et al., 2000).

BK channels are expressed ubiquitously throughout mammalian tissues (including the brain) (Toro et al., 1998) and in neurons have been determined to be responsible for spike

Table 1. Structural and functional differences between the three main types of calcium-activated potassium channels in central neurons.

	BK	**SK**	**IKCa**
Conductance	100-250 pS	10-20 pS	20-90 pS
Protein Structure	7 transmembrane, 1194 amino acids (Jensen et al., 2001)	6 transmembrane, 536 amino acids (Jensen et al., 2001)	6 transmembrane, 427 amino acids (Jensen et al., 2001)
Calcium Dependence	Calcium-dependent	Calcium-dependent	Calcium-dependent
Voltage Dependence	Yes	None	None
Inhibitors	TEA, ChTx, IbTx	Apamin	TRAM-34, NS6180, ChTx, MTx, Senicapoc
Agonists	NS1619	NS309, DC-EBIO	NS309, DC-EBIO
Accessory Protein Interactions	β subunits	CaM, Protein kinase CK2, PP2A	CaM, Hippocalcin?
Functions	fAHP, spike repolarization	mAHP	sAHP?

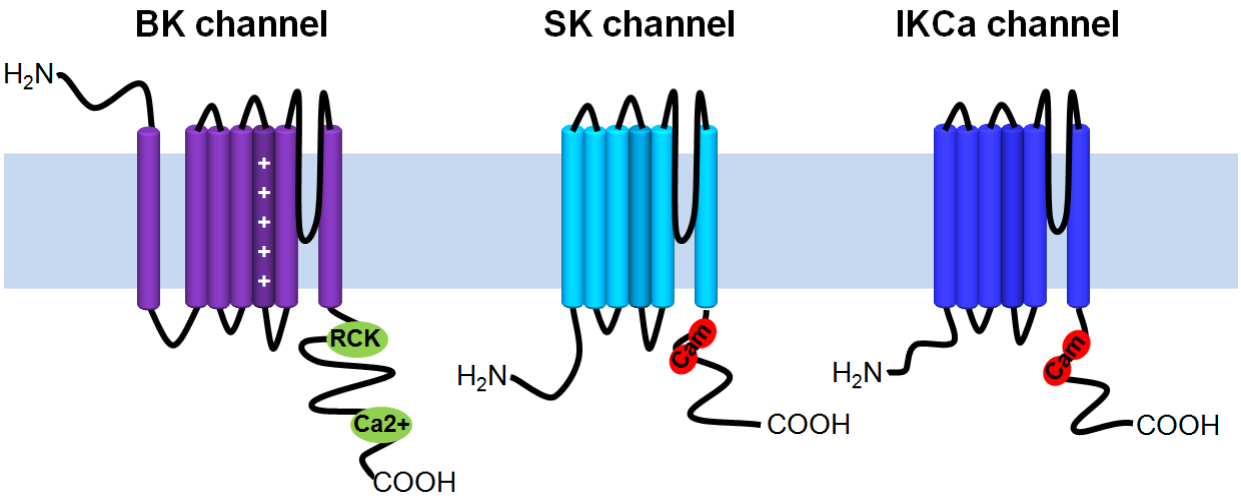

Figure 1. Structural differences between the three types of KCa channel monomers.
BK channels, with 7 TM domains, have a larger C-terminus than both SK and IKCa channels, with its calcium sensing domain contained within the C-terminus. SK and IKCa channels have 6 TM domains and interact with CaM, which is reported to act as a calcium sensor for both proteins. All three types form homotetramers to form functional channels.

repolarization and generation of the fast component of the AHP (fAHP), which lasts 2-5 ms (Storm, 1987, 1990; Gu et al., 2007). BK channels also contribute to the modulation of dendritic calcium spikes and release of neurotransmitters (Berkefeld et al., 2010). While essential for maintenance of spike generation and maintaining fast repetitive discharge, these roles contribute only minimally to setting neuronal firing rates on a longer time scale (Faber and Sah, 2002).

Neuronal BK channels have been reported to form complexes with voltage-gated calcium channels (Cav), and indeed their calcium source is derived from the activation of these channels (Berkefeld et al., 2010; Engbers et al., 2013; Rehak et al., 2013). This is important in that it allows BK channels to be activated by local calcium sources, removes the need to sense bulk calcium levels in the cell, and functionally separates BK channel activation from other calcium-dependent processes. However, the dynamics of calcium sensing by BK channels are still under investigation, as new information has recently come to light suggesting that multiple BK-Cav complexes can function together to shift calcium sensing to that of a microdomain (Engbers et al., 2013).

1.2.2 SK channels

The SK family of KCa channels has been established as a major contributor to the control of excitability in the CNS. There are three major isoforms, SK1-3, all of which have messenger RNA (mRNA) and protein expressed in the brain but with different patterns of expression (Stocker and Pedarzani, 2000; Sailer et al., 2002). The three isoforms are similar in core structural sequence, with differences in the N- and C- termini imparting isoform-specific properties (Adelman et al., 2012). As mentioned above, SK pore-forming subunits have 6 transmembrane (TM) domains (S1-S6) with a pore region between S5 and S6 and cytosolic N- and C-terminals (**Table 1**, **Fig. 1**). Like BK channels, SK pore-forming subunits assemble in homomeric tetramers to form a functional channel (Kohler et al., 1996). Unlike BK channels, SK channels are voltage independent and sensitive to internal calcium, with a half-maximal activation of approximately 300 nM (Adelman et al., 2012). SK channels are often reported to be inward rectifying due to divalent cation block at high voltages, but recently this effect has been shown to be due to an intrinsic mechanism in the channel, challenging the view that SK channels

are voltage independent (Li and Aldrich, 2011). SK channel activation requires an accessory protein called calmodulin (CaM) that is constitutively bound to the SK intracellular C-terminus directly after the S6 TM domain to induce a conformational change in the channel upon binding calcium (Schumacher et al., 2001). When this occurs, SK channels contribute a hyperpolarizing outward potassium current, with SK channels being shown to mediate an apamin-sensitive medium AHP (mAHP) in a wide range of central neurons, including pyramidal neurons of the CA1 region of the hippocampus (Sah, 1996; Stocker et al., 1999; Bond et al., 2004). The mAHP lasts 50-100 ms (Storm, 1987) and has been shown to reduce excitability in many central neurons, with block by apamin increasing firing rate and overall excitability (Sah, 1996; Adelman et al., 2012). However, the role of SK in generating the mAHP in the CA1 region of the hippocampus has been called into question with the finding that Kv7 and HCN channels generate the mAHP in CA1 pyramidal cells (Gu et al., 2005). This study found that calcium-free media did not affect the mAHP, and that XE-991, a potent Kv7 blocker, and not apamin, eliminated the mAHP. Therefore, the molecular identity underlying the mAHP is not quite resolved, but SK may play a significant role in generating the mAHP in central neurons.

SK channels have been shown to be activated by a multitude of calcium sources, including Cav channels activated during the upstroke of an action potential (Marrion and Tavalin, 1998), and internal calcium stores through calcium-induced calcium release (CICR) (Adelman et al., 2012). These calcium sources generate calcium microdomains for activation of SK (Fakler and Adelman, 2008). In addition, SK channels have been reported to form close associations with N-methyl-D-aspartate (NMDA) receptors (NMDARs) in dendritic spines of pyramidal cells in the CA1 region of hippocampus (Ngo-Anh et al., 2005). These channels are therefore in a crucial position to regulate and reduce the size of synaptic potentials received by CA1 pyramidal neurons. Therefore, SK channels are important for regulating multiple aspects of neuron membrane excitability in response to increases in calcium levels inside the cell, including partial generation of the mAHP and modulating synaptic processing.

1.2.3 Biophysical properties, expression profile, and roles of IKCa in non-neuronal peripheral cells

The last class of KCa channel, IKCa, is more similar to SK channels than BK in terms of its biophysical properties. Like SK channels, IKCa channels have six TM domains (S1-6), and are maximally activated at internal calcium levels of ~500 nM, with a half-maximal activation of ~ 300 nM free internal calcium (Ishii et al., 1997; Joiner et al., 1997; Logsdon et al., 1997). The IKCa channel also has a CaM-binding domain on the C-terminus of each pore-forming subunit that are thought to constitutively bind CaM (**Table 1**, **Fig. 1**) (Fanger et al., 1999; Keen et al., 1999; Joiner et al., 2001). Like SK channels, IKCa channels expressed in heterologous systems are reported to be voltage-independent (Joiner et al., 1997; Logsdon et al., 1997) or inwardly rectifying (Ishii et al., 1997). Conflicting reports of IKCa inward rectification could be due to differences in experimental procedures, such as different expression systems (ex. Chinese hamster ovary cells (Joiner et al., 1997), human embryonic kidney (HEK) cells (Logsdon et al., 1997), or oocytes (Ishii et al., 1997)), and different IKCa clone origins (ex. human placenta (Joiner et al., 1997), human T lymphocytes (Logsdon et al., 1997), or human pancreas (Ishii et al., 1997)). In addition, a recent report showed a variant of IKCa in human erythroleukemia (HEL) cells that demonstrated voltage dependence, where the current generated by IKCa became larger at more positive voltages due to a measureable change in channel open probability (Stoneking et al., 2013). This group also recently showed that the same variant in HEL cells is blocked in a voltage dependent manner by internal magnesium ions that compete with calcium for binding to the channel, to reduce its unitary conductance and probability of opening (Stoneking and Mason, 2013). Differences in the concentration of internal magnesium can therefore potentially explain the different reports of IKCa inward rectification. The extent to which IKCa channels exhibit voltage dependence is thus still open to question, and potentially cell-specific.

Despite these similarities with SK channels, there are significant differences between these channels that provide IKCa with a unique profile used by multiple types of cells for different roles. In addition to a larger single channel conductance (~20-90 pS) than SK channels, IKCa channels exhibit a slower activation by calcium due to a balance of phosphorylation/dephosphorylation mediated by resident levels of adenosine 3', 5'-cyclic

monophosphate (cAMP)-dependent protein kinase A (PKA) (Vogalis et al., 2002b). Also, the IKCa protein sequenced from human pancreas is 427 amino acids long and has only ~45% identity to the human SK channel sequences (Begenisich et al., 2004).

IKCa has been reported to be absent from neurons of the CNS. However, this channel is well known to be expressed in peripheral tissues such as the blood and immune systems, including red blood cells where IKCa was identified as the molecular basis for the Gardos channel, the first KCa channel recognized (Hoffman et al., 2003). In these cells, IKCa regulates water volume regulation, where a calcium-activated potassium efflux results in dehydration of the cell. IKCa is also expressed in immune cells such as activated microglia (Ghanshani et al., 2000; Wong and Schlichter, 2014b) and lymphocytes (Logsdon et al., 1997) where it assists in hyperpolarizing the cell membrane to facilitate calcium influx, which is important for proliferation, cytokine production, and immune function (Logsdon et al., 1997). In fluid-secreting epithelial cells, IKCa assists in hyperpolarizing the membrane to facilitate Cl^- efflux (Takahata et al., 2003). IKCa channels are also important in regulating the proliferation of smooth muscle cells, fibroblasts and T and B cells (Wulff et al., 2007). By hyperpolarizing the membrane of these cells, IKCa causes an influx of calcium, which is involved in activation of proliferation machinery.

1.2.4 IKCa has a distinct pharmacological profile

IKCa channels exhibit a distinct pharmacological profile from BK or SK channels (**Table 1**). Indeed, a "toolbox" of pharmacological agents has been established against the various KCa channels, providing an important means of isolating each group of KCa to determine their functional roles in neurons. BK channels are blocked by nanomolar concentrations of the scorpion small peptide toxins iberiotoxin (IbTx) (Galvez et al., 1990) and charybdotoxin (ChTx) (Miller et al., 1985; Reinhart et al., 1989), as well as by submillimolar concentrations of tetraethylammonium (TEA - IC_{50}~80-330 µM) (Coetzee et al., 1999). SK channels, on the other hand, are potently inhibited by the highly selective bee venom peptide apamin at nanomolar concentrations (IC_{50} ~27 pM to 12 nM, depending on the isoform) (Pedarzani and Stocker, 2008). These channels have also been reported to be blocked by a scorpion toxin called

scyllatoxin, as well as the organic compound bicuculline that is often used to block gamma-aminobutyric acid (GABA)ergic inhibition (Pedarzani and Stocker, 2008).

IKCa channels are insensitive to apamin and only blocked by TEA at high concentrations (IC_{50} = 24 mM) (Wulff et al., 2007), the two main agents used to block SK and BK channels, respectively. Importantly, highly selective inhibitors of the IKCa channel were recently identified, including TRAM-34 (IC_{50} = 20 nM) (Wulff et al., 2000), a derivative of clotrimazole, and NS6180 (IC_{50} = 9 nM in cloned human IKCa) (Strobaek et al., 2013). TRAM-34 and NS6180 are lipophilic molecules with very different structures that bind to IKCa at the same location on the cytoplasmic side of the channel (residues T250 and V275) (Wulff et al., 2001; Strobaek et al., 2013). Another molecule capable of blocking IKCa is Senicapoc (IC_{50} = 11 nM), which has been used to treat sickle cell disease due to excessive potassium efflux through IKCa and subsequent dehydration of red blood cells (Ataga and Stocker, 2009). IKCa has also been shown to be potently blocked by the small peptide scorpion toxins maurotoxin (MTx, IC_{50} = 1 nM) (Castle et al., 2003) and ChTx (IC_{50} = 5 nM) (Miller et al., 1985; Rauer et al., 2000). However, both ChTx and MTx have been shown to be non-selective in their block of IKCa. ChTx is also a potent blocker of BK channels, as well as certain Kv channels including Kv1.3 (IC_{50} = 5 nM) (Wulff et al., 2007). MTx also blocks other Kv channels, including Kv1.2, and at a higher potency than IKCa (Kv1.2 IC_{50} = 100 pM) (Castle et al., 2003). Since these non-specific actions can lead to confusing results when trying to elucidate the roles of IKCa, MTx and ChTx can only be used under conditions suitable for interpreting effects on IKCa channels in isolation.

In addition to the inhibition of IKCa channels, there are pharmacological agents that can be used to activate or enhance IKCa activity. The most potent activators include NS309 (EC_{50} = 27 nM) (Strobaek et al., 2004) and dichloro-EBIO (DC-EBIO, EC_{50} = 750 nM) (Singh et al., 2001; Wulff et al., 2007). However, it is important to consider that these compounds also activate SK channels, with a slight selectivity for IKCa over SK channels (Strobaek et al., 2004), requiring additional block of SK channels if used in the context of IKCa channel agonists.

1.3 Evidence for neuronal IKCa expression

1.3.1 IKCa in the peripheral nervous system

Despite the dogma created by early reports of a lack of IKCa mRNA in the brain (Ishii et al., 1997; Joiner et al., 1997; Logsdon et al., 1997), there is a growing line of evidence for IKCa presence in the nervous system. Outside of the CNS, early reports demonstrated the presence of a calcium-activated potassium channel of intermediate conductance that was insensitive to TEA or apamin in rat visceral sensory neurons of the nodose ganglion, with a single channel conductance of approximately 60 pS (Hay and Kunze, 1994). In the enteric nervous system, IKCa has been shown to be present in myenteric neurons where it generates the slow component of the AHP (sAHP) (Vogalis et al., 2002a; Neylon et al., 2004). An apamin-resistant, calcium-activated potassium current was elicited by a large depolarization causing multiple action potentials, and had a single channel conductance of 30 pS. Block of this slow current using clotrimazole (an IKCa blocker) completely eliminated the sAHP (Vogalis et al., 2002a; Neylon et al., 2004). These IKCa channels were shown to have very slow inactivation properties, with slow calcium dissociation and second messenger influences such as phosphorylation by PKA. In addition to electrophysiological evidence of IKCa expression, IKCa immunolabel was demonstrated in enteric neurons of humans and rats (Furness et al., 2003; Neylon et al., 2004). In rat dorsal root ganglion (DRG) neurons of the spinal cord, immunocytochemical labeling demonstrated expression of IKCa in IB4-expressing nociceptor neuron populations (Mongan et al., 2005). Importantly, like peripheral sensory neurons, several other types of central neurons also exhibit a slow, apamin-insensitive AHP current (Sah and Davies, 2000).

1.3.2 Evidence from the CNS

There have also been findings that implicate IKCa expression in the CNS. IKCa was first shown to be present in the CNS in magnocellular neuroendocrine neurons in the supraoptic nucleus of the rat hypothalamus, where it contributes to a sAHP current (Greffrath et al., 1998). This study showed that the sAHP was insensitive to apamin, IbTx, and margatoxin (MgTx, a BK channel blocker), but was blocked by ChTx. Also, IKCa immunolabel and expression was reported in human and cultured rat spinal cord neurons, and regulated by inflammatory

neurotrophic factors (Boettger et al., 2002). More recent evidence of IKCa expression in the CNS was obtained in cultured mouse anterior pituitary corticotrophs (Liang et al., 2011). In these cells, a TRAM-34-sensitive IKCa current was shown to control the release of adrenocorticotropic hormone (ACTH). Application of TRAM-34 resulted in an increase in excitability by inducing a higher rate of action potential firing and, as a result, higher ACTH release. IKCa knockout mice also demonstrated stress hyperresponsiveness, implicating IKCa's role in functioning of the hypothalamus-pituitary-adrenal (HPA) axis (Liang et al., 2011). Therefore, for an extensive period of time, IKCa was demonstrated to be present in excitable cells of the peripheral and enteric nervous systems and the CNS, but not in central neurons.

The strongest and most unequivocal evidence for IKCa expression in central neurons was recently demonstrated by the Turner laboratory. The first CNS recordings of these channels in cerebellar Purkinje cells demonstrated that there are KCa channels with a single channel conductance of approximately 36 pS that are sensitive to the IKCa blocker TRAM-34 as well as the BK and IKCa inhibitor ChTx (Engbers et al., 2012a). In Purkinje cells, it was found that IKCa is responsible for the generation of a synaptically-evoked sAHP that suppresses temporal summation, hyperpolarizes membrane voltage, and reduces spike output. This sAHP was found to exhibit some of the same properties as synaptically-evoked sAHPs found elsewhere in the CNS such as slow kinetics, prolonging of excitatory post-synaptic potential (EPSP) rate of decay, activation by calcium, and potassium selectivity. However, unlike in the CA1 hippocampus, the Purkinje cell sAHP is activated by calcium from T-type calcium channels in tight nanodomains, allowing the hyperpolarizing current generated by IKCa to act as a high-pass filter that suppresses low frequency parallel fiber inputs (noise). IKCa is therefore a major player in the Purkinje cells' abilities to process meaningful sensory information passed on from parallel fibers of cerebellar granule cells. This is the first direct evidence that IKCa is not only expressed in neurons of the CNS, but also that it plays an indispensible role in controlling neuron excitability and information processing. Challenge to the persistent thought that IKCa is absent from central neurons leads to the possibility that this channel may have considerable influence in other regions of the brain.

In addition to electrophysiological data, IKCa mRNA expression was detected in Purkinje cells using single-cell reverse transcriptase polymerase chain reaction (RT-PCR). Using immunocytochemistry, the Turner lab also found the expression of IKCa to be much more widespread than previously anticipated and present in multiple regions of the CNS including the cerebellum, cortex, hippocampus, and thalamus (Turner et al., 2014). IKCa immunolabel was identified using a monoclonal antibody that was established to exhibit IKCa specificity compared to all SK isoforms. In the hippocampus, IKCa immunolabel was detected in the CA1 and CA3 regions, while expression was relatively lacking in CA2.

1.4 The slow AHP in hippocampal CA1 pyramidal cells

1.4.1 Hippocampus: roles and characteristics

The hippocampus is a well-studied brain region that has received much attention since the fascinating case study of HM, in which the anterior two-thirds of this patient's hippocampi were removed as a treatment for severe seizures. After the procedure, HM had severe anterograde amnesia, where he could not form new memories post-surgery. In addition, the hippocampus has a well-known and relatively simple connectivity, making it a desirable target for study of synaptic processes. It is divided into four main regions: the dentate gyrus (DG), the subicular complex, the hippocampus proper, and the entorhinal cortex (EC). The hippocampus proper is further divided into three main regions, called the Cornu Ammonis (CA1-3). The CA1 region has been used to study pyramidal neuron properties in the hippocampus due to its pivotal roles in the trisynaptic circuit. This is the excitatory circuit from the EC to the DG along the perforant path, then to the CA3 through granule cell mossy fibers, which then project to the CA1 via Schaeffer collaterals, and finally to the subiculum, the major output of the hippocampus (Amaral and Witter, 1989). Pyramidal neurons of the CA1 are major players in this circuit, and their connection with the CA3 region is often studied due to the consistency and strength of their synaptic connection, which makes it relatively easy to evoke synaptic activity by stimulation for study.

The hippocampus has long been thought to contribute to spatial memory since it was observed that hippocampal damage in rodents gave rise to an inability to perform during spatial

tasks (O'Keefe and Dostrovsky, 1971). Early studies also showed that hippocampal lesions impaired a rat's ability to navigate a water maze (Morris et al., 1982), and lesions to the rat dorsal hippocampus produced deficiencies in spatial learning (Moser et al., 1995; Hollup et al., 2001).

Therefore, the hippocampus is an important brain structure that has critical roles in multiple behaviours, and figuring out the underlying mechanisms for these functions is an important endeavor. One crucial mechanism for controlling excitability that is used widely in neurons of the hippocampus is the calcium-activated AHP.

1.4.2 Properties of the hippocampal CA1 sAHP

The nature of the AHPs following a spike or train of spikes in CNS neurons has long been studied in hippocampal pyramidal cells (Madison and Nicoll, 1984; Lancaster and Adams, 1986; Lancaster and Nicoll, 1987; Lancaster et al., 2001). This cellular phenomenon has since been extended to other neurons of the brain, including pyramidal cells of the cortex (Pineda et al., 1999; Abel et al., 2004). AHPs represent an inhibitory response with multiple components and factors that can modulate their impact on the membrane potential over different time frames. Three different calcium- and voltage-dependent AHPs are recorded following action potential discharge in CA1 pyramidal cells (**Fig. 2**). As mentioned above, an initial fAHP of 1-2 ms is largely mediated by BK and Kv channels (Lancaster and Adams, 1986; Shao et al., 1999; Sah and Faber, 2002), whereas a mAHP lasting up to 100 ms is mediated by SK channels, and specifically KCa2.2 (Stocker et al., 1999; Bond et al., 2004). Despite thorough characterization of the fAHP and mAHP in CA1 pyramidal cells, the channels responsible for a third AHP called the slow AHP (sAHP) that can last seconds, has defied explanation for almost 30 years. This is important because the sAHP exerts the largest influence on neuronal excitability and represents one of the primary targets for neuromodulation by a wide range of transmitters. At the cellular level the sAHP contributes to important measures of cell excitability and information encoding behaviours such as spike frequency adaptation (Sah and Faber, 2002; Vogalis et al., 2003), neuronal gain (Higgs et al., 2006) and synaptic integration (Lancaster et al., 2001; Wu et al., 2004; Fernandez de Sevilla et al., 2007). At the circuit level the amplitude of the sAHP, which is influenced by neuromodulation, has been linked to the expression of a wide range of behaviours that include synaptic plasticity, learning, spatial orientation, aging, learning, and stress (Joels and

de Kloet, 1989; Borde et al., 2000; Giese et al., 2001; Martin et al., 2001; Wu et al., 2002; Tombaugh et al., 2005; Brosh et al., 2006; Gant and Thibault, 2009; Farmer and Thompson, 2012). In addition, the sAHP may assist in preventing cell death due to excessive calcium influx (Vergara et al., 1998).

It is widely accepted that in the CA1 the sAHP current (Is*AHP*) is mediated by a KCa channel, as it has been shown to be calcium-activated, is potassium-selective, voltage-independent, and is activates and decays over a duration of seconds. Calcium dependence was demonstrated when addition of internal calcium chelators such as EGTA or BAPTA (Madison and Nicoll, 1984; Lancaster and Nicoll, 1987), or block of calcium influx through high voltage-activated calcium channels using cadmium (Cd^{2+}) (Madison and Nicoll, 1984; Lancaster and Adams, 1986; Lancaster and Nicoll, 1987; Storm, 1989) eliminated the sAHP. The channels responsible for the sAHP have been shown to be potassium-selective in that the reversal potential of the sAHP follows the reversal potential of potassium (Lancaster and Adams, 1986). The amplitude of the sAHP is directly related to the levels of action potential firing (Madison and Nicoll, 1984; Lancaster and Adams, 1986; Storm, 1989), as expected for a graded increase in calcium influx with a neuron's level of firing. Chloride has been repeatedly shown to not play a role in the sAHP, as its reversal potential does not follow E_{Cl} (Alger and Nicoll, 1980; Hotson and Prince, 1980). The sAHP has also been reported to be voltage-independent (Lancaster and Adams, 1986) and to have a long time scale in CA1 cells (Hotson and Prince, 1980; Madison and Nicoll, 1984; Lancaster and Adams, 1986; Lancaster and Nicoll, 1987). Other properties of the sAHP include TEA, ChTx, and apamin insensitivity (Storm, 1989), with TEA insensitivity being shown at 200 μM, and ChTx insensitivity when tested at 25 nM (Lancaster and Nicoll, 1987). These results were interpreted to show that the sAHP is not mediated by BK, SK, or IKCa channels.

1.4.3 Roles of the sAHP in behaviour

While important for single cell and synaptic processing of information, the sAHP in CA1 pyramidal neurons has been implicated in learning behaviours in animals. The hippocampus

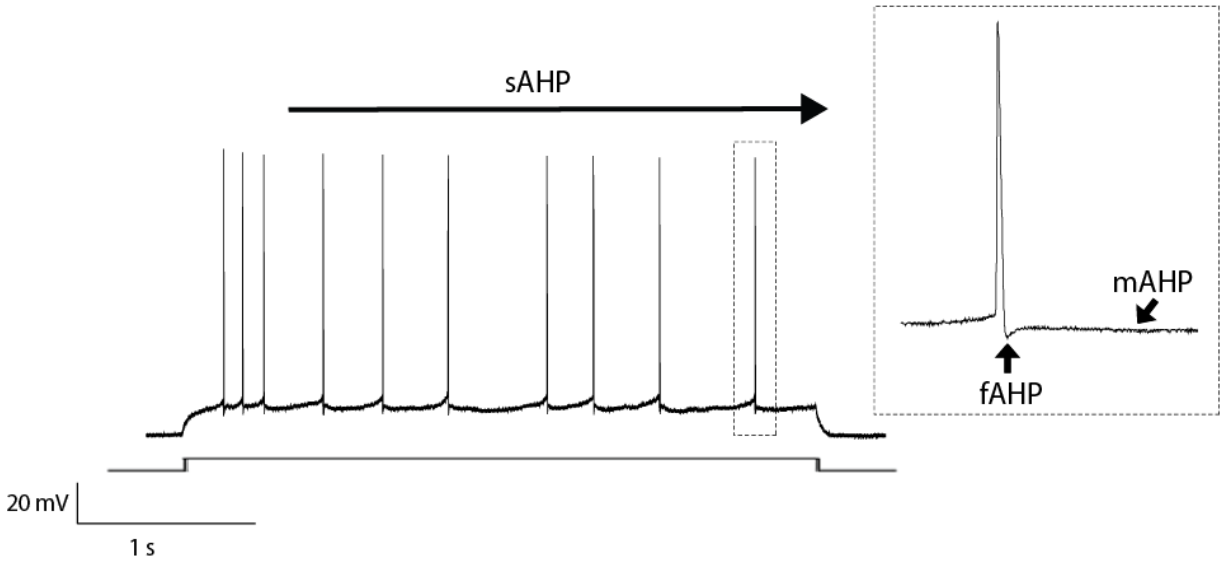

Figure 2. Properties and functions of the three types of AHP in CA1 pyramidal cells.
The various AHPs found in the CNS are classified based on their durations. The fAHP (shown in *inset* - enlarged single spike) is evoked directly after a spike, and lasts approximately 1-2 ms. The mAHP also occurs after an action potential or burst of action potentials, and lasts approximately 100 ms. The sAHP is the longest lasting AHP, at a range of 500 ms to multiple seconds, and results in spike accommodation (growth of time between consecutive spikes - large *black arrow* above trace) during current injection to a CA1 pyramidal cell. Loss of either or both the mAHP and sAHP results in increased excitability and decreased spike accommodation.

itself has long been known to be crucial for spatial learning tasks (Morris et al., 1982; Morris et al., 1990). In rabbits, the magnitude of the sAHP has been correlated with hippocampus-dependent temporal learning tasks such as eye blink conditioning (Moyer et al., 1996), as well as hippocampus-dependent spatial learning tasks such as the Morris water maze in rats (Oh et al., 2003). In both tests, successful learning of the task in question results in a reduction in the post-burst AHP and increased excitability of CA1 neurons, while non-learners show no reduction compared to control animals. Decline of spatial memory in aging rats has been described previously (Gage et al., 1984; Deupree et al., 1991), and an inability to modulate the sAHP has been suggested as a mechanism to describe such a loss (Campbell et al., 1996; Landfield, 1996). These reports suggest that aged rats demonstrate increased calcium influx through L-type calcium channels, responsible for activating the sAHP. This increases putative KCa channel activity, resulting in a larger sAHP and loss of hippocampus-dependent spatial memory. In fact, aged rats trained in the Morris water maze displayed larger sAHP magnitudes (and not mAHP magnitude) when they did not successfully learn the task versus when young or aged rats learned the task successfully (Tombaugh et al., 2005). The authors also found enhanced L-type calcium spikes in aged animals that were unable to learn the task.

Interestingly, spatial learning is also influenced by β-adrenergic signalling through the β1-receptor. This was shown by the finding that β-adrenergic receptor antagonists prevented retrieval, but not consolidation, of contextual and spatial memories in mice (Murchison et al., 2004). In addition, dopamine β-hydroxylase ($Dbh^{-/-}$) knockout mice, which do not produce noradrenaline, showed impairments in spatial memory (Murchison et al., 2004). Further studies demonstrated that noradrenaline's effects on spatial memory were through elevation of levels of cAMP, which then activates PKA (Ouyang et al., 2008). PKA is a known inhibitor of the sAHP in CA1 pyramidal neurons (Madison and Nicoll, 1982), which suggests a possible link between the sAHP, neuromodulation by β-adrenergic signalling, and spatial learning.

1.4.4 PKA-mediated regulation of the sAHP

Previous researchers studying the sAHP have had to rely on non-specific pharmacological agents such as noradrenaline, carbachol, and downstream messengers of the pathways activated

by these receptor agonists such as cAMP (Madison and Nicoll, 1982, 1984; Lancaster and Nicoll, 1987; Storm, 1989). The sAHP is blocked by these neuromodulators, with pronounced inhibition by noradrenaline through β-adrenergic receptors (Madison and Nicoll, 1982) and other transmitters that activate PKA through increases in levels of cAMP (Madison and Nicoll, 1986; Andrade and Nicoll, 1987; Pedarzani and Storm, 1993). However, it is necessary to identify the molecular basis of the sAHP if we are to gain the opportunity to directly target this response using genetic manipulations and more specific pharmacological blockers.

1.4.5 What channel underlies the sAHP?

Although multiple candidate channels have been suggested to underlie the sAHP, there exists substantial evidence that points toward IKCa channels being a major contributing factor. Firstly, several aspects of the pharmacological profiles of IKCa and the sAHP overlap (**Table 2**). Being insensitive to blockers of BK and SK channels (TEA and apamin, respectively) (Lancaster and Nicoll, 1987; Shah and Haylett, 2000), the sAHP was thought to be created by apamin-insensitive SK channels because initial noise analysis reported single channels of low conductance (Sah and Isaacson, 1995). However, this theory was disproved when transgenic animals with all of the isoforms of SK knocked out still showed a sAHP (Bond et al., 2004). Also, the reported apamin-insensitivity of one of the SK isoforms was put into question (Shah and Haylett, 2000). Interestingly, the sAHP in cultured hippocampal neurons, just like in enteric neurons, was blocked by clotrimazole and its derivatives (such as UCL2027), which also block IKCa channels (Shah et al., 2001; Shah and Haylett, 2002; Shah et al., 2006). Another piece of supporting evidence that IKCa is involved in generating the sAHP is that single channel analysis in rat hippocampal pyramidal cells from *in vitro* brain slices reported that the channel responsible for the sAHP had a single channel conductance of around 19 pS, an intermediate conductance level (Lima and Marrion, 2007). However, no efforts were made to isolate this channel to identify its molecular basis. Also, in the enteric nervous system, where IKCa has been shown to be involved in generating the sAHP (Vogalis et al., 2002a; Vogalis et al., 2002b), IKCa has been shown to be strongly modulated by PKA, which is a hallmark characteristic of the sAHP in hippocampus.

Table 2. The hippocampal CA1 sAHP and IKCa have similar pharmacological properties. Various inhibitors tested on IKCa channels in previous studies expressed in a range of systems (*left column*) and the sAHP of hippocampal CA1 pyramidal neurons (*right column*).

IKCa			**sAHP**	
Inhibitors	**[ ]**		**Inhibitors**	**[ ]**
TRAM-34	20 nM		8-bromo-cAMP	100 µM
NS-6180	20 nM		Isoproterenol	4 µM
Senicapoc	20 nM		Noradrenaline	10 µM
ChTx	5 nM		Histamine	1 µM
Maurotoxin	1 nM		Carbachol	50 µM
Nitrendipine	900 nM		Nitrendipine	20 µM
PKA*Cat*	100 U/ml		PKA*Cat*	100 U/ml
Nimodipine	1 µM		Histamine	1 µM
Barium	10 mM		Rp-CaMPs	500 µM
Flufenamic acid	10 µM			
Camstatin	5 µM			
Resveratrol	50 µM			
Clotrimazole	70 nM			
Caffeneic acid	1.3 µM			
Cyclosporin A	10 µM			
Insensitive to:			**Insensitive to:**	
Apamin	100 nM		Apamin	100 nM
TEA	25 mM			
Activators			**Activators**	
DC-EBIO	300 nM		DC-EBIO	1 µM
Riluozole	2.5 µM		Alkal. Phosphatase	20 U/ml
SKA-31	1 µM			
NS-309	27 nM			

Despite this evidence for IKCa being responsible for the sAHP, there is also evidence suggesting otherwise. Firstly, the sAHP was reportedly insensitive to ChTx (Lancaster and Nicoll, 1987; Shah and Haylett, 2000; Shah and Haylett, 2002). Also, no one had successfully detected IKCa signal (mRNA or protein) in the CNS (Jensen et al., 2001; Andrade et al., 2012), a problem that may be due to the quality of the sensors being used to detect the signal.

There have been multiple studies that suggest other candidate channels that seem to be at least partially responsible for the sAHP. Currently, the primary candidate is the KCNQ family of Kv7 channels, which are voltage-dependent potassium channels responsible for M-current (I_M). This channel has been shown to be partly responsible for the sAHP in granule cells of the dentate gyrus in the hippocampus, as well as some CA3 pyramidal cells (Tzingounis and Nicoll, 2008; Tzingounis et al., 2010; Andrade et al., 2012). However, blockers of Kv7 channels, such as linopirdine and XE-991, have either not blocked or only partially blocked the sAHP in CA1 pyramidal cells (Schnee and Brown, 1998; Marx et al., 2002; Gu et al., 2005; Soh and Tzingounis, 2010). Also, Kv7 channels are not activated by calcium, and have even been reported to be inhibited by calcium (Selyanko and Brown, 1996) and enhanced by PKA, a potent blocker of the sAHP (Marx et al., 2002). Therefore, Kv7 channels are not considered to contribute substantially to the sAHP in CA1 pyramidal cells. Calcium-activated chloride channels (CaCC) have also been suggested to play a role in the CA1 sAHP (Huang et al., 2012), but this body of evidence rests on non-selective blockers of these channels, including niflumic acid (NFA), flufenamic acid, and 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB); all of which also block IKCa channels (Fioretti et al., 2004; Olivan-Viguera et al., 2013). Another candidate is the Na^+/K^+ ATPase, which was recently reported to generate a very long-lasting sAHP in CA1 pyramidal cells at 35°C, (Gulledge et al., 2013). However, this interpretation relied on using ouabain, a non-selective blocker of the Na^+/K^+ ATPase, as well as quite intense and non-physiological stimuli.

All in all, the molecular identity of the sAHP in CA1 pyramidal cells is still undecided. Although there is substantial evidence pointing towards IKCa being responsible for I*sAHP*, some results contradict the role for IKCa and suggest other channels. A likely possibility is that the sAHP is caused by not just one channel, but rather that multiple channels can produce a sAHP

under different cellular backgrounds or with different molecular surroundings (Andrade et al., 2012).

1.4.6 Synaptic activation of the sAHP in CA1 pyramidal neurons

Calcium sources that have been attributed to activation of the sAHP in CA1 pyramidal cells have been restricted to L-type calcium channels (Moyer et al., 1992), and not N-type calcium channels, T-type calcium channels, or ryanodine-sensitive calcium stores (Marrion and Tavalin, 1998; Borde et al., 2000; Lima and Marrion, 2007; Kaczorowski, 2011). It is worth noting however, that the dihydropiridines used to block L-type channels in these tests are also highly effective as direct blockers of IKCa channels (Ellory et al., 1994). Since the sAHP is generated by bulk rises in cytoplasmic calcium (and not just increases in calcium microdomains), the magnitude of the sAHP is proportional to the level of increase in bulk calcium (Abel et al., 2004). Most reports have used somatic depolarization through current injection as a tool to generate the sAHP, which creates a burst of action potentials that cause an influx of calcium through voltage-gated calcium channels. However, other potent sources of calcium are the NMDA and AMPA receptors (Alford et al., 1993; Sah and Bekkers, 1996; Kovalchuk et al., 2000; Fernandez de Sevilla et al., 2007), the primary ligand-activated receptors triggered in CA1 pyramidal cells by excitatory synaptic input. This source of calcium is important as it reflects the level of synaptic activity of the CA1 pyramidal cell in question. It has been shown that calcium influx from excitatory synaptic activation through NMDA channels can elicit a robust sAHP (Lancaster et al., 2001; Wu et al., 2004), with a protocol consisting of 50 Hz suprathreshold stimulation delivered to the stratum radiatum (SR) of CA1 hippocampus provoking a large sAHP (Wu et al., 2004). The sAHP after a train of subthreshold synaptic stimulation, by contrast, is significantly smaller (**Fig. 3a**) as noted previously (Wu et al., 2004). This may be due to activation of L-type calcium channels closer to the soma by action potentials. In addition, the sAHP induced by calcium influx through NMDA channels has been shown to contribute to the postsynaptic response to excitatory synaptic inputs. Indeed, block of the sAHP

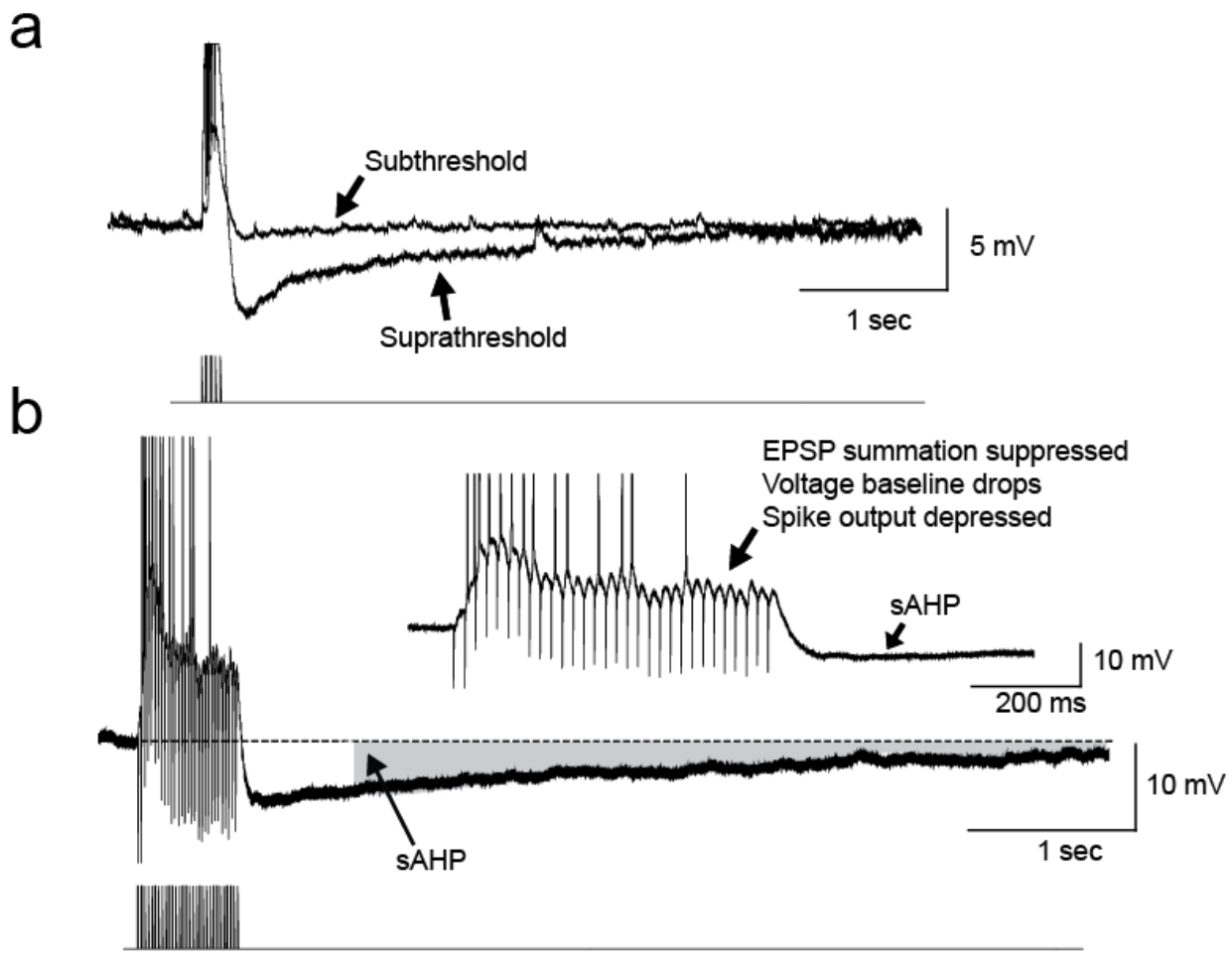

Figure 3. The synaptically-evoked sAHP controls spike output and decreases postsynaptic excitability in CA1 pyramidal cells.

(a) A short train of five EPSPs evoked by stimulating SR inputs at 50 Hz (stimuli represented by *bottom trace*) produces a long-lasting sAHP. Suprathreshold synaptic stimulation evokes a sAHP of larger magnitude and duration than a train of subthreshold EPSPs. **(b)** A longer train of 30 SR stimuli at 50 Hz (stimuli represented by *bottom trace*) results in a large sAHP, illustrated with *gray shading* under the baseline voltage (*dashed line*, -65 mV), as well as a suppression of EPSP summation. All recordings were conducted in the presence of 100 nM apamin, 100 µM XE991, and 50 µM picrotoxin.

using β-adrenergic agonists such as isoprenaline and calcium chelators influences the shape of the depolarizing envelope of a train of EPSPs, and boosts temporal integration to uncover an NMDA-mediated afterdepolarization (Lancaster et al., 2001; Wu et al., 2004). It has also been shown that a synaptically-evoked sAHP can reduce the initial spike-burst response and suppress temporal summation of EPSPs (Sah and Bekkers, 1996; Borde et al., 1999; Lancaster et al., 2001; Wu et al., 2004; Fernandez de Sevilla et al., 2007). Therefore, calcium influx necessary to activate the sAHP can come from synaptic sources.

Just as the molecular identity of the KCa channel responsible for the sAHP evoked by direct current or voltage-clamp commands at the soma remains a mystery, the identity of channels underlying the synaptically-evoked sAHP is also elusive. This is likely because the same channel (or complement of channels) is responsible for both types of sAHP, as they share identical properties such as activation by calcium, potassium-selectivity, slow kinetics, and inhibition by β-adrenergic agonists. The reasons that suggest that IKCa may be responsible for the sAHP in pyramidal cells of the hippocampus CA1 region also suggest that IKCa may also play a role in synaptic processing in these cells. Characterization of the role for IKCa channels is therefore important in understanding a neuron's ability to process synaptic information.

The background data existing in the field and briefly summarized above led me to propose the following:

Hypothesis: The calcium-activated potassium channel of intermediate conductance IKCa is expressed in CA1 hippocampal pyramidal neurons to generate the sAHP, which reduces membrane excitability in response to synaptic or somatic excitation.

OBJECTIVES

By testing this hypothesis in CA1 hippocampal neurons I will move towards completing the following important objectives:

1. **To determine if IKCa channels are expressed in pyramidal cells of the CA1 region of hippocampus.**
2. **To determine the role of IKCa current in regulating the excitability of pyramidal cells, and specifically how it contributes to the sAHP.**

SIGNIFICANCE

These experiments will provide novel insight into a prominent and long-studied yet poorly understood neuronal phenomenon. The sAHP is one of the strongest modulators of neuron excitability and firing properties that may influence information processing in the hippocampus and other areas of the CNS, and is in turn modulated by important second messenger and hormonal influences. Given this, my findings will be highly significant to understanding underlying mechanisms of control over neuronal excitability and its neuromodulation.

Chapter Two: METHODS

2.1 Animal care and tissue dissection

Experiments were conducted on P18-24 male Sprague-Dawley rats (Charles River) raised from timed-pregnant dams or breeding colonies of P25-50 C57BL/6 *wt* mice, or P25-60 KCa3.1-/- mice (UC Davis mouse facility) (Si et al., 2006; Turner et al., 2014). Animals were anaesthetized by isoflurane inhalation until unresponsive to ear pinch, and coronal (240 µm) dorsal hippocampus slices were prepared in ice-cold sucrose-based cutting solution composed of (in mM): 215 Sucrose, 25 $NaHCO_3$, 20 D-glucose, 2.5 KCl, 0.5 $CaCl_2$, 1.25 NaH_2PO_4 and 3 $MgCl_2$ preoxygenated by carbogen (95% O_2, 5% CO_2) gas. The slices were then incubated for 10-15 min at 34°C in artificial cerebrospinal fluid (aCSF) composed of (in mM): 125 NaCl, 3.25 KCl, 1.5 $CaCl_2$, 1.5 $MgCl_2$, 25 $NaHCO_3$, and 25 D-glucose preoxygenated by carbogen (95% O_2, 5% CO_2) gas. Slices were then stored in carbogen-gassed aCSF at room temperature before recordings were obtained at 32-34 ºC as a submerged slice preparation maintained on the stage of either a Zeiss Axioskop II or Olympus BH-50/BX51W1 microscope.

2.2 Transgenic animals

The transgenic mouse line used contains a functional knockout (KO) of IKCa (IKCa-/-), with a neomycin resistance gene cassette inserted in place of exon 4, which contains the channel pore of IKCa (Si et al., 2006). These animals were obtained from the UC Davis mouse facility (Si et al., 2006; Turner et al., 2014).

2.3 Recording solutions

The composition of external solutions were as follows (in mM): aCSF - 3.25 KCl, 125 NaCl, 25 $NaHCO_3$, 1.5 $CaCl_2$, 1.5 $MgCl_2$, 25 D-glucose. For current clamp recordings, electrodes were filled with a $KMeSO_4$-based internal solution with composition (in mM): 130 $KMeSO_4$, 10 HEPES, 0.1 EGTA, 7 NaCl, 0.3 $MgCl_2$, 2 Tris-ATP, 0.5 Na-GTP, and 5 d-t-creatine phosphate, pH = 7.3 with KOH, providing an E_{Cl} of -75 mV and E_K of -97 mV. Outside-out excised patch voltage clamp recordings were obtained using an electrolyte of 140 mM KCl, 2.83 mM $MgCl_2$ (1

mM [Mg]i), 10 mM HEPES, 5 mM EGTA and 4.25 mM $CaCl_2$ (1 µM free [Ca]i, Maxchelator program, http://maxchelator.stanford.edu/index.html, Ca/Mg/ATP/EGTA Calculator v1), pH 7.3 with KOH, providing an E_{Cl} of +4 mV and E_K of -99 mV. Since IKCa channels from the periphery have been reported to be fully activated by ~500 nM free [Ca]i (Ishii et al., 1997; Joiner et al., 1997; Logsdon et al., 1997), 1 µM internal calcium was chosen to ensure maximal activation of the channel.

Perforated patch recordings used an electrolyte of 10 mM KCl, 135 mM K-Gluconate, 10 mM HEPES, 1 mM $MgCl_2$, and 75 µg/ml gramicidin prepared in dimethylsulfoxide (DMSO) (< 0.01% DMSO in electrolyte). aCSF with elevated [Ca]o of 2.5 mM, as well as 1.25 mM KH_2PO_4, 1.25 mM KCl, and 16 mM D-Glucose was used for the perforated patch recordings in **Fig. 14** when testing the effects of NS-309 as per methods described by Pedarzani and co-workers (Pedarzani et al., 2005). Di-tris-creatine phosphate (5 mM), 2 mM Tris-ATP and 0.5 mM Na-GTP were added daily from frozen stock solutions to all whole-cell and outside-out patch recording electrolytes.

On-cell recordings were obtained with an electrolyte of HEPES-buffered aCSF with 150 mM NaCl, 3.25 mM KCl, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM HEPES and 20 mM D-glucose, pH 7.3 with NaOH. IKCa channels were isolated by including in the electrode TTX, apamin, XE-991, TEA, 4-AP, CsCl and excitatory synaptic blockers. Bath solution had the same composition as the electrode solution except that excitatory synaptic blockers were omitted.

Chemicals were obtained from Sigma-Aldrich Corp. unless otherwise noted. Where indicated, the following drugs were used to block specific ion channels in order to isolate IKCa for recording (see **Table 3**): BK (TEA, 5 mM; IbTx, 100 nM), SK (apamin, 100 nM), Kv7 (XE-991, 10 µM), Kv4.x (4-AP, 5 mM external and 2 mM internal), Kv1.x (5 mM TEA, ChTx, 100 nM; MTx, 100 nM), voltage-gated sodium (TTX, 1 µM), high voltage-activated (HVA) calcium ($CdCl_2$, 30 µM), low voltage-activated (LVA) calcium (Ni^{2+}, 300 µM), HCN (CsCl, 2 mM). Ligand-gated receptors were blocked by bath application of: GABA-A (picrotoxin, 50 µM), GABA-B (CGP55485, 1 µM, Tocris), NMDA (DL-AP5, 25 µM, Ascent Scientific), and AMPA/KA (DNQX, 10 µM, Tocris). $NiCl_2$, $CdCl_2$, CsCl, TEA, 4-AP, and picrotoxin were prepared daily from stock solutions and all other drugs daily from frozen aliquots. DNQX was dissolved in DMSO prior

Table 3. Blockers used to isolate IKCa channel current.

Shown are the various toxins, compounds, and inorganic ions and their concentrations that are used to block contaminating current and isolate IKCa current.

Internal	External	Channel / receptors blocked
2 mM 4-AP		Kv4.x, Kv3.x
	1 µM TTX	Nav1.x (except Nav1.5)
	5 mM TEA	Kv1.x, Kv3.x, BK, Kv7.x
	5 mM 4-AP	Kv4.x, Kv3.x
	100 nM apamin	SK1, SK2, SK3
	10 µM XE-991	Kv7.x
	2 mM CsCl	HCN
	300 µM Ni^{2+}	All LVA Cav3 isoforms
	30 µM Cd^{2+}	All HVA Ca channels
	10 µM DNQX	AMPAR
	25 µM DL-AP5	NMDAR
	50 µM picrotoxin	GABA-A
	1 µM CGP55485	GABA-B

to use (final DMSO concentration < 0.1%) and apamin dissolved in 50 mM acetic acid before preparing stock solution. Senicapoc was a generous gift of H. Wulff (UC Davis, California).

2.4 Patch recording configurations

Pipettes were constructed from 1.5 mm O.D. fiber-filled glass (A-M Systems) with resistance of 4-8 MΩ. Patch recordings were made using Multiclamp 700B amplifiers and Digidata 1440A with DC-10 kHz band pass filter and pClamp software. A sampling rate of 40 kHz was used for all recordings. Series resistance was compensated with bridge balance circuitry for current clamp recordings and by up to 80% compensation during voltage clamp recordings. Negative bias current of < 200 pA was applied during current clamp recordings to maintain a subthreshold resting potential at ~-65 mV.

Whole-cell and outside-out recordings were conducted on cells with access resistance of 8-15 MΩ, with cells rejected for any drift in access resistance of > 20%. For whole-cell experiments, control recordings were made >5 min after break in to promote full stabilization with the internal solution. Access resistance was continuously monitored during perforated patch recordings after ensuring that access resistance was stable ~5-10 min after a GΩ seal was obtained.

For on-cell recordings, mean single channel conductance was calculated for hyperpolarizing potentials where channel amplitude was best delineated. Recordings were obtained with a 10 kHz cutoff filter and processed offline by filtering at 240-500 Hz (Bessel 8-pole).

2.5 Stimulation

Synaptic input was evoked using a concentric bipolar electrode (Frederick Haer, CBCMX75(JL2)) positioned in the mid SR and driven by a stimulus isolation unit (Digitimer**,** 0.1-0.2 ms pulse width), with stimulus patterns controlled within PClamp software.

2.6 Drug applications

The lipophilic drugs TRAM-34, Senicapoc, and NS-6180 all target an internal binding site on the IKCa channel and thus must first be internalized (Wulff et al., 2001; Stocker et al., 2003; Strobaek et al., 2013). For bath perfusion of these compounds we found that channel activity was blocked in on-cell recordings in 10-15 min while whole-cell recordings of the sAHP or I*sAHP* could take 20-30 min to achieve a full block. Although all three drugs were designed to target the same intracellular site on IKCa channels we found that the fastest and most reliable block was obtained with TRAM-34 and Senicapoc as compared with NS-6180. Time-matched control recordings in the absence of drug perfusion ruled out internal wash-out or a change in access resistance in whole-cell or perforated patch recordings as potential secondary contributors to drug effects (**Fig. 6**). In contrast we found that internal perfusion of 1 µM TRAM-34 by exchanging the electrode solution (ALA Instruments, CA) achieved a rapid and consistent block of IKCa channels (<10 min). Drug application in this manner was also beneficial in restricting drug effects to the postsynaptic cell. For this reason the majority of tests in whole-cell recording configuration were conducted using internal perfusion of 1 µM TRAM-34. Toxins applied by local pressure ejection were carried in a HEPES (10 mM)-buffered aCSF containing 150 mM NaCl, 3.25 mM KCl, 1.5 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM HEPES, and 20 mM D-glucose, pH 7.3 with NaOH. Pressure electrodes used for drug ejections included bovine serum albumin (BSA) (0.1%) to prevent non-specific adhesion of the drugs, and food coloring (1:100) to visualize the region of drug ejection.

2.7 Data analysis and statistics

sAHP area was measured as the area from 200 ms after the protocol, which was chosen to ensure exclusion of the mAHP (shown to last ~100 ms), to 7 secs after the protocol under baseline, which was calculated as the mean voltage level preceding the current injection or stimulus protocol in current clamp mode. In voltage clamp mode, I*sAHP* area from 200 ms after the protocol to 7 secs after the protocol was taken over baseline, the mean current level preceding the voltage protocol. Amplitude of the sAHP was measured as the voltage level 200 ms after the current or stimulus protocol. Data were analyzed using Clampfit 10 software and

custom Matlab R2007B scripts. Statistical analysis was carried out in OriginPro 8. Paired-sample Student t-tests were used to determine significance, unless otherwise indicated. Average values are expressed as mean ± S.E.M.; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and NS, not significant.

Chapter Three: ELECTROPHYSIOLOGICAL AND PHARMACOLOGICAL CHARACTERIZATION OF IKCa IN CA1 HIPPOCAMPAL PYRAMIDAL CELLS

3.1 The validity of IKCa channel sAHP tests.

We first investigated the validity of our pharmacological methods used to distinguish between the contributions of IKCa and those of other ion channels. To test the functional role of IKCa channels, other ion channels were blocked or their activations minimized. Any potential contribution by SK channels was prevented by recording in the presence of 100 nM apamin, and Kv7 channels were blocked with 10 µM XE-991 (Tzingounis et al., 2010). It is known that IKCa channels are apamin insensitive (Wulff et al., 2007), and we confirmed through expression of KCNN4 cDNA in tsA-201 cells that 10 µM XE-991 had no significant effect on IKCa channels (**Fig. 4a**). It has further been established that XE-991 does not impede SR-evoked synaptic transmission in the slice preparation (Petrovic et al., 2012). Any contribution from the Na^+/K^+ pump was minimized by recording at 32ºC, a temperature that has been shown to decrease the contribution of the Na^+/K^+ pump and show more contribution by a calcium-activated potassium channel to the sAHP (Gulledge et al., 2013). In addition, when we tested the protocol used by Gulledge *et al*. to evoke the sAHP mediated by Na^+/K^+ pump, we found that the majority of the large magnitude sAHP following the stimulus was TRAM-34-sensitive (**Fig. 5**).

Recordings of I*sAHP* using whole-cell or perforated patch configurations were stable over 30 min time (**Fig. 6,** $n = 7$), revealing minimal influence of washout or a change in access resistance that could account for the actions of applied drugs. To focus on excitatory synaptic potentials we applied 50 µM picrotoxin to block GABAergic transmission. The combination of apamin, XE-991, and picrotoxin slightly reduced but did not block spike accommodation evoked during current pulse injection in rat and mouse pyramidal neurons (**Figs. 7a, 10a, 11a**; $n = 8$ and 5, respectively).There was also little qualitative change in the firing response to SR stimulus trains, although the early phase of the subsequent AHP (SK-mediated) was blocked and the sAHP was slightly more prominent, likely due to an increase in calcium influx in the absence of SK channels (**Fig. 10b, e**; $n = 8$). Recording the I*sAHP* following a step command or a burst of SR stimuli also established that these compounds blocked only an early component of outward current consistent with an SK-mediated response (**Fig. 7d, e**, $n = 8$). As previously reported the

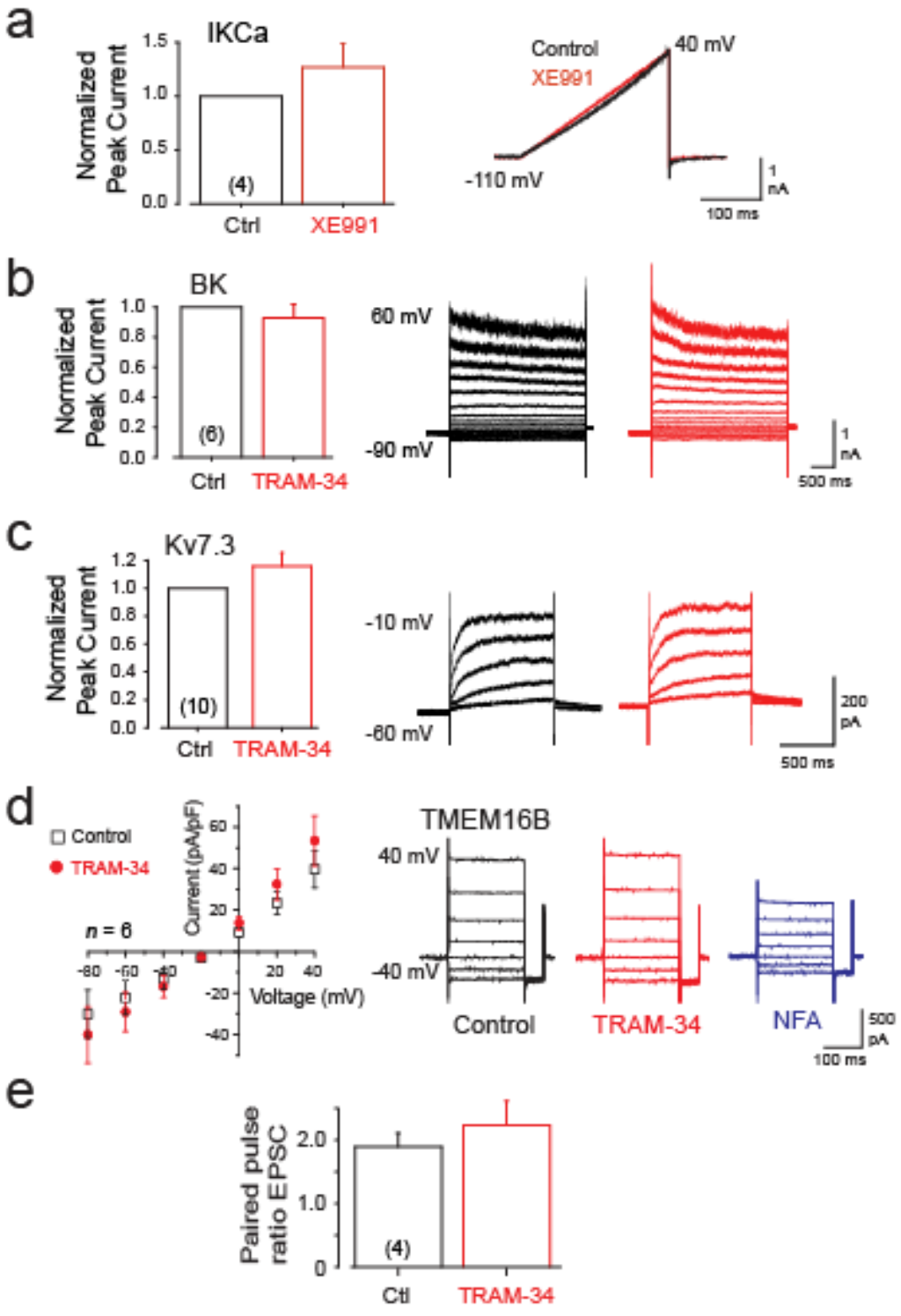
a
IKCa
Normalized Peak Current
1.5
1.0
0.5
0.0
(4)
Ctrl
XE991
Control
XE991
40 mV
-110 mV
1 nA
100 ms
b
BK
Normalized Peak Current
1.0
0.8
0.6
0.4
0.2
0.0
(6)
Ctrl
TRAM-34
60 mV
-90 mV
1 nA
500 ms
c
Kv7.3
Normalized Peak Current
1.2
1.0
0.8
0.6
0.4
0.2
0.0
(10)
Ctrl
TRAM-34
-10 mV
-60 mV
200 pA
500 ms
d
Control
TRAM-34
n = 6
Current (pA/pF)
60
40
20
-20
-40
-80
-60
-40
20
40
Voltage (mV)
TMEM16B
40 mV
-40 mV
Control
TRAM-34
NFA
500 pA
100 ms
e
Paired pulse ratio EPSC
2.0
1.0
0
(4)
Ctl
TRAM-34

Figure 4. Pharmacological isolation of IKCa channels.

Whole-cell recordings obtained with alpha subunits of the indicated proteins transiently expressed in tsA-201 HEK cells and recorded at room temperature. **(a)** The Kv7 channel blocker XE-991 (10 µM) does not affect IKCa channels coexpressed with calmodulin for currents recorded in 1 µM [Ca]i. **(b, c)** TRAM-34 (1 µM) has no significant effect on BK channels **(b)** or Kv7.3 channels **(c)** transiently expressed in tsA-201 cells. **(d)** TRAM-34 (1 µM) has no significant effect on TMEM-16B steady-state chloride current recorded in 100 nM [Ca]i, with subsequent block by 300 µM NFA. **(e)** TRAM-34 (1 µM) does not significantly affect the mean amplitude of a SR-evoked EPSP or the excitatory post-synaptic current (EPSC) paired pulse ratio (20 ms interstimulus interval). Average values are mean ± SEM with sample values for both control and test recordings shown in brackets. Also see (Strobaek et al., 2013) for more information on these tests.

Recordings displayed in panels **(a-d)** partially obtained by T. Bartoletti, N.C. Heath, S. Dykstra, and A. Rizwan. Data obtained in **(e)** by S. Dykstra.

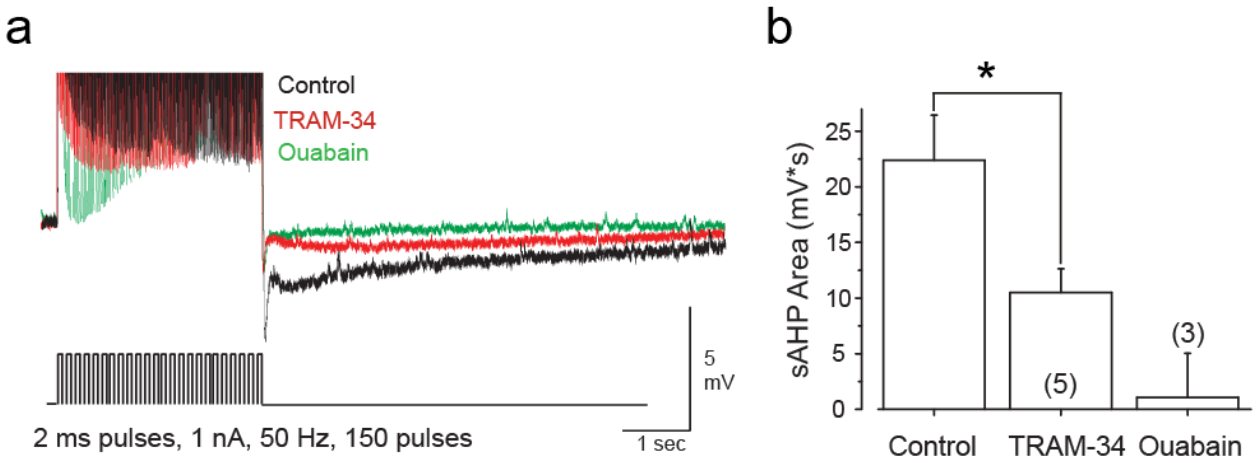

Figure 5. The majority of the putative Na^+/K^+ pump-mediated sAHP is TRAM-34-sensitive. **(a)** A representative whole-cell recording from a rat CA1 pyramidal cell applying a stimulus protocol consisting of 1 nA, 2 ms current pulses delivered at 50 Hz for 3 sec to test the role for the Na^+/K^+ pump in generating the sAHP at 35ºC. Infusion of TRAM-34 (1 µM) blocks the majority of the sAHP, compared to < 15% by subsequent perfusion of 20 µM ouabain. Recordings were conducted in the presence of 100 nM apamin, 10 µM XE-991, and 50 µM picrotoxin. **(b)** Summary bar plot of mean sAHP area following the end of the current pulse train. Average values are mean ± S.E.M. *, $P < 0.05$.

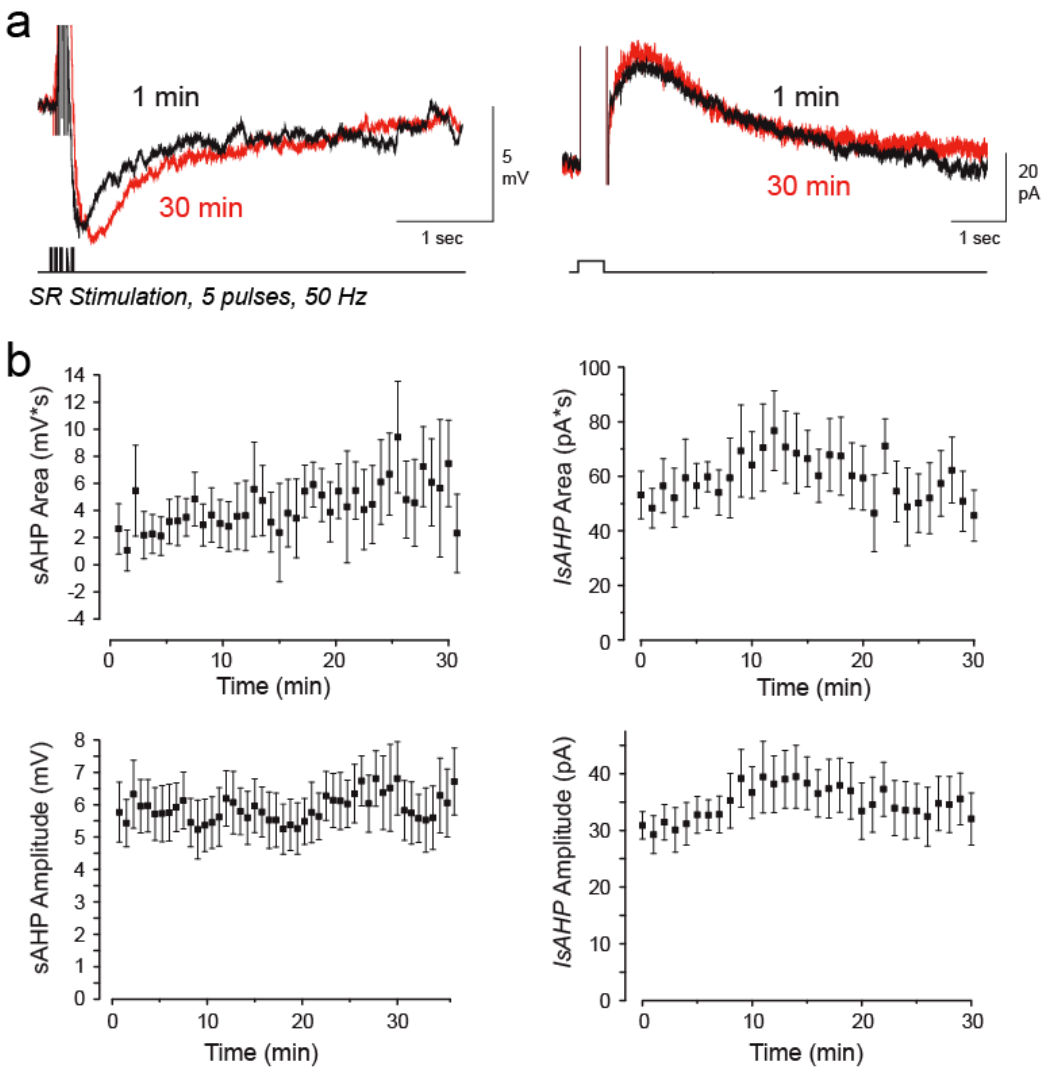

Figure 6. The sAHP in whole-cell or perforated patch configurations resists wash out. **(a)** Whole-cell current clamp (*left*) and perforated patch voltage clamp (*right*) recordings from P18-22 rat CA1 pyramidal cells. Applying a stimulus protocol consisting of either 5 suprathreshold SR stimuli (50 Hz) (*left*) or a 500 ms step to +60 mV to evoke I*sAHP* (*right*) revealed sAHP currents that were stable for over 30 min. Recordings were started after 5 min stabilization time post-break in or perforation, and shown here comparing initial recordings (0 min, *black*) and after 30 min (*red*). **(b)** Summary plots of mean sAHP or I*sAHP* areas and peak magnitudes after the end of the stimulus (n = 7 for both conditions).

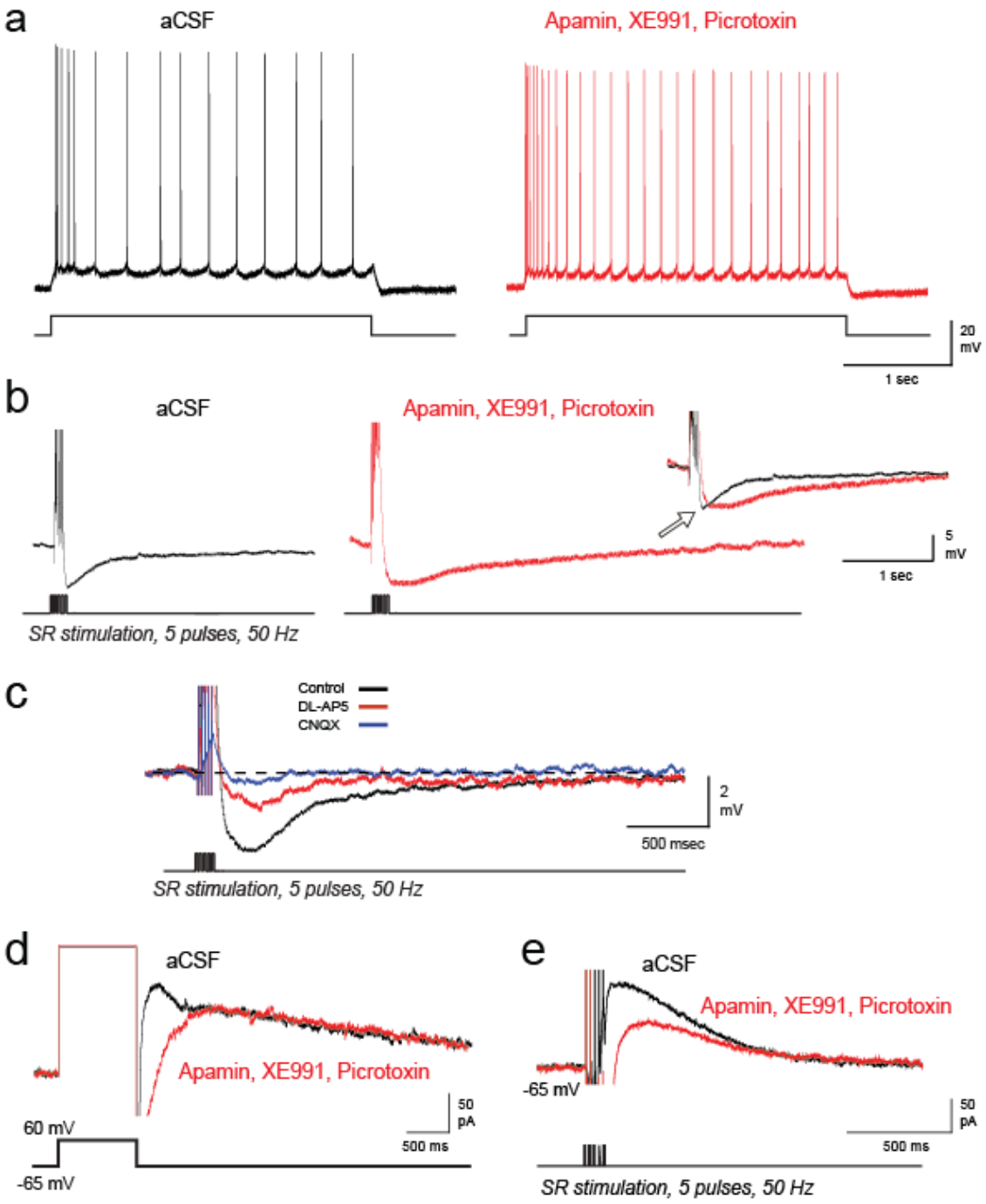
a
aCSF
Apamin, XE991, Picrotoxin
20 mV
1 sec
b
aCSF
Apamin, XE991, Picrotoxin
5 mV
1 sec
SR stimulation, 5 pulses, 50 Hz
c
Control
DL-AP5
CNQX
2 mV
500 msec
SR stimulation, 5 pulses, 50 Hz
d
aCSF
Apamin, XE991, Picrotoxin
50 pA
500 ms
60 mV
-65 mV
e
aCSF
Apamin, XE991, Picrotoxin
-65 mV
50 pA
500 ms
SR stimulation, 5 pulses, 50 Hz

Figure 7. Background channel blockers have minimal effects on the sAHP and I*sAHP*. **(a)** Perfusion of 100 nM apamin, 10 µM XE-991, and 50 µM picrotoxin to block SK and Kv7 channels and GABAergic transmission slightly reduce but do not eliminate spike accommodation during current pulse injection. **(b)** The early phase of the sAHP following a train of SR input is blocked (*arrow*) and the sAHP is more evident after perfusion of apamin, XE-991, and picrotoxin (*inset*). **(c)** The sAHP evoked by a train of SR stimuli is progressively reduced by block of NMDA receptors (DL-AP5, 25 µM) and AMPA receptors (DNQX, 10 µM). **(d)** Recording of I*sAHP* evoked as a tail current under whole-cell voltage clamp using a +60 mV depolarizing step command (shown *below* traces), before (*black trace*) and after (*red trace*) application of 100 nM apamin, 10 µM XE-991, and 50 µM picrotoxin. **(e)** Recording of I*sAHP* evoked as a tail current under whole-cell voltage clamp using a SR stimulation command (shown *below* traces), before (*black trace*) and after (*red trace*) application of 100 nM apamin, 10 µM XE-991, and 50 µM picrotoxin.

Data displayed in panel **(c)** obtained by S. Dykstra.

amplitude of the evoked sAHP could differ between cells, with up to 20% reportedly lacking a sAHP (Wu et al., 2004). The current study focused on those cells exhibiting a detectable sAHP.

The role of IKCa channels can be distinguished using the selective blocker TRAM-34, a lipophilic compound that acts at a site near the inner pore (Wulff et al., 2000; Wulff et al., 2001). The selectivity of TRAM-34 has been thoroughly established, with IKCa channels exhibiting far greater sensitivity to 1 µM TRAM-34 than either SK or BK channels (Wulff et al., 2000; Wulff et al., 2001; Wulff et al., 2007). Control tests confirmed that 1 µM TRAM-34 had no significant effect on BK channels or Kv7.3 channels expressed in tsA-201 cells, or the SR-evoked EPSC paired pulse ratio in tissue slices (**Fig. 4e**). Also, previous work has proposed that Na-activated potassium (Slack) channels contribute to a sAHP in lamprey spinal neurons (Wallen et al., 2007). Therefore, to assess whether these channels contribute to the synaptically-evoked sAHP that we studied, we applied 1 mM quinidine, a sodium channel blocker (and by extension, Slack channels), during the 50 Hz SR stimulation protocol and found that there was no contribution of these channels to the sAHP (n = 4) (**Fig. 10**). With these controls we were confident in our ability to examine the sAHP and I*sAHP* evoked by trains of SR inputs, just suprathreshold current injections, and step or ramp commands to identify the expression and functional role of IKCa channels.

3.2 CA1 pyramidal cells show outward current with a pharmacology consistent with IKCa channels.

To test the membrane expression of IKCa channels we carried out pharmacological tests on outside-out recordings obtained from CA1 pyramidal cell somata. The internal solution for recording was buffered to 1 µM calcium to provide a consistent [Ca]i, and 30 µM Cd^{2+} and 300 µM Ni^{2+} were included in the external aCSF to block HVA and LVA calcium channels. Recordings were further conducted in the presence of drugs to block SK, BK, Na, Kv1, Kv4, Kv7, HCN channels, and GABA-A and -B and glutamate receptors (for full list of blockers applied, see **Table 3** and **Methods**). A ramp command was then applied under voltage clamp from a holding potential of -110 mV to +60 mV (500 ms) every 30 secs over 5 min to ensure stable recordings. Internal perfusion of the electrode with 1 µM TRAM-34 reduced the total

outward current by 38 ± 5.4 % in 7/8 recordings (measured at +40 mV, $P < 0.01$) (**Fig. 8a**). The TRAM-34 sensitive current gave rise to a linear I-V plot that reversed at -90.1 ± 4.0 mV ($n = 7$), a value that approached the predicted value of -99 mV for E_K (**Fig. 8a**). The scorpion toxins ChTx and MTx are known to block IKCa channels, although both toxins also target Kv1.x isoforms (MTx, Kv1.2; ChTx, Kv1.3) and ChTx is a potent blocker of BK channels (Castle et al., 2003; Wulff et al., 2007). In the presence of 5 mM TEA to block BK and Kv1 channels we tested the effects of pressure ejecting 100 nM ChTx or MTx using outside-out recordings and 1 µM [Ca]i. ChTx reduced total current by 33 ± 7.5 % ($n = 7$, measured at +40 mV, $P < 0.01$), with ChTx-sensitive current reversing on mean I-V plots at -81.3 ± 8.8 mV ($n = 7$) (**Fig. 8b**). MTx reduced outward current by 40 ± 7.6% ($n = 5$, $P < 0.01$) and reversed at -94.1 ± 6.7 mV ($n = 5$) (**Fig. 8c**). Finally, internal perfusion of the electrode with the catalytic subunit of PKA (PKA*Cat*, 100 u/ml) blocked 35 ± 4.3% of outward current in 5/6 cells ($P < 0.001$) that reversed at -97.6 ± 4.7 mV ($n = 5$) (**Fig. 8d**). A comparison of these results revealed that the degree of block by each of the compounds TRAM-34, ChTx, MTx, and PKA*Cat* were not significantly different (one-way ANOVA, $P = 0.4$). These results reveal a potassium current in pyramidal cells that can be evoked with a buffered internal [Ca]i of 1 µM with the unique pharmacological profile of IKCa channels.

3.3 CA1 pyramidal cells exhibit calcium-dependent intermediate conductance potassium channels.

To determine if calcium-activated potassium channels of intermediate conductance could be recorded in pyramidal cells we obtained on-cell somatic patch recordings to preserve intracellular contents and native calcium buffering mechanisms. To isolate putative IKCa channels, on-cell recording electrodes contained a HEPES-buffered aCSF with both electrode and bath aCSF supplemented with blockers of SK, BK, Na, Kv7, Kv4, and HCN channels, and both glutamatergic and GABAergic transmission (see **Methods** and **Table 3**). Calcium channels were not blocked in order to promote calcium conductance across the patch by delivering a set of depolarizing spike-like commands through the electrode (50 Hz, 20 pulses, 5 ms, 80 mV). The membrane potential was subsequently stepped to a set of steady-state potentials from -60 to +30

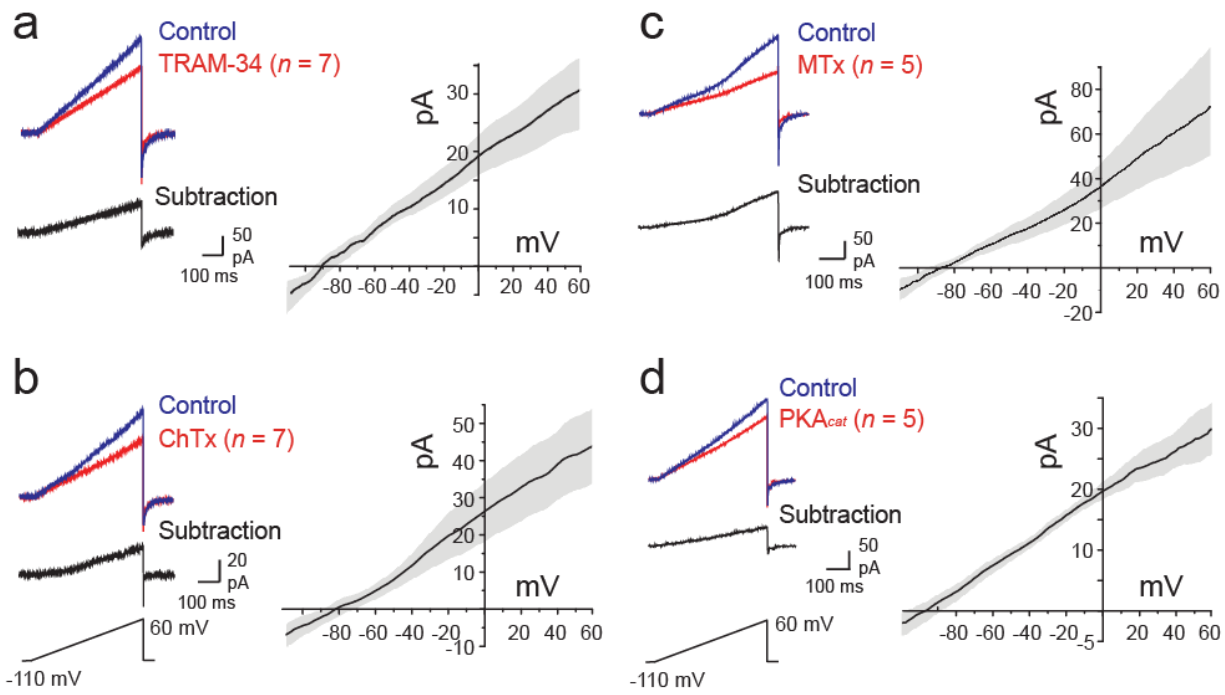

Figure 8. CA1 pyramidal cells exhibit an outward current with a pharmacological profile consistent with IKCa channels.

Outside-out recordings from pyramidal cell somata in the presence of 1 µM [Ca]i to a 500 msec ramp command from -110 mV to +60 mV. Mean I-V plots reflect currents blocked by the indicated pharmacological agents following subtraction of test from control recordings. SEM values for I-V plots are indicated by the *shaded area*. Recordings were conducted in the presence of 100 nM apamin, 5 mM TEA, 5 mM 4-AP, 1 µM TTX, 10 µM XE-991, 30 µM Cd^{2+}, 300 µM Ni^{2+}, 2 mM Cs^{+}, and synaptic blockers. The internal solution included 2 mM 4-AP. In each case outward current is voltage-independent and reduced by **(a)** TRAM-34 (1 µM), **(b)** ChTx (100 nM), **(c)** MTx (100 nM) or **(d)** PKA*Cat* (100 u/ml). ChTx and MTx were focally pressure ejected while TRAM-34 and PKA*Cat* were internally perfused in the patch electrode. Sample numbers are indicated in brackets.

mV with respect to the resting state to examine evoked currents. Under conditions of recording with 3.25 mM potassium in the on-cell electrode we assumed that the resting membrane potential under the patch in the same conditions approached that recorded in a separate set of perforated patch recordings of -64 ± 1.5 mV (*n* = 12). To simplify interpretation, outward current with respect to the cell interior is presented as upward in all on-cell recordings.

We found that the train of spike-like commands was followed by activation of non-inactivating channels (*n* = 4) or even macropatch current (*n* = 6) (**Fig. 9a, b**). Single channel events recorded at different step potentials revealed a mean conductance of 30.4 ± 5.8 pS (*n* = 4) (**Fig. 9a**), a value within the range of ~30-40 pS reported in other cell types for IKCa channels (Ishii et al., 1997; Logsdon et al., 1997; Jensen et al., 1998; Neylon et al., 1999). Both single channels (*n* = 4) and macropatch currents (*n* = 6) were rapidly blocked by bath perfusion of 1 µM TRAM-34, which will cross the cell membrane in regions outside of the on-cell patch (**Fig. 9a,b**). The I-V plot for the TRAM-34-sensitive macropatch currents evoked by the spike-like stimulus train reversed ~10 mV more negative than the native resting potential and could exhibit slight inward rectification at more hyperpolarized potentials (*n* = 4/6) (**Fig. 9b**).

To determine the ion selectivity of macropatch currents we obtained on-cell recordings with equimolar [K] across the patch by using a HEPES-buffered electrolyte containing 140 mM KCl, setting E_K to 0 mV. By comparison, E_{Cl} under these conditions was predicted to rest at -90 mV across the patch. All other blockers in the electrode and bath were as described above for a low external potassium electrolyte. To apply the series of spike-like depolarizing commands over a range that would promote calcium influx through the patch the holding potential was set to -65 mV and the membrane stepped to potentials after the spike-like depolarizations over a range of -40 mV to +120 mV (Δ mV) with respect to -65 mV. As before, we recorded non-inactivating macropatch currents that were blocked by 1 µM TRAM-34 (*n* = 6) (**Fig. 9c**). Importantly, the TRAM-34-sensitive currents were confirmed as representing a potassium conductance by reversing through 0 mV (E_K) on the I-V plot (**Fig. 9c**).

If the currents evoked under these conditions reflect IKCa channels they should also exhibit calcium-sensitive activation. The calcium sensitivity of these currents was established when 1 µM 1-EBIO increased channel open probability and peak macrocurrent in 6/9 cells in the

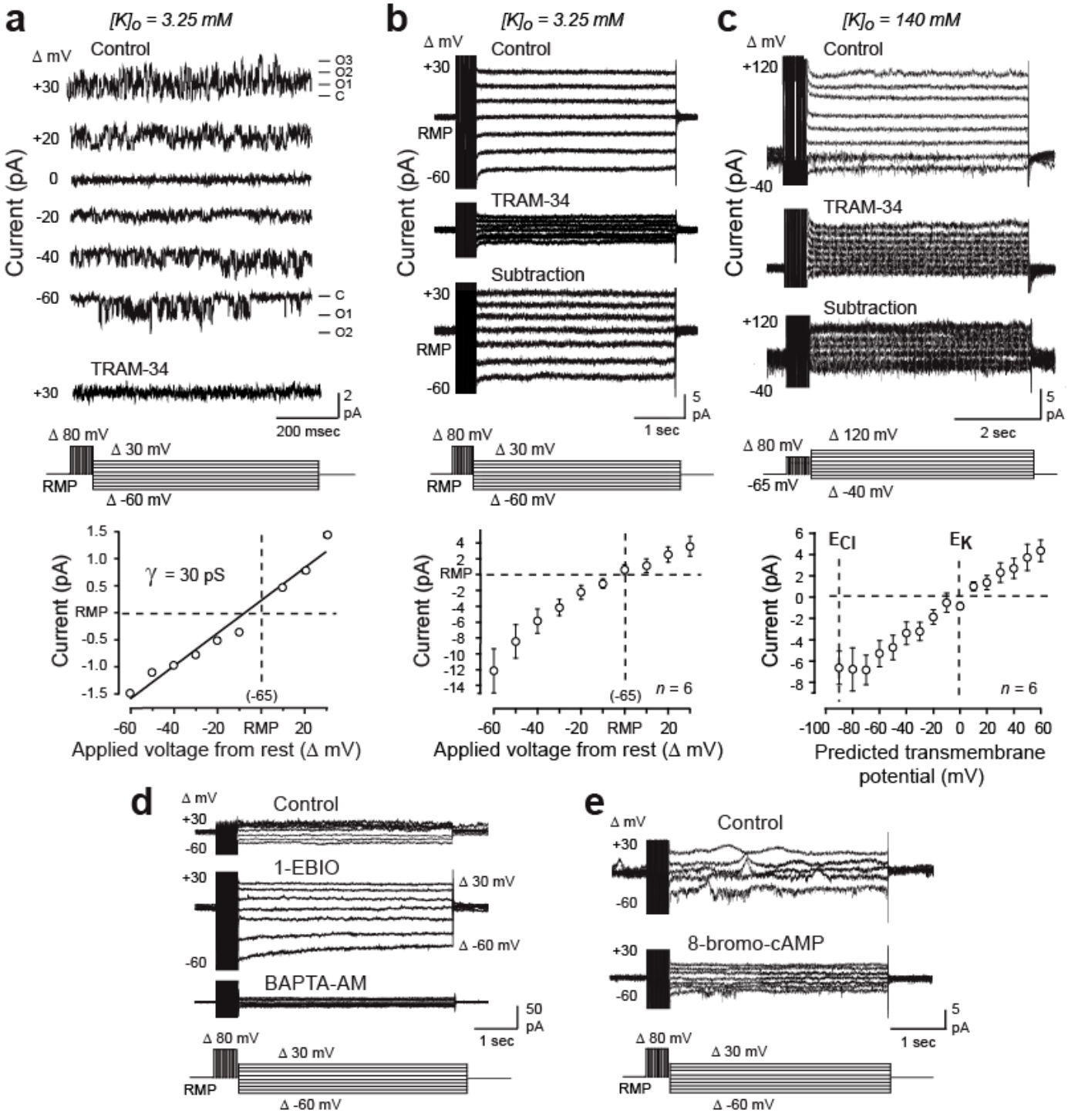
a
[K]o = 3.25 mM
Control
TRAM-34
Current (pA)
Δ mV
+30
+20
0
-20
-40
-60
O3
O2
O1
C
2 pA
200 msec
Δ 80 mV
Δ 30 mV
RMP
Δ -60 mV
γ = 30 pS
(-65)
Applied voltage from rest (Δ mV)
b
[K]o = 3.25 mM
Control
TRAM-34
Subtraction
1 sec
n = 6
c
[K]o = 140 mM
+120
-40
Δ 120 mV
-65 mV
Δ -40 mV
2 sec
ECl
EK
Predicted transmembrane potential (mV)
d
1-EBIO
BAPTA-AM
50 pA
e
8-bromo-cAMP

Figure 9. Intermediate conductance calcium-gated potassium channels are expressed in CA1 pyramidal cells.

Shown are on-cell somatic recordings using a HEPES-buffered aCSF electrolyte, with calcium influx through a patch evoked using a repetitive spike-like command (50 Hz, 20 pulses, 5 ms, 80 mV depolarized from rest) followed by steps to different steady-state potentials. On-cell electrodes and bath perfusate contained 1 µM TTX, 100 nM apamin, 5 mM TEA, 5 mM 4-AP, 2 mM CsCl, and 100 nM XE-991 to isolate IKCa channels, and 25 µM DL-AP5, 10 µM DNQX, 50 µM picrotoxin, and 1 µM CGP-55845 to block glutamatergic and GABAergic transmission. Note that the RMP across the patch for on-cell records in **(a, b, d, e)** that use 3.25 mM [K]o is presumed to be ~-65 mV, while that for 140 mM [K]o in **(c)** was set at -65 mV as a steady-state holding potential. Voltage commands reflect the voltage step applied to the electrode and displayed with net depolarizing commands upward. Current polarity is displayed with respect to the cell interior with outward current as an upward deflection. I-V plots in **(b, c)** reflect the TRAM-34-sensitive currents calculated by subtraction of test from control records. **(a, b)** On-cell recordings using 3.25 mM [K]o in the electrolyte reveals either single channel activity **(a)** or macropatch current **(b)** evoked following the pulse train. Current reversed at a potential ~-10 mV more hyperpolarized than resting state and could exhibit inward rectification at hyperpolarized potentials on I-V plots. Bath perfusion of 1 µM TRAM-34 rapidly blocked both single channel and macropatch currents. **(c)** On-cell recordings using equimolar [K]o exhibits reversal of TRAM-34-sensitive current through 0 mV, verifying potassium as the primary conductance. **(d)** The on-cell recorded macropatch current is enhanced by the KCa3.1 agonist 1-EBIO (1 µM) and blocked by 10 mM BAPTA-AM. **(e)** On-cell macropatch current is blocked by bath perfusion of 100 µM 8-bromo-cAMP. Records in **(c)** show every second record for clarity. Traces in **(a, c-e)** were filtered at 400-500 Hz and traces in **(b)** were filtered at 240 Hz (Bessel 8-pole). Average values are mean ± SEM. HP, holding potential.

All data recorded by A. Rizwan

presence of 100 nM apamin (**Fig. 9d**). Subsequent perfusion of the membrane permeable calcium chelator BAPTA-AM (10 mM) fully blocked evoked currents (n = 3) (**Fig. 9d**). PKA-mediated phosphorylation is well known to block the sAHP of CA1 pyramidal cells (Lancaster and Nicoll, 1987; Pedarzani et al., 1998) and also IKCa channels (Klein et al., 2009; Wong and Schlichter, 2014a). We found that the membrane permeable 8-bromo-cAMP (100 µM) rapidly blocked channel activity or macropatch outward current in on-cell recordings under these conditions (n = 5) (**Fig. 9e**). These data reveal that in the presence of SK and BK channel blockers, a calcium-dependent intermediate conductance potassium channel can be recorded that is blocked by TRAM-34, an internal calcium chelator, or elevation of PKA, all properties consistent with IKCa channels.

3.4 The sAHP of CA1 pyramidal cells.

A key role for the sAHP is in mediating spike accommodation, where a slow activation of the sAHP progressively reduces spike frequency during an injected current pulse and triggers a post-stimulus AHP (Madison and Nicoll, 1984; Lancaster and Nicoll, 1987). The sAHP can also be evoked synaptically by inputs from the SR using short stimulus bursts (5-30 pulses, 50 Hz), with the sAHP developing during the stimulus train and generating a large sAHP immediately following the stimulus as a hyperpolarized voltage in current clamp (**Figs. 10, 12, 13**) (Nicoll and Alger, 1981; Lancaster et al., 2001; Shah and Haylett, 2002; Wu et al., 2004; Fernandez de Sevilla et al., 2007). I*sAHP* is often recorded under voltage clamp as a tail current following a step command and can also be evoked for study using a stimulus burst to SR inputs (**Figs. 7, 15c, d**).

3.5 IKCa channels contribute to spike accommodation and temporal summation.

Internal infusion of 1 µM TRAM-34 blocked spike accommodation and the sAHP evoked by current pulse injection or during a 30 pulse train of SR stimuli in rat and mouse CA1 pyramidal cells (**Figs. 10a, b, 11, 13a**). The initial suppression of temporal summation of EPSPs during SR stimulation under control conditions could be extensive, with the membrane potential during a 50

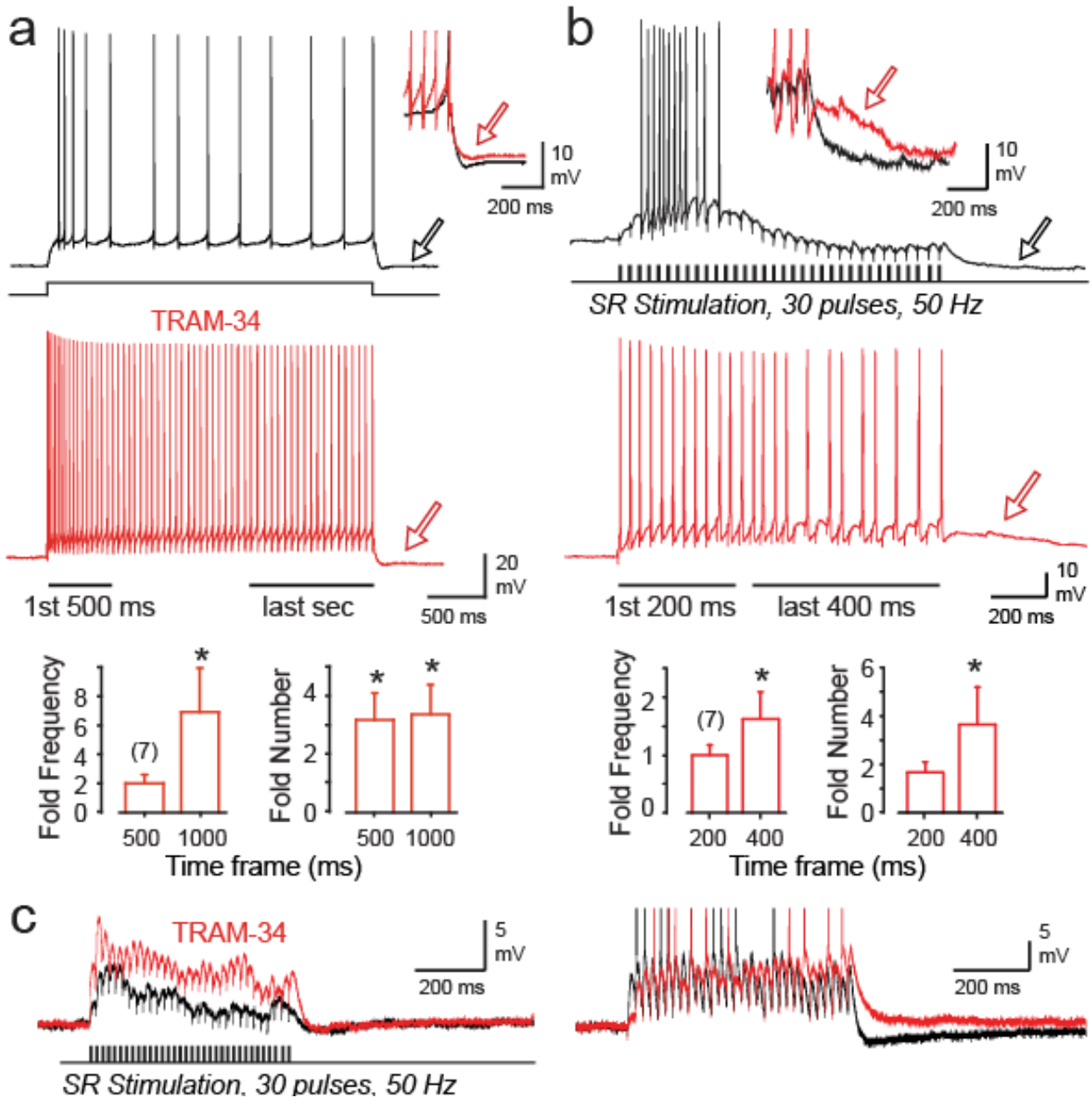
a
10
mV
200 ms
TRAM-34
20
mV
1st 500 ms
last sec
500 ms
Fold Frequency
(7)
*
500
1000
Fold Number
*
*
500
1000
Time frame (ms)
b
10
mV
200 ms
SR Stimulation, 30 pulses, 50 Hz
10
mV
1st 200 ms
last 400 ms
200 ms
Fold Frequency
(7)
*
200
400
Fold Number
*
200
400
Time frame (ms)
c
TRAM-34
5
mV
200 ms
SR Stimulation, 30 pulses, 50 Hz
5
mV
200 ms

Figure 10. IKCa channels contribute to the sAHP in rat CA1 pyramidal cells.
(a) Spike accommodation is blocked by bath applied 1 µM TRAM-34 to increase spike number and frequency. **(b)** Repetitive SR stimulation (30 pulses, 50 Hz) in the same cell as **(a)** is associated with spike accommodation and a loss of temporal summation of EPSPs that are blocked by 1 µM TRAM-34. *Open arrows* and *inset* illustrate the effects of TRAM-34 on the sAHP that follows a synaptic train. Mean bar plots of the fold increase in spike frequency and spike number for current pulse injections and synaptic trains are shown below for the time frames indicated by *horizontal bars* in **(a, b)**. Values were normalized to control recordings in each cell. **(c)** Repetitive SR stimulation (30 pulses, 50 Hz) at sub- or suprathreshold (*left and right panels*, respectively) intensity in the *absence* of picrotoxin to preserve inhibitory inputs reveals a significant effect by TRAM-34 (1 µM) on the degree of temporal summation of EPSPs. All recordings in **(a, b)** were obtained in 100 nM apamin, 10 µM XE-991, and 50 µM picrotoxin, while those in **(c)** did not include picrotoxin. Sample numbers for mean values are shown in brackets. Average values are mean ± SEM; *$P < 0.05$.

Data in panels **(a, b)** partially obtained by T. Bartoletti, N.C. Heath, S. Dykstra, and A. Rizwan. Statistics carried out by T. Bartoletti.

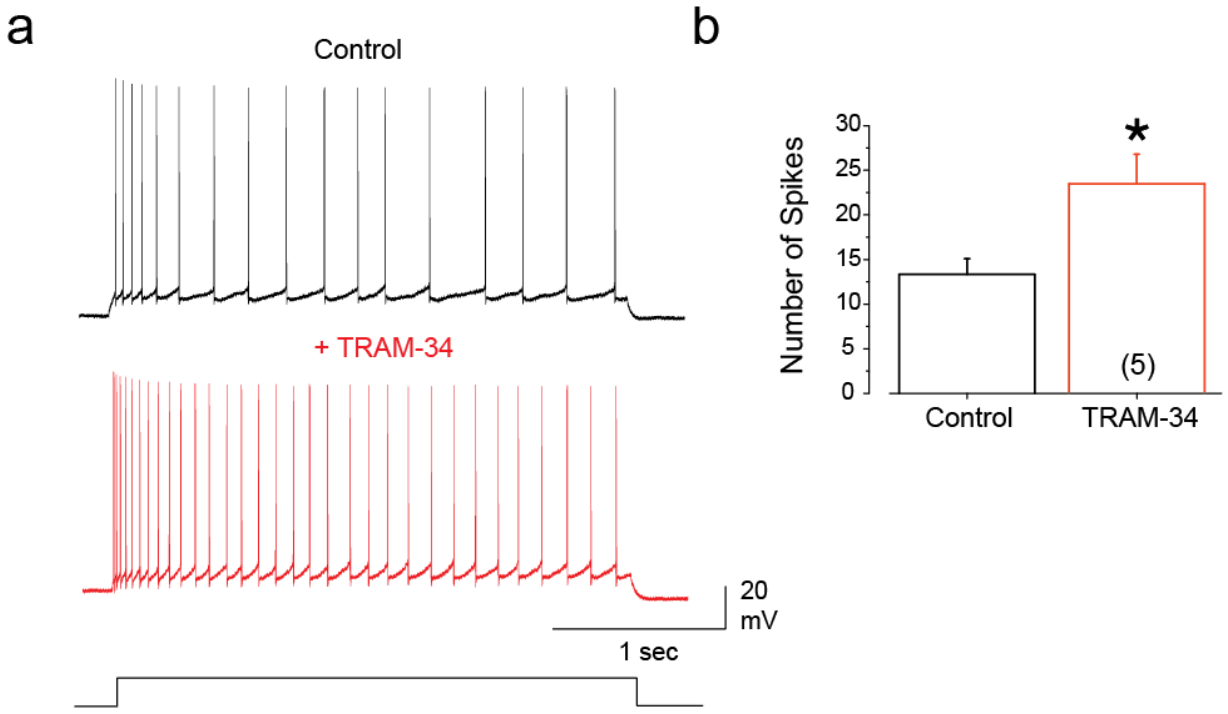

Figure 11. Block of IK_{Ca} with internally perfused TRAM-34 reduces spike accommodation in mice.

(a) Somatic current injection for 3 s at 75 pA in the presence of 100 nM apamin, 10 µM XE-991, and 50 µM picrotoxin results in spike accommodation in mouse wild-type CA1 pyramidal neurons (control trace in *black*), which is reduced by internal perfusion of 1 µM TRAM-34 (*red trace*). **(b)** The number of spikes increases significantly with internal perfusion of 1 µM TRAM-34 (* $P < 0.05$, paired t-test).

Hz SR stimulus train often approaching or even falling below the initial resting potential (**Fig. 10b**). Block of the sAHP by TRAM-34 led to a maintained depolarization during SR-evoked synaptic trains (**Fig. 10b**), with spike accommodation during current pulse injection or SR stimulation accompanied by an increase in the total frequency and number of spikes (**Fig. 10a, b**). The increase in frequency and number of spikes discharged was evident from the initial phase of either stimulus (**Fig. 10a, b**), promoting an increase in spike output within the first few stimuli. A block of the sAHP by TRAM-34 was also reflected in a slower rate of repolarization of membrane potential or an afterdepolarizing potential immediately following a stimulus (**Fig. 10a, b,** *insets*). The magnitude of a post-stimulus TRAM-34-sensitive sAHP was more prevalent for SR-evoked trains compared to just threshold current-evoked spike discharge (**Figs. 10a, b, 12a**). This result is expected given a known direct relation between spike frequency and number with sAHP amplitude (Hotson and Prince, 1980; Madison and Nicoll, 1984), with threshold current injection evoking an average spike frequency of 12.7 ± 2.3 Hz (n = 7) compared to 36.1 ± 5.3 Hz for 50 Hz SR stimulation (**Fig. 12a**). The larger post train sAHP evoked by SR stimulation was also apparent when stimuli were adjusted to evoke an equivalent number of spikes as that evoked by a short duration current pulse (**Fig. 12b,c**). We repeated the tests with SR stimulation in a subset of recordings in which picrotoxin was excluded to determine if the effects of TRAM-34 could be detected when feedforward and feedback inhibitory GABAergic inputs were intact. These tests confirmed that TRAM-34-sensitive currents are effective when GABAergic inputs are intact by modulating temporal summation and spike accommodation for either subthreshold or suprathreshold SR stimulation (**Fig. 10c**).

3.6 The synaptically-evoked sAHP is reduced in KCa3.1-/- mice

We next examined the effects of SR stimulus trains in wild type (*wt*) and KCa3.1-/- mice. Trains of SR synaptic input in *wt* mice (30 pulses, 50 Hz) were followed by a pronounced sAHP that peaked immediately following the train and lasted for several seconds (**Fig. 13a**). By comparison, recordings conducted in KCa3.1-/- mice revealed at most a small amplitude late sAHP following a similar SR stimulus train (**Fig. 13b**), presumably carried by the Na^+/K^+ pump

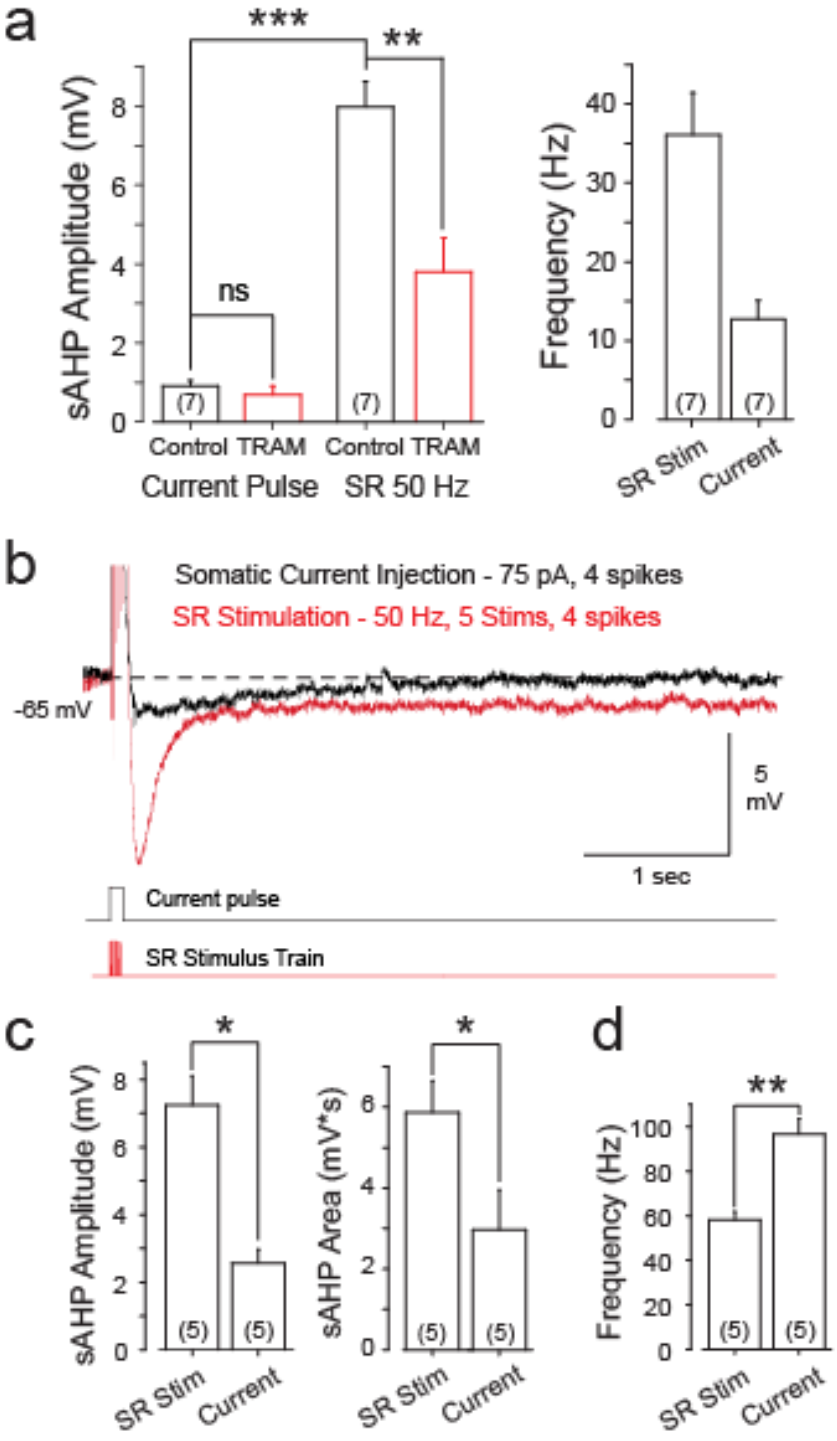
a
***
**
ns
sAHP Amplitude (mV)
0
2
4
6
8
(7)
(7)
Control TRAM Control TRAM
Current Pulse SR 50 Hz
Frequency (Hz)
0
10
20
30
40
(7)
(7)
SR Stim
Current
b
Somatic Current Injection - 75 pA, 4 spikes
SR Stimulation - 50 Hz, 5 Stims, 4 spikes
-65 mV
5 mV
1 sec
Current pulse
SR Stimulus Train
c
*
sAHP Amplitude (mV)
0
2
4
6
8
(5)
(5)
SR Stim
Current
*
sAHP Area (mV*s)
0
2
4
6
(5)
(5)
SR Stim
Current
d
**
Frequency (Hz)
0
20
40
60
80
100
(5)
(5)
SR Stim
Current

Figure 12. The synaptically-evoked sAHP has a greater magnitude than the somatically-evoked sAHP in mouse CA1 pyramidal neurons.

(a) The synaptically-evoked sAHP is larger in magnitude and more sensitive to TRAM-34 than the sAHP elicited by a current step at the soma. This is expected given that SR stimulation evokes a higher frequency of action potentials (*right* plot). **(b)** Representative traces from the same neuron demonstrating the size of both the somatically-evoked sAHP (*black trace*), evoked using a 50 ms, 75 pA current step to elicit 4 spikes (shown below trace), and the synaptically-evoked sAHP (*red trace*), evoked using 5 SR stimuli with intensity adjusted to also elicit 4 spikes (shown below trace). **(c)** Mean peak amplitude, area below the respective sAHPs, and frequency of spikes during recordings in **Fig. 10**. The synaptically-evoked sAHP is larger than that evoked by current injection, yet produces a lower frequency of spike firing (* $P < 0.05$, ** $P < 0.01$, paired t-test).

(Gulledge et al., 2013). Moreover, infusion of 1 µM TRAM-34 into the electrode rapidly reduced the sAHP in *wt* mice but not in KCa3.1-/- mice (**Fig. 13a, b**). Closer examination of the baseline membrane voltage during the SR stimulus train revealed a pronounced suppression of temporal summation of EPSPs that was greater in *wt* mice than in KCa3.1-/- animals (**Fig. 13c**). Infusion of TRAM-34 in the electrode of recordings from *wt* mice promoted temporal summation during the 30 pulse stimulus train and raised the baseline voltage (EPSPs) to within the range recorded in KCa3.1-/- animals (**Fig. 13c**).

These data provide strong evidence that IKCa channels contribute to the synaptically evoked sAHP of CA1 pyramidal cells. Further evidence was obtained when the sAHP following a 5 pulse 50 Hz SR stimulus train was reduced by the alternate IKCa channel blocker Senicapoc (100 nM) (sAHP area reduced by 61 ± 17%, $n = 6$, $P < 0.05$) (**Fig. 13d**). Conversely, sAHP area was enhanced by 1-EBIO (1 µM) (241 ± 47%, $n = 5$, $P < 0.05$) or SKA-31 (1 µM) (363 ± 108%, $n = 6$, $P < 0.01$), two agonists that increase the sensitivity of IKCa channels to [Ca]i (**Fig. 13d**) (von Hahn et al., 2001; Sankaranarayanan et al., 2009). Since all of these recordings were conducted in 100 nM apamin, the effects of 1-EBIO and SKA-31 are consistent with an action on IKCa-mediated current.

3.7 Synaptic activation of IKCa channels and the sAHP

To test for potential synaptic activation of IKCa channels in relation to the sAHP, we applied blockers of SK, BK, Kv7, Kv4 and HCN channels and GABA-A inhibition (see **Methods**). We again used on-cell recordings at the soma to avoid disrupting internal calcium buffering mechanisms, short trains of suprathreshold SR stimuli (5 pulses, 50 Hz), and applied a 60 mV steady-state holding potential above the RMP to the patch to increase driving force for potassium current. The role of IKCa channels was then distinguished using TRAM-34.

In 5/27 cases on-cell recordings yielded single channels that were activated during or by the end of a 5 pulse SR stimulus train, followed by a high rate of opening immediately following the train and then a persistent flickering state for several seconds (**Fig. 15a**). Alternatively, a macropatch outward current could be recorded that had a rapid onset following the SR stimulus

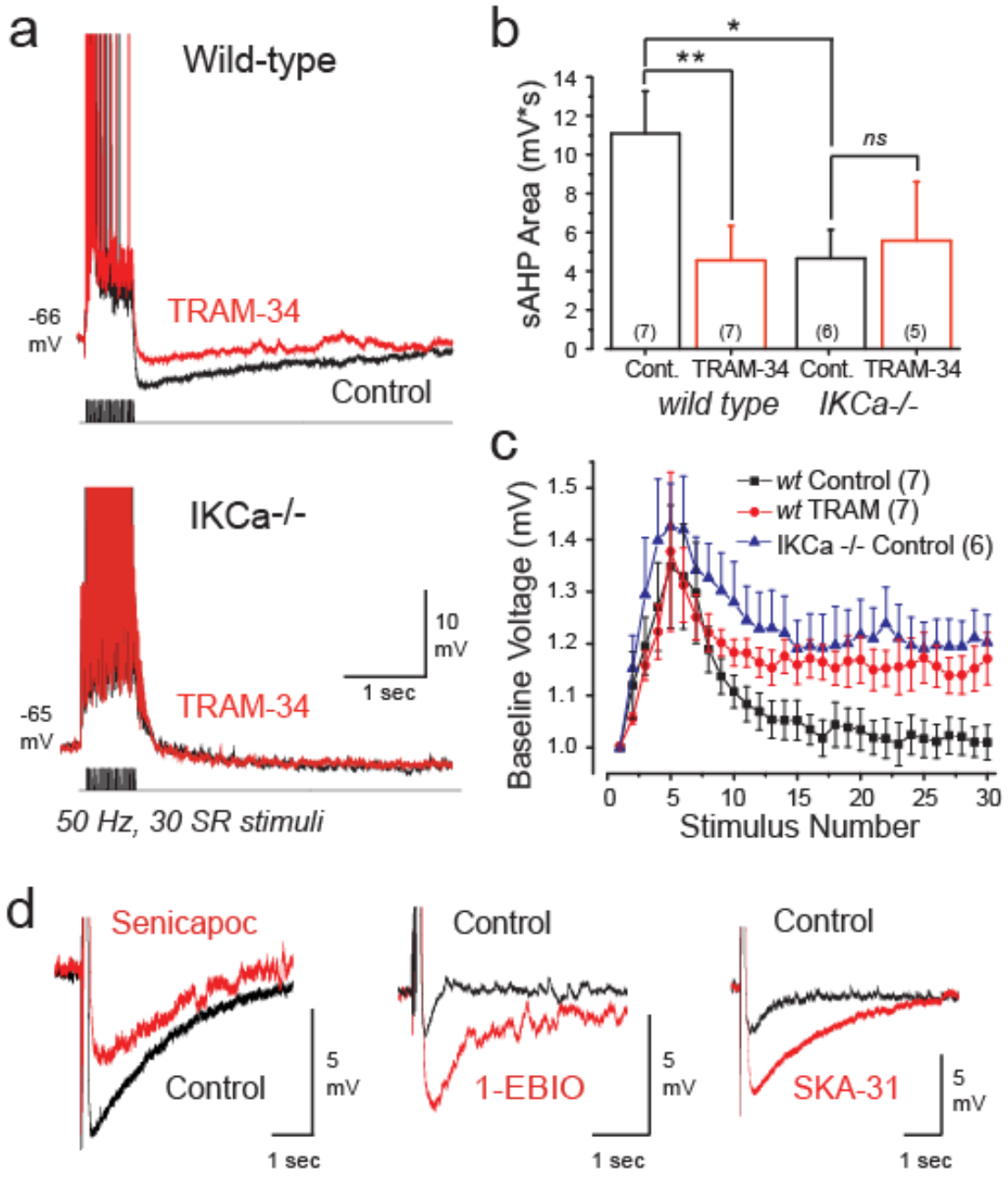
a
Wild-type
-66 mV
TRAM-34
Control
IKCa-/-
-65 mV
TRAM-34
10 mV
1 sec
50 Hz, 30 SR stimuli
b
sAHP Area (mV*s)
*
**
ns
(7)
(7)
(6)
(5)
Cont.
TRAM-34
Cont.
TRAM-34
wild type
IKCa-/-
c
Baseline Voltage (mV)
wt Control (7)
wt TRAM (7)
IKCa -/- Control (6)
Stimulus Number
d
Senicapoc
Control
5 mV
1 sec
Control
1-EBIO
5 mV
1 sec
Control
SKA-31
5 mV
1 sec

Figure 13. IKCa channels contribute to the sAHP to control temporal summation of synaptic input.

(a) Responses in mouse CA1 pyramidal cells to SR stimulus trains (30 pulses, 50 Hz) at 34ºC reveal a substantial sAHP in *wt* but not KCa3.1-/- animals that is blocked by internal infusion of 1 µM TRAM-34 in *wt* animals. **(b)** Mean sAHP area in *wt* and IKCa-/- animals before and after TRAM-34. **(c)** Mean plots of baseline membrane voltage normalized to the voltage at the start of repetitive SR stimulation (as in **a**) reveal a suppression of EPSP summation in *wt* animals that is reduced to that of IKCa-/- animals by 1 µM TRAM-34 infused through the electrode. All recordings were obtained in 100 nM apamin, 10 µM XE-991, and 50 µM picrotoxin. **(d)** The sAHP following 5 pulse 50 Hz SR stimulus trains in rat pyramidal cells is reduced by the IKCa blocker Senicapoc (100 nM), or enhanced by the IKCa agonists 1-EBIO (1 µM) or SKA-31 (1 µM). Average values are mean ± SEM; *P < 0.05, ** P < 0.01, ns, not significant, two way ANOVA. Sample size for mean values are shown in brackets. Spikes in **(a)** and **(d)** are truncated.

Recordings in panel **(d)** partially obtained by A. Rizwan and N.C. Heath.

train and remained open for 2-20 secs before spontaneously terminating with variable duration from stimulus to stimulus (n = 5) (**Fig. 15b**). Calculation of an ensemble average of the SR-evoked macropatch currents revealed an outward current that peaked immediately following the SR stimulus train and then slowly dissipated over 4-5 secs (**Fig. 15b**), as expected for a sAHP. Bath application of 1 µM TRAM-34 rapidly blocked SR-evoked single channels (n = 5) or macrocurrents (n = 5) (**Fig. 15a, b**). These results are important in providing the first evidence for synaptic activation of an identified potassium channel with activity that correlates to the sAHP of CA1 pyramidal cells.

The apamin- and TEA-insensitive component of calcium-activated outward current underlying I*sAHP* is most often recorded as a tail current under voltage clamp following a depolarizing command step of ~ 1 sec (Pedarzani et al., 2005). We directly compared the sensitivity of the step-evoked and SR-evoked I*sAHP* to TRAM-34 application. Perforated patch recordings were used to preserve intracellular buffering mechanisms to compare outward currents evoked by 5 SR stimuli (50 Hz) to that of the I*sAHP* evoked by a step command to +60 mV (500 ms) (**Fig. 15c**). All recordings were again conducted in the presence of 100 nM apamin, 10 µM XE-991, and 50 µM picrotoxin from a holding potential of -65 mV. Under these conditions an outward current followed the train of SR stimuli in 7/8 cells with an amplitude of 47 ± 5 pA and duration of 6.8 ± 1.5 secs (n = 7). In the same cells a step command to +60 mV evoked an I*sAHP* of 28 ± 11 pA and 6.3 ± 1.2 secs (n = 7) (**Fig. 15c**). Perfusion of 1 µM TRAM-34 blocked both the SR- and step-evoked outward current (n = 7) (**Fig. 15**). We also considered testing the drug NS-309, which can augment SK or IKCa current by increasing channel sensitivity to internal calcium (Strobaek et al., 2004). However, we could not reliably test NS-309 given additional effects on the inward calcium tail current that follows a step command (**Fig. 17**). If IKCa channels underlie I*sAHP* then the outward current should also be blocked by ChTx. We thus recorded I*sAHP* evoked by a step command in perforated patch recordings in the presence of 5 mM TEA to first block BK channels. These tests showed that the apamin- and TEA-insensitive I*sAHP* was blocked by pressure ejection of 100 nM ChTx (n = 7) (**Fig. 15d**).

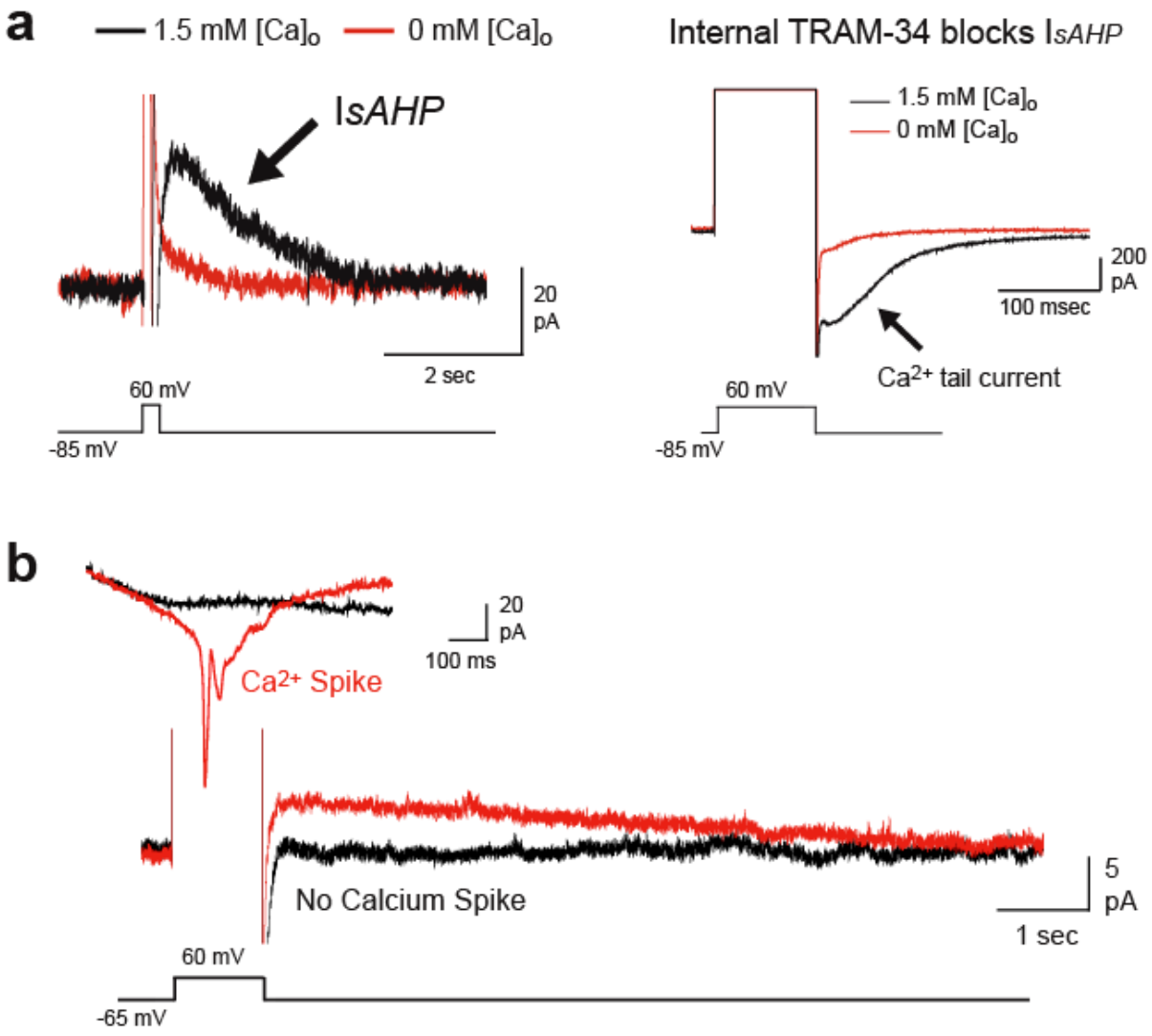
a
1.5 mM [Ca]o
0 mM [Ca]o
IsAHP
20 pA
2 sec
60 mV
-85 mV
Internal TRAM-34 blocks IsAHP
1.5 mM [Ca]o
0 mM [Ca]o
200 pA
100 msec
Ca2+ tail current
60 mV
-85 mV
b
20 pA
100 ms
Ca2+ Spike
No Calcium Spike
5 pA
1 sec
60 mV
-65 mV

Figure 14. A significant component of I*sAHP* in CA1 pyramidal cells is calcium-dependent. **(a)** Representative whole-cell recordings of I*sAHP* (*left*) evoked by a step command to +60 mV at 34ºC is blocked by perfusion of low extracellular calcium, as is an inward tail current recorded in another cell in the presence of internal 1 µM TRAM-34 (*right*). **(b)** A perforated patch whole-cell recording at the soma records an I*sAHP* of 6 sec duration following a step command to +60 mV at 22 ºC. The I*sAHP* is dependent upon generation of calcium current during the step command, visible here as an all-or-none unclamped calcium spike (*inset*). Recordings in **(a)** were conducted using a KMeSO4-based electrolyte with 0.1 EGTA, ATP, GTP, creatine, and 1 µM TRAM-34 (*right* frame). External medium for recordings in **(a)** included 200 nM TTX, 100 nM apamin, 5 mM TEA, 2 mM CsCl and both glutamate and GABA-A receptor blockers. Recordings in **(b)** used an external medium of 0.5 µM TTX, 1 mM TEA and external 2.5 mM $CaCl_2$ to match the solutions used by Pedarzani and coworkers (Pedarzani et al., 2005).

Data in **(a)** recorded by J.D.T. Engbers.

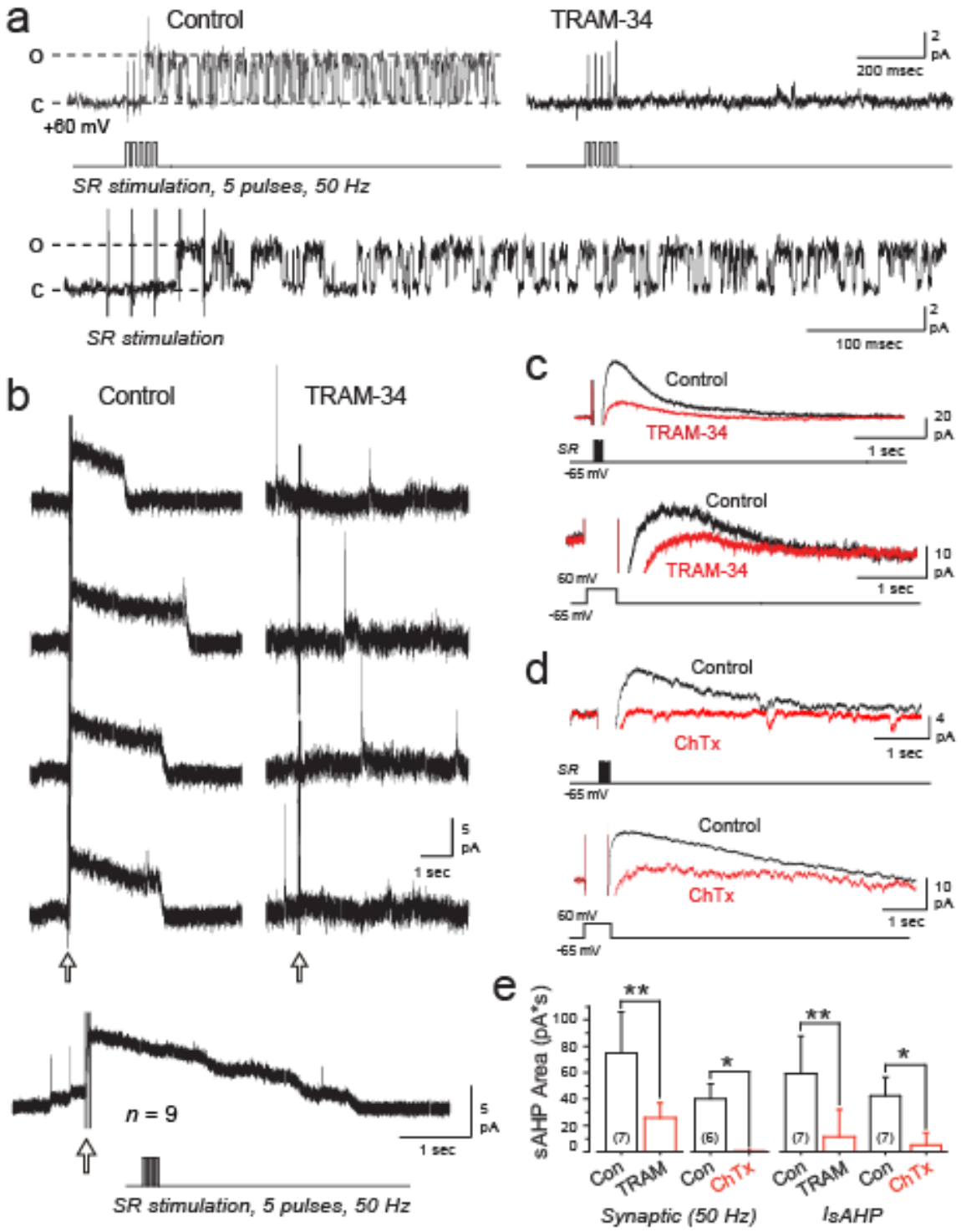
a
Control
TRAM-34
o
c
+60 mV
2 pA
200 msec
SR stimulation, 5 pulses, 50 Hz
SR stimulation
2 pA
100 msec
b
Control
TRAM-34
5 pA
1 sec
n = 9
5 pA
1 sec
SR stimulation, 5 pulses, 50 Hz
c
Control
TRAM-34
20 pA
SR
1 sec
-65 mV
Control
TRAM-34
10 pA
60 mV
1 sec
-65 mV
d
Control
ChTx
4 pA
SR
1 sec
-65 mV
Control
ChTx
10 pA
60 mV
1 sec
-65 mV
e
sAHP Area (pA*s)
100
80
60
40
20
0
**
*
**
*
(7)
(6)
(7)
(7)
Con
TRAM
Con
ChTx
Con
TRAM
Con
ChTx
Synaptic (50 Hz)
IsAHP

Figure 15. IKCa channels are evoked in CA1 pyramidal cells by synaptic stimulation. **(a, b)** On-cell recordings using a HEPES-buffered aCSF electrolyte (3.25 mM K^+). Currents are illustrated with respect to the cell interior (outward current upward). Both bath aCSF and electrolyte contained 100 nM apamin, 5 mM TEA, 5 mM 4-AP, 10 µM XE-991, 2 mM CsCl, 1 µM CGP-55845, and 50 µM picrotoxin. Currents were evoked using a 50 Hz, 5 pulse SR stimulus train with a net 60 mV depolarized holding potential to increase driving force for potassium across the patch. **(a)** A single channel that is activated during and immediately after a 5 pulse SR stimulus train flickers for extended periods of time and is blocked by bath perfusion of 1 µM TRAM-34. Horizontal dashed lines depict open (o) and closed (c) states, with the same control example expanded below. **(b)** On-cell recordings of an outward macropatch current that opens for prolonged but variable periods of time following separate SR stimulus trains (open arrows), and is blocked by TRAM-34. An ensemble average from 9 separate SR stimulus trains in control recordings is shown in the lowest trace, revealing an average time course equivalent to a sAHP. Transients in **(b)** reflect capacitive transients from spontaneous discharge of spikes in the cell. **(c - e)** Comparison of outward current evoked by SR stimulation to I*sAHP* evoked by a step command using perforated patch recordings in the presence of 100 nM apamin, 10 µM XE-991, and 50 µM picrotoxin, with 5 mM TEA included to block BK channels for tests with ChTx **(d, e)**. Currents were evoked by either 5 suprathreshold SR stimuli (50 Hz) or a 500 ms step to 60 mV to evoke I*sAHP* and bath apply 1 µM TRAM-34 or pressure eject 100 nM ChTx. TRAM-34 results in **(c)** are from the same cell and ChTx in **(d)** from a separate cell. Mean bar plots in **(e)** illustrate the effects of TRAM-34 and ChTx on the SR-evoked response or I*sAHP*. Traces in **(a, b)** were filtered at 500 Hz and the expanded trace in **(a)** at 1 kHz (8 pole bessel). Average values are mean ± SEM; *P < 0.05, ** P < 0.01. Sample numbers for mean values are shown in brackets.

Data in panels (**a, b**) recorded by A. Rizwan.

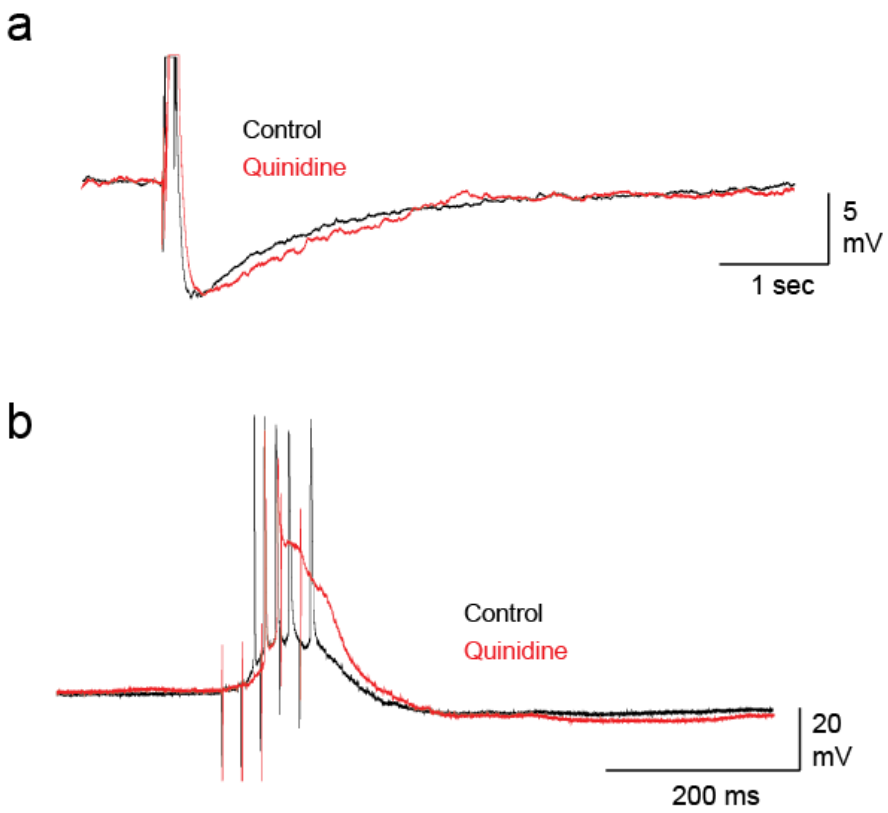

Figure 16. Slack channels do not contribute to the sAHP in CA1 pyramidal neurons. **(a)** A 50 Hz, five pulse SR stimulus evokes a large magnitude sAHP that is not affected by bath application of 1 mM quinidine, a sodium channel blocker previously used to block sodium-activated potassium (Slack) channel activity (*n* = 4). However, quinidine uncovers a presumed calcium spike during the protocol (*n* = 3/4), confirming a probable direct action on sodium channels **(b)**. Action potentials are truncated in **(a)**.

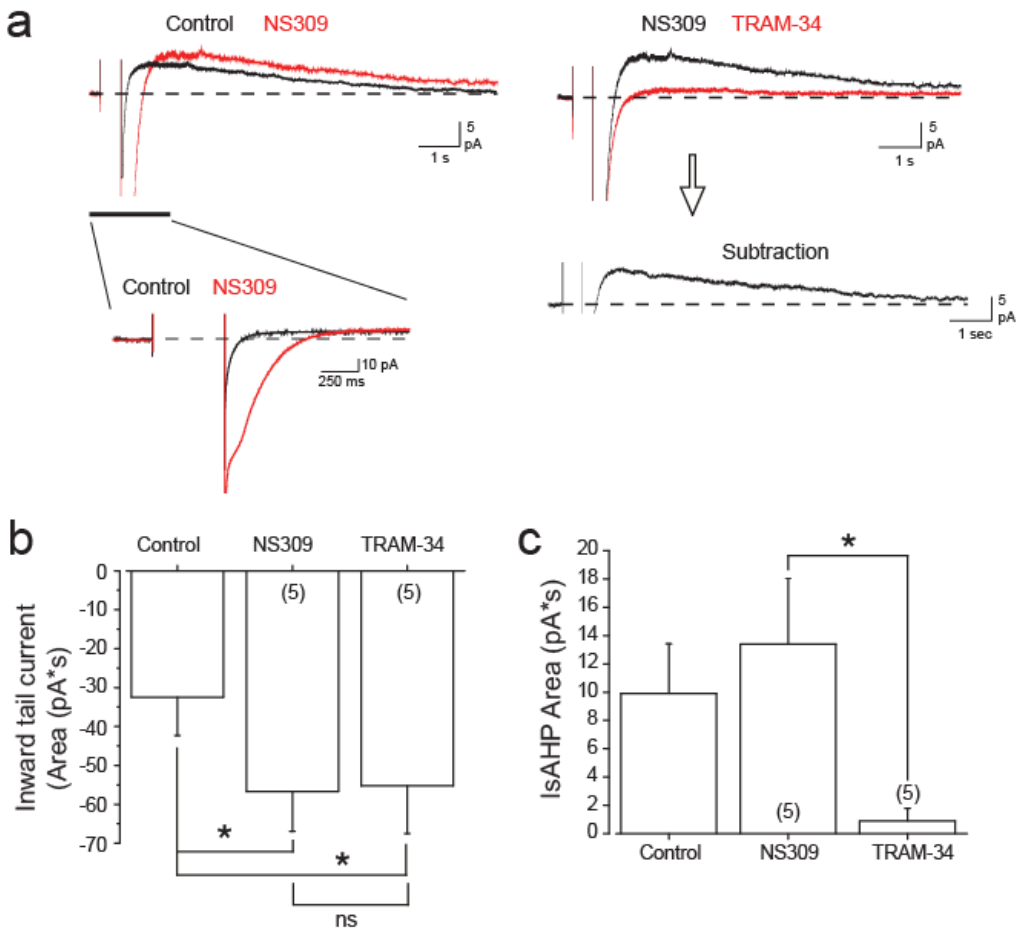

Figure 17. NS-309 augments the calcium tail current and Is*AHP*.

(a) Perforated patch whole-cell recording from the soma of the Is*AHP* evoked by a 500 ms step to +60 mV at 22ºC before and after perfusing 10 µM NS-309 and 1 µM TRAM-34. NS-309 is non-specific in augmenting the calcium tail current following the step, confusing interpretation of an increase in the Is*AHP* and TRAM-34-sensitive current. TRAM-34-sensitive currents are shown by subtraction of test from control recordings in the *right panel*. **(b)** Mean bar plots of the area of inward current under baseline (*dashed lines* in **a**) over 800 ms from the end of the +60 mV step (*left plot*). Also mean bar plots of Is*AHP* as the area above baseline from 1 sec following the voltage step to 5 sec after the voltage step (Is*AHP* - *right plot*). Recordings were conducted in the presence of 0.5 µM TTX, 100 nM apamin, 5 mM TEA, 5 mM 4-AP, 10 µM XE-991, 2 mM CsCl, and 50 µM picrotoxin. Sample numbers are shown in brackets. Average values are mean ± SEM; *P < 0.05, **P < 0.01 repeated measures one-way ANOVA.

Chapter Four: Discussion

The current thesis investigated the hypothesis that IKCa is not only expressed in the CNS, specifically the CA1 region of hippocampus, but also that this ion channel plays a significant role in reducing pyramidal neuron excitability by generating the calcium-dependent sAHP. We have shown that outside-out patches pulled from pyramidal cell somata display an outward current that exhibits the same pharmacological profile as IKCa channels (**Fig. 8**), and that single channels isolated during on-cell recordings reflect a potassium-selective current with a single channel conductance of ~30 pS (**Fig. 9**). In addition, the apamin- and XE-991-insensitive sAHP and I*sAHP* from these cells were sensitive to blockers of IKCa channels, including TRAM-34, ChTX, MTx, and Senicapoc (**Figs. 10, 12, 13, 15**), and were increased by the IKCa/SK agonists SKA-31 and 1-EBIO in the presence of apamin (**Fig. 13**). IKCa knockout animals displayed a reduced sAHP that had no TRAM-34 sensitivity (**Fig. 13**). This thesis also shows that other sAHP-generating candidate ion channels either have separate roles (for example the Na^+/K^+ pump, **Fig. 5**) or were confirmed to have no role in generating I*sAHP* or sAHP under the conditions examined here (i.e. SK, Kv7 channels, Slack channels, **Fig. 16**).

It is well established that IKCa channels are expressed in endothelial cells of the cerebrovasculature and in activated microglial cells in the CNS (Wulff et al., 2007). However, the current findings have wide ranging impact by challenging previous thought that IKCa channels are not expressed in CNS neurons, and identifying the ionic basis for a phenomenon that has been recognized for over 30 years but has continually eluded explanation. The sAHP is one of the strongest modulators of neuron excitability in many cell types, including CA1 pyramidal neurons. Identifying IKCa channels as the underlying basis for the sAHP thus helps us to understand how a crucial physiological component of one of the most widely-studied circuits, the trisynaptic circuit in the hippocampus, is generated and contributes to signal processing. The sAHP also has a powerful role in controlling neuron excitability, a key factor in keeping the circuit in check and to prevent epileptic episodes. Given other evidence that IKCa channels are expressed in numerous regions of the brain (Turner et al., 2014), the findings here also raise questions as to how IKCa channels influence neuron excitability in other regions such as the CA3 division of hippocampus, the thalamus, and cortex. Since this study only focused on CA1

pyramidal neurons, these other regions remain as future objectives for studying the influence of IKCa channels.

4.1 Expression of IKCa in the CNS

Previous evidence has suggested that IKCa is indeed expressed in the CNS, despite the persistent dogma that it is not. A recent report established that IKCa contributes to synaptic processing in cerebellar Purkinje cells (Engbers et al., 2012b). The distribution of IKCa immunolabel and GFP expression tied to the IKCa promoter further suggested that IKCa protein is expressed in multiple regions of the brain, including the hippocampus (Turner et al., 2014). The current study presents evidence that IKCa channels are functionally expressed in hippocampal CA1 pyramidal cells. Yet many of the results obtained here contradict earlier work investigating the ionic basis for the sAHP in these cells.

4.1.1 Discrepancies between previous work and the present study

IKCa channels were first detected in peripheral tissues where they were cloned from various human cells such as placental cells (Joiner et al., 1997), T lymphocytes (Logsdon et al., 1997), and pancreas (Ishii et al., 1997). Importantly, these initial studies concluded based on a lack of RNA that IKCa channels are not expressed in CNS neurons, a belief that held fast for almost three decades. These first results could be due to the methods used to probe for the presence of IKCa channels by relying on Northern Blot RNA analysis of human tissue (Ishii et al., 1997; Joiner et al., 1997; Logsdon et al., 1997). This method could be subject to degradation of sample and may not be sensitive enough to detect levels of IKCa RNA found in the brain. It is possible that the RNA sequence of IKCa channels in the brain is modified from that of other parts of the body to the point where it may not be detected by the probes used. Our initial work with single cell RT-PCR and sequencing suggest substantial conformity of the sequence of IKCa mRNA that can be extracted from pyramidal cells with that reported in peripheral cells, at least in the region of the channel pore (data not shown). Therefore, future work on the sequence of IKCa in central neurons will be important for establishing any molecular differences that might exist. However, the work presented here is entirely in line with the distribution pattern recently reported for IKCa

channel immunolabel and green fluorescent protein (GFP) expression tied to IKCa promoter activity in a transgenic line of mice (Turner et al., 2014). Together, these localization studies and the recordings here argue persuasively that IKCa channels are expressed in CA1 hippocampal neurons.

Second, several key pharmacological tests conducted in the past that would have identified IKCa channels differ from some of the results found here. Early studies on the CA1 sAHP reported that it was not sensitive to ChTx, a well-known blocker of both BK and IKCa (Lancaster and Nicoll, 1987; Wulff et al., 2007). However, this test was only conducted at a maximum of 25 nM ChTx, which may not be high enough to affect IKCa, as compared to the 100 nM used here. Early studies on IKCa showed that the channel was inhibited by approximately 75% at approximately 30 nM ChTx (Ishii et al., 1997; Joiner et al., 1997; Logsdon et al., 1997). Since the sequence of IKCa in CA1 cells is not fully known, it is possible that differences in the protein structure of IKCa investigated in neurons may confer less sensitivity to known inhibitors. As an example of this possibility, a calcium-activated potassium channel of intermediate conductance was reported in visceral sensory afferent neurons that exhibited all of the properties of IKCa channels except that it was insensitive to 50 nM ChTx (Hay and Kunze, 1994). Differences in TRAM-34 effectiveness may also relate to the fact that IKCa has an internal TRAM-34 binding site, and thus requires sufficient time for the drug to cross the membrane and block the channel. We thus found that internal perfusion of TRAM-34 introduced less variability than bath perfusion. Moreover, the least variability was observed using TRAM-34 in on-cell or perforated patch recordings, two recording configurations that preserve intracellular calcium buffering mechanisms, as compared to the whole-cell patch recording configuration used in most previous studies.

Third, most early studies on the sAHP in CA1 hippocampus used a somatic current injection protocol to elicit the sAHP following spike discharge. The present study showed that a synaptic activation protocol is more effective at eliciting the sAHP (and the TRAM-34-sensitive sAHP) than direct current injection (**Fig. 12**), potentially accounting for considerable variability in the occurrence and amplitude of sAHP reported in previous studies (Wu et al., 2004). Indeed, given the magnitude of the difference between direct or synaptically-evoked sAHPs (**Fig. 12**), it is even possible that IKCa channels may be more effectively activated by ligand-gated receptors.

Whether this could reflect any form of direct association between receptor and channel is currently unknown. It could also result from a difference in the relative magnitude of calcium influx evoked via a ligand-gated receptor as compared to primarily somatic spike discharge that would accompany a current pulse injected at the soma. Our comparison of the ability for TRAM-34 or ChTx to block the I*sAHP* evoked using either a conventional step command or SR stimulus trains in voltage clamp (**Fig. 15**) further establishes that the current studied here corresponds to that recorded in previous studies. Therefore, discrepancies between the data presented here and some previous studies may be due to their not using a sufficient concentration of inhibitors, the delivery method employed, or the recording mode used.

4.1.2 Properties of IKCa expressed in CA1 pyramidal neurons

The channel that is proposed here to underlie the CA1 pyramidal cell sAHP shares many of the previously reported properties of IKCa, such as an intermediate conductance level of ~30 pS (**Fig. 5**) (previously reported to be 12-42 pS as measured at the inwardly rectifying section of the I-V plot) (Ishii et al., 1997; Joiner et al., 1997; Logsdon et al., 1997), potassium selectivity (**Fig. 5**) (Joiner et al., 1997; Logsdon et al., 1997), activation by 1 µM calcium (Ishii et al., 1997; Joiner et al., 1997; Logsdon et al., 1997) (**Fig. 4**), and an apparent inward rectification profile in its single channel I-V relationship (**Fig. 5**) (Ishii et al., 1997; Logsdon et al., 1997). There have been conflicting reports in the past with regards to the I-V relationship of IKCa channels. In addition to the above mentioned studies, others have reported IKCa inward rectification (Christophersen, 1991) while still others report outward rectification (Joiner et al., 1997; Gao et al., 2010) or even voltage dependence (Stoneking et al., 2013).

Clues to explain the differences in these findings can be found in the present study. The I-V relationship that was discovered in outside-out patches from CA1 pyramidal cells was for the most part linear (**Fig. 8**). However, single channels from the same cells recorded using the on-cell mode revealed an inwardly rectifying profile (**Fig. 9b**). The difference could be due to the presence or absence of certain calcium sensors, ion species such as magnesium (Stoneking and Mason, 2013), and/or phosphorylating components. For example, in outside-out patches IKCa was constitutively activated with a buffered level of 1 µM internal calcium. However, second

messengers (i.e. PKA, CaM) may not be present in the patch in necessary amounts to influence IKCa channel activity. Single channel on-cell recordings preserved both the internal cellular environment and calcium buffering of the cell, as well as magnesium ions that have previously been shown capable of imparting different I-V relationships and opening probabilities in IKCa channels from HEL cells (Stoneking and Mason, 2013). In this study, 4/6 cells showed single IKCa channels with an I-V relationship that was inwardly rectifying (**Fig. 9**). Also, accessory proteins that interact with IKCa are still present in on-cell recordings. This difference could explain how different protocols and patch configurations can influence the I-V relationship of IKCa channels.

4.1.3 IKCa in other central neurons

Interestingly, the recent immunocytochemical report from the Turner lab (Turner et al., 2014) demonstrated that immunolabel for IKCa channels and GFP expression tied to the IKCa promoter were present at a greater relative strength in interneurons than pyramidal neurons of the CA1. Preliminary data have also shown that interneurons from the hippocampus have a large calcium-activated hyperpolarizing current that is TRAM-34 sensitive (data not shown). The presence of IKCa channels in interneurons has far-reaching implications for neuron excitability. These channels may have roles in not only modulating the excitability of pyramidal excitatory neurons, but also inhibitory neurons in the circuit in response to increases in calcium, and modulated by second messengers.

Also identified in the study by Turner et al., 2014 were neurons from various other brain regions in rats and mice that exhibit IKCa immunolabel and GFP fluorescence tied to IKCa promoter activity. These include pyramidal neurons in the cortex and the thalamus, other brain regions responsible for important information processing related to various behaviours. A calcium-activated sAHP has been described in these neurons, including the rat neocortex (Lorenzon and Foehring, 1993, 1995) and thalamic paraventricular neurons (Zhang et al., 2009). In pyramidal neurons of the neocortex, for example, the sAHP was found to be sensitive to the same neuromodulators as in pyramidal neurons of the CA1 such as isoproterenol (Abel et al., 2004). This indicates that the same sort of modulation and possibly the same channels underlie

the sAHP. Given the findings presented here, it would be a reasonable hypothesis that IKCa channels underlie the sAHP in these other neurons. However, some more experiments need to be carried out in these regions of the brain to test this hypothesis, as not all evidence points to IKCa being the mediator of the sAHP in the abovementioned neurons. In the thalamus for example, the sAHP is insensitive to most known calcium-activated potassium channel blockers, including apamin, TEA, ChTx, and IbTx (Zhang et al., 2009). Also, it is known that latter parts of the sAHP in cortical neurons are mediated by sodium-activated potassium channels (Foehring et al., 1989; Schwindt et al., 1989). Although the present study shows that these sodium-activated potassium (Slack) channels do not contribute to the sAHP in CA1 pyramidal neurons (**Fig. 16**), the relative contribution of Slack channels versus IKCa channels is not currently known, and remains a future direction for this field.

4.2 Channels underlying the sAHP in CA1 hippocampal pyramidal cells

The sAHP in the CA1 region of hippocampus, as mentioned previously, has been studied for over 30 years. In this time, many potential channels have been suggested to be responsible for generating this event. However, one set of facts has been consistently recognized to be true: the sAHP is generated by a calcium-activated potassium channel that has slow kinetics (Alger and Nicoll, 1980; Hotson and Prince, 1980; Madison and Nicoll, 1982, 1984) and is blocked by PKA and its corresponding neurotransmitter activators (Madison and Nicoll, 1986; Pedarzani and Storm, 1993; Haug and Storm, 2000; Lancaster et al., 2006). Many of the candidates referred to above do not fit these well-established requirements, while IKCa fits this profile quite well.

The sAHP was first thought to be generated by apamin-insensitive SK channels after fluctuation analysis suggested the single channel conductance of the sAHP channel to be around 2-5 pS (Sah and Isaacson, 1995). However, this was later discounted as SK KO animals continued to show a large calcium-activated sAHP, while SK2 KO animals lacked an apamin-sensitive mAHP (Bond et al., 2004). Since SK channels were definitively shown to not be responsible for the sAHP, other possible contributors for the sAHP included CaCCs such as TMEM16B (Huang et al., 2012), Kv7 channels (responsible for I_M) (Tzingounis and Nicoll, 2008; Tzingounis et al., 2010), sodium-activated potassium channels such as the Slack channel (Schwindt et al., 1989; Zhang et al., 2010), and finally the Na^+/K^+ pump (Gulledge et al., 2013).

However, convincing lines of evidence show that these other candidate channels do not contribute to the calcium-activated sAHP in CA1 pyramidal neurons. Ever since its initial observation, the sAHP has been shown to follow E_K and not E_{Cl} (Hotson and Prince, 1980; Lancaster and Adams, 1986), demonstrating that it is potassium-selective and not mediated by a chloride channel. In this study, we provide the best evidence to date that the single channels underlying the sAHP are potassium selective (**Fig. 9**). In addition, outside-out patch recordings show that there is a calcium-activated outward current that reverses near E_K and is blocked by TRAM-34, ChTx, MTx, and PKA (**Fig. 8**). Together these data strongly argue for the expression of IKCa in pyramidal cells and its contribution to the sAHP. In addition, previous work suggesting that CaCCs contribute to the sAHP have relied on non-specific pharmacological agents such as NFA and NPPB (Huang et al., 2012), both of which have been shown to also block IKCa (Fioretti et al., 2004; Olivan-Viguera et al., 2013). To test if TRAM-34 is non-selective and able to block CaCCs, we also confirmed that TMEM16B channels expressed in isolation in HEK cells are not blocked by TRAM-34 (**Fig. 4d**).

The strong calcium-dependence of sAHP activation (**Figs. 9d, Fig. 14**) rules out multiple candidate channels such as sodium-activated potassium channels (i.e. Slack), which are inhibited by increases in calcium (Joiner et al., 1998). Nonetheless, we investigated the role of Slack channels in generating the synaptically-evoked sAHP by applying quinidine, and established that Slack channels do not contribute to the sAHP under our recording conditions (**Fig. 16**). The inhibition by increased calcium is also true for Kv7 channels (Andrade et al., 2012), which have been shown to be important in generating the sAHP in CA3 neurons but less so in CA1 (Tzingounis and Nicoll, 2008; Tzingounis et al., 2010). As evidence, KCNQ5 dominant negative-expressing animals reportedly display a reduced I*sAHP* in CA3 but not CA1 pyramidal cells (Tzingounis et al., 2010), and KCNQ2 and KCNQ3 KO animals show a reduced mAHP and sAHP in CA3 but not CA1 pyramidal cells (Tzingounis and Nicoll, 2008). In addition, the current study shows that applying a combination of apamin, picrotoxin, and XE-991, a potent Kv7 channel blocker (Zaczek et al., 1998), does not reduce I*sAHP* in CA1 pyramidal cells, and all of the control recordings in this study were conducted in the presence of XE-991. We also directly confirmed that TRAM-34 does not act on Kv7 channels expressed in isolation in HEK

cells (**Fig. 4c**). Altogether, the evidence suggests that neither Slack nor Kv7 channels contribute to the sAHP in CA1 pyramidal neurons under our conditions.

A recent study has shown that the Na^+/K^+ pump can generate a long-lasting hyperpolarization following a long series of action potential-like waveforms at 50 Hz and at 35°C (Gulledge et al., 2013). A significant component of this potential was blocked using ouabain, a drug that blocks the Na^+/K^+ pump. However, oubain is also known to be non-selective and can block internal calcium stores, an effect that could confuse interpretations of its affect on the calcium-activated sAHP (Tian and Xie, 2008). Instead, we found that IKCa is responsible for approximately 70% of the sAHP generated using this protocol (**Fig. 7**). The remaining sAHP, sensitive to ouabain, is very long-lasting (~10 secs) and smaller in amplitude, and could thus be generated by the Na^+/K+ pump. This finding reinforces the idea that multiple channels likely contribute to the sAHP depending on the context of the cellular background and the neuron's excitation state (for example, synaptically activated or not).

4.3 IKCa channels and the sAHP

Despite strong evidence suggesting IKCa's contribution to the sAHP, many questions about the nature the channels' properties in the CA1 remain. For example, the calcium source for activation of the sAHP (and specifically IKCa) is not fully known, and many studies in the past have tried to resolve this question. Also, the exact location of the channels responsible for the sAHP has been a hotly debated topic that this study may be able to shed some light on as well.

4.3.1 Calcium sources for the IKCa-mediated sAHP

Traditionally, HVA calcium channels were thought to be the main source of calcium to activate the sAHP in CA1 pyramidal cells. This has been demonstrated in the past using the non-selective HVA calcium channel blocker, Cd^{2+} which blocked the I*sAHP* (Lancaster and Adams, 1986) and the sAHP evoked by a somatic current step that evoked a burst of action potentials (Lancaster and Nicoll, 1987). More selective L-type channel blockers such as nifedipine (Borde et al., 2000), nimodipine, and isradipine (Lima and Marrion, 2007) also reduced I*sAHP*. Nevertheless, these results need to be balanced off with the fact that nifedipine is also highly

effective as a direct blocker of IKCa channels (Wulff et al., 2007). On the other hand, blockers of N-, P/Q-, R-, or T-type calcium channel blockers had no effect (Marrion and Tavalin, 1998; Lima and Marrion, 2007). In rat and mouse CA1 pyramidal neurons, a reduction of I*sAHP* of ~70% was observed after application of ryanodine, which inhibits release of calcium from internal stores through ryanodine receptors (Borde et al., 2000; van de Vrede et al., 2007). Therefore, it is accepted that L-type voltage-gated calcium channels and internal calcium stores, but not other voltage-gated calcium channels, can contribute to activation of the sAHP.

With the current study, the question arises as to the mechanism of calcium activation driving IKCa channels and the sAHP. As shown in **Fig. 12**, synaptic activation produces a sAHP of larger magnitude than that evoked through somatic current injection. This is consistent with previous studies that show that 50 Hz SR stimulation evokes a large magnitude sAHP that is caused by calcium influx through NMDARs (Shah and Haylett, 2002; Wu et al., 2004; Fernandez de Sevilla et al., 2007). Like these previous studies, the current study shows that application of DL-AP5 (an NMDAR blocker) eliminated the sAHP evoked by synaptic input (**Fig. 9c**). Therefore, it can be concluded from this study that calcium influx through synaptic NMDARs is a significant contributor to the IKCa-mediated sAHP. However, as we did not investigate the role of calcium released from internal stores, we do not know if this source is a major contributor to this phenomenon. Also, because somatic current injection that evokes the same number of action potentials as in the synaptic protocol produces a significantly smaller sAHP (**Fig. 12**), the traditional source of calcium, L-type calcium channels, may not be as significant a source of activation of IKCa. The whole cell, somatically-evoked sAHP was less affected by TRAM-34 than the synaptically-evoked sAHP (**Fig. 12a**), another indicator that L-type calcium channels are not a major player in the IKCa-mediated sAHP. On the other hand, somatic on-cell patches containing TRAM-34- and 1-EBIO-sensitive channels in the presence of apamin (i.e. IKCa) were observed after both somatic and synaptic stimulation (**Figs. 9 and 15a, b**), further indicating that preservation of the internal cellular conditions may affect the activation of IKCa and that L-type calcium channels at the soma may play a role in activating the sAHP.

Traditionally, it has been thought that calcium-permeable AMPARs (CP-AMPARs) were not present in CA1, as the GluA2 subunit that blocks calcium conductance was shown to be present in most AMPARs in pyramidal cells (Wenthold et al., 1996). AMPARs in CA1 neurons

also display a linear I-V relationship, a signature of AMPARs containing GluA2 subunits and thus lacking calcium conductance (Hestrin et al., 1990; Jonas and Sakmann, 1992). However, recent evidence suggests that CP-AMPARs are indeed present in CA1 pyramidal neurons, and activated by inputs evoked from the SR (Mattison et al., 2014). Since all of the synaptic activation carried out in this study was achieved through SR stimulation, the contribution of CP-AMPARs may be relevant to the activation of IKCa channels. We found that the drug DL-AP5 significantly reduced the SR-evoked sAHP and application of DNQX, an AMPAR blocker, further eliminated the sAHP response (**Fig. 9c**). This indicates that although NMDARs contribute to the majority of the calcium-activated synaptic sAHP, CP-AMPARs may also play a smaller role.

The current study found that subthreshold synaptic activation evoked an AHP that is smaller in magnitude than the sAHP evoked using suprathreshold synaptic activation (see **Fig. 3**). This finding was consistent with previous results, where a faster AHP was evoked using subthreshold synaptic activation, but was reportedly not calcium-activated, as it was not blocked by internal perfusion of the calcium chelator BAPTA (Wu et al., 2004). Therefore, IKCa activation requires suprathreshold synaptic activation, which indicates that backpropagating action potentials and concurrent activation of internal calcium stores or voltage-gated calcium channels, or possibly relief of the magnesium block of NMDARs, are important for the activation of this sAHP.

4.3.2 The implications of a calcium-activated sAHP and IKCa-protein interactions

Another important consideration for the calcium-activated sAHP is the nature of calcium dynamics following a stimulus that evokes a sAHP in CA1 pyramidal neurons. Calcium plays a an important regulatory role in neurons, and many calcium-dependent processes would cause harm to the neuron and its surrounding system if bulk calcium levels were maintained at a high level for too long. For example, excitotoxicity, or the buildup of calcium from excessive glutamatergic activation or reduced calcium buffering abilities, can cause activation of enzymes that break down proteins, membranes, and nucleic acids (Dong et al., 2009). The requirement of CA1 pyramidal neurons to modulate the sAHP separately from bulk levels of calcium demonstrates the importance of maintaining this long-duration inhibitory response and its separation from other calcium-dependent processes. BK channels, as a separate example, tightly

couple with voltage-gated calcium channels to activate within calcium nanodomains (Berkefeld et al., 2010; Engbers et al., 2013; Rehak et al., 2013). Also, IKCa channels in Purkinje neurons in cerebellum tightly couple with LVA T-type calcium channels to enable nanodomain interactions that allow the transient nature of T-type calcium channel activation to be imparted on that of potassium efflux (Engbers et al., 2012a). However, in the CA1 there is no evidence to date of a similar molecular association between HVA L-type calcium and IKCa channels.

Previous findings have shown that even with rapid and large increases in calcium levels in the neuron that drop off rapidly, sAHP channels activate and deactivate slowly without a clear match to cellular calcium dynamics (Sah and Clements, 1999). Therefore, unlike the apamin-sensitive mAHP, which activates and turns off in close association with increases and decreases of intracellular calcium (Sah and Clements, 1999), the sAHP has other factors that allow for its activation and deactivation. These other contributing factors are not completely known, but may involve second messenger systems such as PKA. IKCa has been previously shown to be inhibited by PKA (even in the presence of high calcium) in the enteric nervous system, where IKCa channels generate the sAHP (Vogalis et al., 2003). The sAHP in CA1 pyramidal cells, unlike the mAHP and fAHP, has been shown to be sensitive to neuromodulation, through such modulators as noradrenaline (Madison and Nicoll, 1982), histamine (Haas and Konnerth, 1983), and a wide variety of other transmitters that activate $G\alpha_s$-coupled receptors and the cAMP-PKA signaling cascade (Haug and Storm, 2000; Andrade et al., 2012). IKCa channels have been shown to constitutively interact with CaM (Fanger et al., 1999). However, another calcium sensor, hippocalcin, has been proposed as being important for generating the sAHP in CA1 pyramidal neurons (Tzingounis et al., 2007). Future directions for this study may investigate the role of hippocalcin and the nature of its potential interactions with IKCa to generate the sAHP. In addition, as the sAHP is modulated by PKA, there may be a dynamic interaction between PKA, CaM, hippocalcin, and consequent IKCa mediation of the calcium-dependent sAHP. Preliminary data even suggest that increases in calcium levels in CA1 neurons promote binding of hippocalcin to IKCa channels (H. Asmara, unpublished observations). Small increases in calcium invoked by subthreshold synaptic activation, may not be enough to make the switch between CaM and hippocalcin. In addition, PKA may prevent interactions between CaM and IKCa and inhibit the sAHP, as recently reported for IKCa channels from microglia (Wong and

Schlichter, 2014b). In these cells, mutating one serine residue in human IKCa reduced PKA-dependent regulation of the channel. Further studies involving tests for these hypotheses in neurons will be needed to clarify these important issues.

4.3.3 Location of IKCa

Another debated issue in the sAHP field is the location of the channels responsible for generating the sAHP. Early studies suggested that sAHP channels are located in the apical dendrites of CA1 pyramidal neurons based on a comparison to the known locations of GABAergic responses Further studies, where CA1 neuron processes were cut off demonstrated that only about 30% of the current from these channels were located in the apical dendrites, with none present in the axon (Bekkers, 2000). This study also concluded that there was no sAHP current located in the soma of these cells, but this was based on a lack of single channel current, which is restricted by the small chance of obtaining a single channel within the cell-attached patch. More recently, a study showed indirectly that sAHP channels are in fact present in the soma and proximal apical dendrites of CA1 pyramidal neurons (Fernandez de Sevilla et al., 2007). This conclusion came from the finding that NMDA-mediated EPSPs were shunted at the soma of dually patch clamped (soma and apical dendrite) CA1 pyramidal neurons when synaptically evoked at the SR input. Therefore, it was concluded that sAHP channels at the soma are in an ideal location to modulate the strength of synaptic input to the pyramidal neuron.

Recent findings from the Turner lab have shown that there is IKCa immunolabel present in the somata and proximal dendrites of CA1 pyramidal neurons (Turner et al., 2014). The current study shows that sAHP, and more specifically TRAM-34-sensitive, current was found in 9 out of 27 patches from CA1 pyramidal cell somata, providing direct evidence that IKCa is present in the soma (**Fig. 9**). In addition, outside-out patches pulled from the somata of these neurons showed calcium-activated outward potassium current that was sensitive to 1 μM TRAM-34 or 100 nM ChTx (**Fig. 8**). Therefore, it can be concluded from this study that at least some IKCa channels are located at the soma. Further studies investigating the potential functional dendritic localization of IKCa in CA1 pyramidal neurons will be required. Potentially, dual patch clamp of the soma and the proximal apical dendrites in the presence of TRAM-34 may show a lack of

EPSP shunting when measured at the soma. This would help to resolve this long-standing question of the location of sAHP channels.

4.3.4 Implications of IKCa's role in generating the calcium-activated sAHP in the hippocampus

The ability of the sAHP found in CA1 pyramidal neurons to modulate EPSP strength (Fernandez de Sevilla et al., 2007), as well as modulate neuron firing and excitability on a long time scale, gives rise to the conclusion that the sAHP is important for these neurons' ability to process incoming information. The current study's finding that IKCa is largely responsible for this sAHP leads to the conclusion that IKCa could be an important channel in modulating these processing abilities as well. Therefore, the implications suggest that IKCa is important for such behaviours as spatial learning and memory. In the future, behavioural studies investigating the role of IKCa channels in spatial memory could clarify these roles. However, animals containing a conditional KO of the IKCa channel would be necessary to avoid such confounds as compensations in the neuron circuit leading to these behaviours. Other possible future studies could investigate the role of IKCa in the hippocampus-related tasks by locally applying TRAM-34 in the hippocampus in an awake, behaving animal.

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