PKA Reduces the Rat and Human KCa3.1 Current, CaM Binding and Ca$^{2+}$ Signaling, Which Requires Ser332/334 in the CaM-binding C Terminus

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

The Ca\(^{2+}\)-dependent K\(^+\) channel, KCa3.1 (KCNN4/IK/SK4), is a therapeutic target for several CNS disorders involving microglial activation. While PKA signaling is important in many cells that use KCa3.1, reports of channel regulation by PKA are inconsistent. Here, I found that in microglia, PKA activation with Sp-8-Br-cAMPS decreased the current, and this was prevented by the PKA inhibitor, PKI\(_{14-22}\). Mutating the single PKA site (S334A) in human KCa3.1 abolished the PKA-dependent regulation. CaM-affinity chromatography showed that CaM binding to KCa3.1 was decreased by PKA activation, and this regulation was absent in the S334A mutant. Single-channel analysis showed that PKA decreased the open probability in wild-type but not S334A mutant channels. The same decrease in current for native and wild-type (but not S334A) expressed KCa3.1 channels occurred when PKA was activated through the adenosine A2a receptor. Finally, PKA activation reduced Ca\(^{2+}\)-release-activated Ca\(^{2+}\) (CRAC) entry following activation of metabotropic purinergic receptors.
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1 A fictional cartoon creature created by Al Capp (1909–1979) that is shaped like a plump bowling pin. Similar to a Bobo doll (or knock-down doll), a shmoo always springs back up to a standing position after being knocked over. The term “shmoo” has been incorporated into patch-clamp lingo.
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Publications


Abstract

The Ca\(^{2+}\)-dependent K\(^{+}\) channel, KCa3.1 (KCNN4/IK/SK4), is widely expressed and contributes to cell functions that include volume regulation, migration, membrane potential and electrical excitability. KCa3.1 is now considered a therapeutic target for several diseases, including CNS disorders involving microglial activation; thus we need to understand how KCa3.1 function is regulated. KCa3.1 gating and trafficking require calmodulin binding to the two ends of the CaM-binding domain (CaMBD), which also contains three conserved sites for Ser/Thr kinases. While cAMP protein kinase (PKA) signaling is important in many cells that use KCa3.1, reports of channel regulation by PKA are inconsistent. Here, I first compared regulation by PKA of native rat KCa3.1 channels in microglia (and the microglia cell line, MLS-9) with human KCa3.1 expressed in HEK293 cells. In all three cells, PKA activation with Sp-8-Br-cAMPS decreased the current, and this was prevented by the PKA inhibitor, PKI\(_{14-22}\). Inhibiting PKA with Rp-8-Br-cAMPS increased the current in microglia. Mutating the single PKA site (S334A) in human KCa3.1 abolished the PKA-dependent regulation. CaM-affinity chromatography showed that CaM binding to KCa3.1 was decreased by PKA-dependent phosphorylation of S334, and this regulation was absent in the S334A mutant. Single-channel analysis showed that PKA decreased the open probability in wild-type but not S334A mutant channels. The same decrease in current for native and wild-type (but not S334A) expressed KCa3.1 channels occurred when PKA was activated through the adenosine A2a receptor. Finally, by decreasing the KCa3.1 current, PKA activation reduced Ca\(^{2+}\)-release-activated Ca\(^{2+}\) (CRAC) entry following activation of
metabotropic purinergic receptors.


Abstract

Microglia rapidly respond to CNS injury and disease and can assume a spectrum of activation states. While changes in gene expression and production of inflammatory mediators have been extensively described after classical (LPS-induced) and alternative (IL4-induced) microglial activation, less is known about acquired de-activation in response to IL10. It is important to understand how microglial activation states affect their migration and invasion; crucial functions after injury and in the developing CNS. We reported that LPS-treated rat microglia migrate very poorly, while IL4-treated cells migrate and invade much better. Having discovered that the lamellum of migrating microglia contains a large ring of podosomes - microscopic structures that are thought to mediate adhesion, migration and invasion - we hypothesized that IL4 and IL10 would differentially affect podosome expression, gene induction, migration and invasion. Further, based on the enrichment of the KCa2.3/SK3 Ca$^{2+}$-activated potassium channel in microglial podosomes, we predicted that it regulates migration and invasion. We found both similarities and differences in gene induction by IL4 and IL10 and, while both cytokines increased migration and invasion, only IL10 affected podosome expression. KCa2.3 currents were recorded in microglia under all three activation conditions and KCNN3 (KCa2.3) expression was similar. Surprisingly then, of three KCa2.3 inhibitors (apamin, tamapin, NS8593), only NS8593 abrogated the increased migration and invasion of IL4 and IL10-treated microglia (and invasion of unstimulated microglia). This discrepancy was explained by the
observed block of TRPM7 currents in microglia by NS8593, which occurred under all three activation conditions. A similar inhibition of both migration and invasion was seen with a TRPM7 inhibitor (AA-861) that does not block KCa2.3 channels. Thus, we conclude that TRPM7 (not KCa2.3) contributes to the enhanced ability of microglia to migrate and invade when in anti-inflammatory states. This will be an important consideration in developing TRPM7 inhibitors for treating CNS injury.
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List of abbreviations

1-EBIO – 1-ethylbenzimidazolinone  
A2aR – adenosine 2a receptor  
ATP – adenosine triphosphate  
BDNF – brain-derived neurotrophic factor  
CaM – calmodulin  
CaMBD – calmodulin-binding domain  
cAMP – cyclic adenosine monophosphate  
CHO – Chinese hamster ovary  
CNS – central nervous system  
CRAC – Ca\(^{2+}\)-release-activated Ca\(^{2+}\)  
CSF – colony stimulating factor  
dbAMP – dibutyl cyclic adenosine monophosphate  
EC\(_{50}\) – half maximal effective concentration  
EF hand – helix-loop-helix structural domain  
G\(_i\) – G-protein (inhibitory)  
GPCR – G-protein coupled receptor  
G\(_s\) – G-protein (stimulatory)  
GST – glutathione S-transferase  
HEK – human embryonic kidney  
hKCa3.1 – human intermediate conductance Ca\(^{2+}\)-regulated K\(^+\) channel  
ICH – intracerebral hemorrhage  
IBMX – isobutylmethylxanthine  
IL-4 – interleukin-4  
iNOS – inducible nitric oxide synthase  
KCa2.2 – small conductance Ca\(^{2+}\)-regulated K\(^+\) channel, subtype 2  
KCa2.3 – small conductance Ca\(^{2+}\)-regulated K\(^+\) channel, subtype 3  
KCa3.1 – intermediate conductance Ca\(^{2+}\)-regulated K\(^+\) channel  
K\(_v\) – voltage gated potassium channel  
LPS – lipopolysaccharide  
MAPK – mitogen-activated protein kinase  
MPP\(^+\) – 1-methyl-4-phenylpyridinium  
MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine  
MTMR6 – myotubularin related protein 6  
NO – nitric oxide  
P\(_I\)P3 – phosphatidylinositol 3-phosphate  
PKA – protein kinase A  
PKC – protein kinase C  
PKI – protein kinase inhibitor  
rKCa3.1 – rat intermediate conductance Ca\(^{2+}\)-regulated K\(^+\) channel  
Rp-cAMPS – Rp-8-Br-cAMPS  
Sp-cAMPS – Sp-8-Br-cAMPS  
TRAM-34 – 1-[(2-Chlorophenyl)diphenylmethyl]-1H-pyrazole  
UTP – uridine-5’-triphosphate
1. Introduction

1.1. Microglial activation states and KCa3.1: An overview

Microglia, resident immune cells of the CNS, are major players in CNS immune responses. The true origins of microglia have been a subject of debate for decades, but recent evidence has suggested that these cells arise during early development from progenitors in the embryonic yolk sac that nourish the brain rudiment, and remain there into adulthood (reviewed by Ginhoux et al., 2013). A gradient of activation states of microglia is initiated following nearly any insult to the CNS (Town et al., 2005; Block et al., 2007; Hanisch and Kettenmann, 2007; Perry et al., 2010). Activation states of microglia are important because these cells can have different functional outcomes depending on the state. At one end of the spectrum is the “classical” (pro-inflammatory) activation. This is the fast toxic phase following injury (Colton, 2009), and the activated microglia can contribute to neuron killing; e.g., up-regulation of neurotoxic cellular processes such as iNOS induction and NO production; up-regulation of signaling molecules in pro-inflammatory pathways (Bianca et al., 1999; Melton et al., 2003; Kaushal et al., 2007; Schlichter et al., 2010). At the other end is the “alternative” (anti-inflammatory) activation, which is thought to be the slow repair phase (Martinez et al., 2009). Here, microglia play a beneficial role; e.g., proliferation of microglial cells and their migration to the injury site, where they can phagocytose cellular wastes and antagonize the actions of pro-inflammatory mediators (Hart et al., 1999; Szczepanik et al., 2001; Butovsky et al., 2005; Lively and Schlichter, 2013; Ferreira et al., 2014). The state of the CNS following an insult can be situated at any point between these extremes. My thesis is more concerned with alternative activation (see below and Discussion); here, I examined primary rat microglia that are alternatively activated using
interleukin-4 (IL-4) in order to assess the effects of the cAMP-dependent protein kinase (PKA) on native intermediate-conductance Ca\(^{2+}\)-regulated K\(^{+}\) channels (KCa3.1; SK4).

The KCa3.1 channel was initially studied in red blood cells, but is also expressed in immune cells (Lam and Wulff, 2011), enteric neurons (Vogalis et al., 2003), cardiac and smooth myocytes (Bi et al., 2013; Weisbrod et al., 2013), and some cancer cells (reviewed by Schwab et al., 2007). In the CNS, KCa3.1 has been detected in neurons and astrocytes (Kaushal et al., 2007; Bouhy et al., 2011; Engbers et al., 2011), as well as in microglia (Eder et al., 1997; Khanna et al., 2001; Kaushal et al., 2007; Ferreira et al., 2014). In microglia, our lab has shown that KCa3.1 regulates membrane potential, migration/invasion, and production of inflammatory mediators (Fordyce et al., 2005; Kaushal et al., 2007; Schlichter et al., 2010; Liu et al., 2013; Ferreira et al., 2014). The KCa3.1 channel is a potential therapeutic target in the CNS; KCa3.1 blockers have improved the outcome in animal models of trauma, spinal cord injury, ischemic stroke, MS and Alzheimer’s (Wulff et al., 2003; Beeton et al., 2001; Mauler et al., 2004; Reich et al., 2005; Toyama et al., 2008; Bouhy et al., 2011; Chen et al., 2011; Shineman et al., 2011; and recently reviewed by Maezawa et al., 2012). Because these disorders involve inflammation and microglial activation, KCa3.1 block might improve the outcome by altering the inflammatory state. The Schlichter lab previously found that KCa3.1 blockers inhibit the pro-inflammatory classical activation of microglia, reducing production of reactive oxygen and nitrogen species and their ability to kill neurons \textit{in vitro} and \textit{in vivo} (Kaushal et al., 2007).

The KCa3.1 channel rectifies inwardsly (15–35 pS in symmetrical K\(^{+}\) solutions), and the gating is voltage independent (Mahaut-Smith and Schlichter, 1989; Grissmer et al., 1993; Schlichter et al., 1993). Instead, calmodulin (CaM) regulates the channel’s gating; KCa3.1 requires CaM binding to a region of the C-terminus, usually called the CaM-binding domain.
(CaMBD), which opens the channel (see Fig. 1), and facilitates channel assembly and trafficking to the plasma membrane (Fanger et al., 1999; Khanna et al., 1999; Joiner et al., 2001). A highly conserved protein of 148 amino acids, CaM is shaped like a dumbbell and consists of two lobes (or domains) connected by a linker region (Babu et al., 1985; Babu et al., 1988). Each lobe contains two EF hands (helix-loop-helix motifs), and each binds to a Ca\(^{2+}\) ion (Crivici and Ikura, 1995). So for every molecule of CaM, up to four Ca\(^{2+}\) ions can bind to it. However, the conformational plasticity and flexibility of CaM is well known, and CaM can take on conformations in which Ca\(^{2+}\) can only bind at one lobe (see below).

CaM in the Ca\(^{2+}\)-free state (apo-CaM) is structurally compacted, and the lobes are packed closely together (Cohen and Klee, 1988). However, following Ca\(^{2+}\) binding, CaM changes to an open state conformation, and this in turn causes the lobes to expose the hydrophobic surfaces of the EF-hand helices. Interestingly, although both lobes of CaM are similar in sequence (Park et al., 2007), the lobe at the C terminus (C-lobe) binds Ca\(^{2+}\) ~10 fold more tightly (K\(_d\) ~0.2 \(\mu\)M) compared to the N-lobe (K\(_d\) ~2 \(\mu\)M) (Potter et al., 1983). Because the K\(_d\) of Ca\(^{2+}\) binding to CaM is affected by cooperativity, when the C-lobe of CaM is Ca\(^{2+}\)-loaded, the K\(_d\) for Ca\(^{2+}\) at the N-lobe becomes <1 \(\mu\)M (Ogawa, 1989). Nuclear magnetic resonance spectroscopy showed that the central linker region of CaM with bound Ca\(^{2+}\) (Ca\(^{2+}\)-CaM) displays considerable flexibility, which enables the lobes to freely move (Barbato et al., 1992). And so, by engaging its C- and N-lobes in a differential manner (Persechini and Kretsinger, 1988; Vetter and Leclerc, 2003), CaM can interact with its targets, such as the KCa3.1 channel, in a variety of ways.

Extensive crystal structure studies have examined the related small-conductance Ca\(^{2+}\)-regulated K\(^+\) channel, KCa2.2; and specifically the isoforms, KCa2.2a and KCa2.2b (Schumacher et al., 2001; Schumacher et al., 2004; Zhang et al., 2012). The structure of
Figure 1. CaM binding to KCa3.1 is required for channel gating. Schematic diagram depicting KCa3.1 in the closed state when CaM is Ca$^{2+}$-free ("Apo-CaM"; left) and the opened state when CaM is Ca$^{2+}$-bound ("Ca$^{2+}$-CaM"; right). Note the role of CaM in crosslinking the monomers of KCa3.1 when the channel is opened. S6 refers to the sixth transmembrane domain of KCa3.1.
complexes of Ca\(^{2+}\)-free-CaM (apo-CaM) and Ca\(^{2+}\)-CaM with the CaMBD of KCa2.2a can provide insight into the CaM-dependent gating of this family of channels. Under low Ca\(^{2+}\) conditions, just the C-lobe of CaM is bound to KCa2.2a, and the channel is closed. When intracellular Ca\(^{2+}\) binds to the N-lobe of CaM, dimerization between the channel’s C termini occurs, which results in crosslinking between the monomers, and the channel opens (Schumacher et al., 2004). Interestingly, our lab was the first to show this mechanism, using KCa3.1 channels (Joiner et al., 2001). Although Schumacher et al. (2004) showed with GST proteins of the KCa2.2 CaMBD that Ca\(^{2+}\)-CaM crosslinks between monomers of the channel’s subunits rather than bridge the same monomer, presently, it is unknown why this is the case. One possible explanation is that the CaMBD is in a conformation in which the residues that the N-lobe of CaM binds to are not accessible to the CaM already bound to it.

CaM has extreme plasticity as well as the potential to adjust its interactions with KCa channels. For example, the KCa2.2b isoform, which differs from KCa2.2a by only three residues (A463, R464, L465), is significantly less sensitive to Ca\(^{2+}\) because CaM is in a different conformation. That is, when CaM is in a complex with KCa2.2a, because the C-lobe adapts a conformation in which Ca\(^{2+}\) cannot bind, Ca\(^{2+}\) only needs to bind to the N-lobe to induce a conformation change in CaM, which opens the KCa2.2a channel (see Fig. 2). However, for the CaM-KCa2.2b complex, both lobes of CaM must be Ca\(^{2+}\)-loaded (Zhang et al., 2012). Nevertheless, the KCa2.2a and KCa2.2b isoforms are functionally similar; both isoforms formed dimers at high Ca\(^{2+}\), and monomers at low Ca\(^{2+}\) (Schumacher et al., 2004; Zhang et al., 2012).

It should be noted that despite its name, CaM is not selective for Ca\(^{2+}\). In fact, any divalent cations and even some heavy metals can potentially compete with Ca\(^{2+}\) for the activation site on CaM, although the outcome can vary. Some metal cations, such as La\(^{3+}\), Tb\(^{3+}\), Pb\(^{2+}\) or
Figure 2. Plasticity of CaM. Schematic diagram depicting KCa2.2a (top) and KCa2.2b (bottom), in the closed (left) and opened (right) states. When CaM (purple) is in a complex with KCa2.2a, CaM’s C-lobe adapts a conformation in which Ca\(^{2+}\) cannot bind. However, when CaM (green) is in a complex with KCa2.2b, which only differs from KCa2.2a by three residues, both the N- and C-lobes of CaM must be Ca\(^{2+}\)-loaded. As a result, KCa2.2b is much less sensitive to Ca\(^{2+}\) than KCa2.2a. In the opened state, the monomers of both isoforms of KCa2.2 form dimers due to cross-linkage by CaM, similar to KCa3.1 channels. S6 refers to the sixth transmembrane domain of the channel.
Cd\(^{2+}\), potentiate CaM activity, i.e. induce a conformational change of CaM (Mills and Johnson, 1985), and in the case with KCa3.1, the channel can be opened (Morales et al., 2013). In the Schlichter lab, a PhD student (Doris Lam) has shown that extracellular application of Ba\(^{2+}\) in high concentrations (>1 mM) can also activate KCa3.1 currents in mouse microglia (unpublished observations). Note that bath applied Ba\(^{2+}\) can enter into the cell through non-selective cation channels such as the transient receptor potential cation channels, subfamily M, member 7 (TRPM7) (Penner and Fleig, 2007), which our lab has shown to be expressed and functional in microglia (Jiang et al., 2003; Siddiqui et al., 2014). On the other hand, a high concentration of Mg\(^{2+}\) (>5 mM) was reported to reduce the open probability of the channel (Stoneking and Mason, 2014). This finding is consistent with that of Crouch and Klee (1980); they found the K\(_d\) of CaM binding to Ca\(^{2+}\) to be 3–20 \(\mu\)M, but in the presence of 3 mM Mg\(^{2+}\), the K\(_d\) for Ca\(^{2+}\) increased (5–40 \(\mu\)M). Hg\(^{2+}\) and Cu\(^{2+}\) have also been shown to inactivate CaM (Mills and Johnson, 1985). Because all the cations mentioned above are competitive inhibitors, they bind directly to the active sites on CaM rather than attaching allosterically. Therefore, under physiological conditions, Ca\(^{2+}\) can out-compete them, especially when intracellular Ca\(^{2+}\) is raised (Park et al., 2007). In microglia, cytosolic Ca\(^{2+}\) is elevated following classical activation (Kaushal et al., 2007; Schlichter et al., 2010; Mizoguchi et al., 2014). Because active microglia can exacerbate CNS damage if they are at the wrong place or time, or directed against the wrong targets, Ca\(^{2+}\) is tightly regulated. KCa3.1 contributes to the driving force for Ca\(^{2+}\) entry through calcium release-activated calcium (CRAC) channels (Ohana et al., 2009). This leads to a positive feedback mechanism such that the entering Ca\(^{2+}\) can activate more KCa channels, and the K\(^{+}\) efflux would maintain a negative membrane potential favorable for Ca\(^{2+}\) influx.
Previously, our lab found that the EC$_{50}$ for activation of KCa3.1 in the microglial cell line, MLS-9, is ~7 µM free Ca$^{2+}$ (Ferreira and Schlichter, 2013), which is much higher than the 200–800 nM range reported for other cell types in the literature (Jensen et al., 2001; Pedarzani et al., 2008; Wulff and Zhorov, 2008). Because there is a report of the existence of a dominant-negative splice variant of KCa3.1 in T lymphocytes (Ohya et al., 2011), to determine whether microglia also express this isoform, our lab had the KCa3.1 channels from primary rat microglia sequenced (Vector Core; UHN). However, the results indicated that microglia only expressed the “normal” functional isoform, and this sequence was consistent with what was reported in the literature for other cell types (Vandorpe et al., 1998; Ohya et al., 2011; Lamberts et al., 2012). And so, this did not account for the Ca$^{2+}$-insensitivity of the KCa3.1 channels in microglia.

Besides variations in the conformation of CaM (see above), a likely explanation is the difference in regulation of either KCa3.1 itself, or its associated subunits. However, not much is known about accessory molecules involved in KCa3.1 regulation (see below). Interestingly, although my lab mates and I have activated KCa3.1 currents in MLS-9 cells with positive gating modulators, including riluzole (Liu et al., 2013), 1-EBIO (present study), or NS309 (Ferreira et al., 2014); until recently, neither we nor others could readily activate KCa3.1 currents in primary rat microglia. Methods that activated KCa3.1 in MLS-9 cells, i.e. the activators listed above, or UTP, or 10 µM free intracellular Ca$^{2+}$ (Ferreira and Schlichter, 2013), failed to activate the currents in resting primary microglia. As the molecular differences between MLS-9 cells and primary microglia are unknown, I have two hypotheses to address this discrepancy. The first is that the MLS-9 cells lack inhibitory mechanisms that are present in the primary cells. For instance, given the high concentration of Ca$^{2+}$ needed to activate the current, I hypothesized that there was an inhibitory molecule attached to KCa3.1, and that only with this molecule’s removal...
would the channel activate. This brought my attention to calpain, a ubiquitous Ca\textsuperscript{2+}-dependent, non-lysosomal cysteine protease. Since the EC\textsubscript{50} for Ca\textsuperscript{2+}-dependent activation of calpain (type-1) is the same as KCa3.1 in MLS-9 cells (Kopil et al., 2011), calpain activation might potentially remove this inhibitory molecule from KCa3.1, subsequently allowing KCa3.1 to activate. In MLS-9 cells, I rationalized that if calpain was inhibited, even 10 \(\mu\text{M}\) of free intracellular Ca\textsuperscript{2+} would fail to activate KCa3.1 (note: this is the case in primary microglia). However, my results showed that treating MLS-9 cells with the calpain inhibitor V had no effect; i.e., KCa3.1 currents were activated with 10 \(\mu\text{M}\) of intracellular free Ca\textsuperscript{2+} even with calpain inhibition (data not shown). My second hypothesis was that perhaps the MLS-9 cells contained certain factors or signaling pathways not present in the endogenous primary microglia. Because MLS-9 cells were created by treating primary microglia with CSF-1 (Cayabyab, 2002), it was possible that this growth factor can play a role in activating the channels in primary cells.

Nevertheless, the Schlichter lab recently discovered that when primary rat microglia received long-term (\(>24\text{ hr}\)) treatment of interleukin-4 (IL-4; 20 ng/ml), KCa3.1 currents in primary cells could also be evoked by the methods listed above that activated the KCa3.1 current in MLS-9 cells (Ferreira et al., 2014; lab observations not shown). And so, in my thesis, I examined microglia that are alternatively activated by IL-4. In the CNS, IL-4 is an immunosuppressive cytokine that has been linked to neuroprotection; in IL-4 knock-out mice, there is an increase in lesion size after stroke as well as more disease progression in the EAE mouse model (Xiong et al., 2010; Ponomarev et al., 2007, Zhao et al., 2006). In microglia, activation by IL-4 induced oligodendrogenesis and neurogenesis (Butovsky et al., 2006), and pretreatment of microglia with IL-4 before exposure to lipopolysaccharide (LPS; a strong inducer of classical activation) prevented neuronal cell injury (Chao et al., 1993). The
mechanism by which IL-4 exerts its neuroprotective effect has been proposed to decrease the production of the pro-inflammatory nitric oxide (NO) and tumour necrosis factor alpha (TNF-α) in microglia (Chao et al., 1993). As benefits of IL-4 were inhibited by antagonizing IL-4 receptors (Suzumura et al., 1994), the effects of IL-4 are through its receptors. Interestingly, there is a convergence between the IL-4 signaling pathway and the cAMP/PKA pathway. That is, both IL-4 and cAMP/PKA similarly inhibited oxidative stress in rat microglia and neurons (Savchenko, 2013), and the neuroprotective effect of IL-4 was shown to be through PKA in rat photoreceptor cells (Adao-Novaes, 2009).

As mentioned above, the block of KCa3.1 in rat microglia with TRAM-34 reduced p38 MAPK and classical activation by LPS (Kaushal et al., 2007), so it can be implied that KCa3.1 activation is pro-inflammatory. However, IL-4 stimulation up-regulated KCa3.1 mRNA expression and allowed robust KCa3.1 current activation by positive gating modulators (e.g., 1-EBIO, NS309), which had no effect in resting primary rat microglia (Ferreira et al., 2014). Because IL-4 is thought to confer an anti-inflammatory phenotype in microglia, KCa3.1 activation would seem to contribute to a neuroprotective response. If so, a dual role for KCa3.1 in microglial activation illustrates the concept that the cell’s activation state can determine whether a channel takes on a pro- or anti-inflammatory role.

1.2. KCa3.1 regulation by PKA: Is there a consensus?

Given the broad contributions of KCa3.1 to cell functions, it is important to understand how the channel is regulated. Presently, the literature has reports of four accessory molecules associated with KCa3.1. (1) Myotubularin related protein 6 (MTMR6) inhibits KCa3.1 function (Srivastava et al., 2005). MTMR6 is a lipid PI₃P phosphatase, and this finding suggested that phosphorylated PI₃P near the channel helps maintain functional KCa3.1. (2) AMP-activated protein kinase had
Figure 3. Sequence of KCa3.1. Schematic diagram showing the sequence of a human KCa3.1 channel subunit. S1–S6 labels the transmembrane domains. The section in blue represents the calmodulin-binding domain (CaMBD). Within the CaMBD, the four labeled residues are the nucleoside diphosphate kinase B (NDKB) site, and the three conserved, putative phosphorylation sites, where the PKA site (S334) is highlighted in yellow.
been shown to bind directly to the distal C terminus of KCa3.1 and to inhibit KCa3.1 current (Klein et al., 2009). (3) Nucleoside diphosphate kinase B and (4) protein histidine phosphatase phosphorylate and dephosphorylate H375 of KCa3.1 respectively. This pair was suggested to act as a switch for KCa3.1; i.e., the channel turns on when phosphorylated, and off when dephosphorylated (Srivastava et al., 2006; Srivastava et al., 2008). The known regulatory kinases and phosphatases support the importance of post-translational regulation of KCa3.1.

Within the CaMBD of KCa3.1, there are three putative phosphorylation sites that are conserved across mouse, rat and human KCa3.1 (see Fig. 3); a PKA site (rat S332; human S334), a PKC site (rat T329), and a casein kinase II site (rat S365). Emphasis was on the CaMBD because of the vital role CaM plays in KCa3.1 gating and trafficking (above). PKA-dependent regulation of KCa3.1 is of interest in the CNS and specifically in microglia. While cAMP is ubiquitous in cells, its expression is strongly up-regulated in the injured CNS (Pearse et al., 2004; Spencer and Filbin, 2004). In the inactive state, PKA is a homodimer composed of a pair each of regulatory and catalytic subunits. Following cytosolic cAMP increase, two cAMP molecules bind to each regulatory subunit. The regulatory subunits then undergo a conformational change and the two catalytic subunits are released (see Fig. 4). The active sites on the catalytic subunits are now exposed and can interact with target proteins to phosphorylate Ser or Thr residues (in-depth kinetic and catalytic mechanisms reviewed by Adams, 2001). The Schlichter lab previously found that PKA regulates the amplitude and voltage-dependence of Kv1.3 channels (Chung and Schlichter, 1997), which contribute to microglia proliferation (Schlichter et al., 1996) and their neurotoxic capacity (Fordyce et al., 2005). However, our lab did not examine whether PKA regulates the KCa3.1 channel in microglia, and previous reports from other labs were inconsistent. PKA regulation studies have provided conflicting results for both native and
Figure 4. Mechanism of PKA activation. Schematic diagram depicting the activation of PKA by cAMP. Note that the homodimer of PKA is made up of two regulatory subunits and two catalytic subunits.
cloned KCa3.1 channels. For rat or human KCa3.1 expressed in *Xenopus* oocytes, PKA activation increased the current (Gerlach et al., 2000), decreased it (Neylon et al., 2004) or had variable effects (von Hahn et al., 2001). In HEK293 cells transfected with human KCa3.1, PKA activation had no effect (Schroder et al., 2000). For native KCa3.1 currents, PKA activation inhibited the current in NIH-3T3 fibroblasts (Choi et al., 2012) and activated human T lymphocytes (Chimote et al., 2013) but increased the current in human erythrocytes (Pellegrino and Pellegrini, 1998), rat acinar cells (Hayashi et al., 2004) and T84 epithelial cells (Gerlach et al., 2000). This inconsistency could be due to limitations of experimental design; e.g., using the *Xenopus* oocyte model, and applying forskolin. The *Xenopus* oocyte is an amphibian progenitor cell that might poorly represent mammalian CNS cells. Because the effect of PKA is cell-dependent (see Discussion); these results must be interpreted cautiously. The use of forskolin also has limitations. It activates adenylyl cyclase, which catalyzes the conversion of ATP to cAMP; however, this is often rapidly reversed by phosphodiesterases. Because cAMP is required for the catalytic subunit of PKA to remain active, cAMP degradation would reduce PKA activity. (Schaap et al., 1993)

I was also particularly intrigued by PKA because it phosphorylated the S332 site (a putative PKA site) of a CaMBD fusion protein constructed from rat KCa3.1 (Neylon et al., 2004). This group constructed five mutant GST fusion proteins of the CaMBD of KCa3.1. Four of them had a single mutation from Ser/Thr to Ala at each of the phosphorylation sites, and one had a simultaneous mutation of all four sites. Incubating each isolated construct with PKA and \[^{33}P\]-ATP showed that (compared to the wild-type fusion protein), mutation of just the 332 site decreased phosphorylation as much as the quadruple mutation. Single mutations of the other sites had no effect. This suggested that the 332 site plays a major role in determining the
phosphorylation state of the channel. However, the authors did not conduct electrophysiological studies, so it was not known whether the 332 site has an actual functional role. Thus, although there is evidence that PKA phosphorylates an integral segment of KCa3.1 (the CaMBD); there is no consensus on whether phosphorylation confers a functional effect on KCa3.1.

1.3. Objective of thesis

Given the importance of PKA and KCa3.1 in the CNS immune system, I examined whether PKA regulates KCa3.1 channel activity, and if so, whether the 334 site is responsible for this regulation. Specifically, PKA-dependent regulation of native rat KCa3.1 (in microglia) and cloned human KCa3.1 channels was compared. I used several means to activate and inhibit PKA, used mutational analysis to study the role of S334 (human isoform), examined CaM binding to wild-type and mutant channels, assessed single-channel activity, and determined effects of this KCa3.1 regulation on Ca$^{2+}$ signaling in alternative-activated microglia.
2. Materials and Methods

2.1. Cells. Primary cultured rat microglia and MLS-9 cells (rat microglia cell line) were used to study native channels, while transfected HEK293 cells were used to study wild-type and mutated human KCa3.1 channels.

2.1.1. Primary rat microglia. Cultures of essentially pure microglias were prepared according to the Schlichter lab’s standard protocols (Kaushal et al., 2007; Sivagnanam et al., 2010; Lively and Schlichter, 2013). In brief, the brains of 1–2 day old Sprague-Dawley pups of either sex (Charles River, St. Constant, Quebec, Canada) were harvested, and the brains were minced in cold minimum essential medium (MEM) (Invitrogen, Burlington, ON, Canada) after removing the meninges.

The dissociated tissue was then centrifuged (300g, 10 min) and re-suspended in MEM with 10% fetal bovine serum (FBS) (Wisent, St. Bruno, PQ) and 0.05 mg/mL gentamycin (Invitrogen). Medium was replaced after two days of growth to remove cellular debris and non-adherent cells. After six more days of growth, flasks with the mixed cell cultures were shaken on an orbital shaker (65 rpm, 4–5 h, 37°C, 5% CO₂). The supernatant, containing non-adherent microglia was centrifuged (300g, 10 min), and the cells were re-suspended in MEM with 2% FBS. Microglia were plated at 6×10⁴ cells/coverslip for 24 h and then exposed to 20 ng/mL rat recombinant interleukin-4 (IL-4; R&D Systems Inc., Minneapolis, MN, USA) for six days (37°C, 5% CO₂) before patch-clamp analysis. Our lab has previously shown that this treatment shifts rat microglia from non-activated (Sivagnanam et al., 2010) to an alternative-activated state (Liu et al., 2013; Lively and Schlichter, 2013).

2.1.2. The rat microglia cell line (MLS-9). Many years ago, the Schlichter lab derived the MLS-9 cell line by treating rat microglia cultures harvested from pups of either sex for
several weeks with colony stimulating factor-1. Our lab has used MLS-9 cells extensively to study K⁺ and Cl⁻ channels (Cayabyab et al., 2000; Cayabyab and Schlichter, 2002; Cayabyab et al., 2002; Ducharme et al., 2007; Schlichter et al., 2010; Ferreira and Schlichter, 2013; Liu et al., 2013). MLS-9 cells were cultured (37°C, 5% CO₂) for several days in MEM with 10% FBS and 0.05 mg/mL gentamycin. They were harvested in phosphate buffered saline (PBS) with 0.25% trypsin and 1 mM EDTA, washed with MEM, centrifuged (300g, 10 min), and re-suspended in MEM. Cells were plated at 4.5×10⁴ cells/coverslip for patch-clamp analysis.

2.1.3. HEK293 cells and transfection. Although initially thought to be of fibroblastic, endothelial or epithelial origin, HEK (human embryonic kidney) cells actually have a neuronal lineage (Shaw et al., 2002). In my study, HEK293 cells were grown for several days in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) with high glucose, 10% FBS, 100 mg/L penicillin-streptomycin (Invitrogen). They were harvested in phosphate buffered saline (PBS) with 0.25% trypsin and 1 mM EDTA, washed with MEM, centrifuged (300g, 10 min), and re-suspended in MEM. Cells were plated at 5.5×10⁴ cells/coverslip for patch-clamp analysis. The human KCa3.1 gene (hKCa3.1) was subcloned into the expression vector, pCMV6-XL5 (OriGene, Rockville, MD). The plasmids, pCMV6-XL5-hKCa3.1 and pEF-GFP, were co-transfected into HEK293 cells using LipofectAMINE (Invitrogen) for 36 h according to the manufacturer’s protocol. For site-directed mutagenesis, Ser³³⁴ was mutated to alanine (S334A) using the QuikChange protocol (Agilent Technologies; Santa Clara, CA, USA) forward primer, CATACTCGCAGGAAGGAGGCGCATGCTGCCGCAGGCAT; reverse primer, CCTGCGGGCAGCAGCGCCTCCTTCCGCGAGGTAGT; the underlined letters indicate the Ser 334 point mutation. The mutated construct was sequenced (ABI 3100, University Health Network) and aligned with hKCa3.1 accession number NM002250.2 (BLAST, NCBI).
2.2. Patch-clamp electrophysiology. Whole-cell recordings of KCa3.1 currents were performed on primary cultured rat microglia and MLS-9 cells expressing native channels, and HEK293 cells transfected with wild-type or mutated human KCa3.1. Single-channel recordings were performed on transfected HEK293 cells. To isolate KCa3.1 from other KCa currents, apamin (100 nM; KCa2.1–2.3 blocker) was always present. Also, at the end of each recording, the KCa3.1 specific potent blocker TRAM-34 (1 μM) was added to verify current identity. In whole cell recordings, the KCa3.1 current amplitude was quantified as the TRAM-34 blocked component. In primary microglia and HEK293 cells, a holding potential of 0 mV was used to inactivate voltage-gated K+ channels. Additionally, in whole cell recordings, the KCa3.1 currents evoked by a step and ramp voltage protocol (i.e. +50 mV step, ramp from -100 to +80 mV) displayed voltage- and time-independent gating (shown by the step protocol) and reversal at –84 mV, the Nernst potential for K+ (shown by the ramp protocol). Capacity transients were cancelled, and series resistance was compensated (~80%) as necessary.

2.2.1. Whole cell recordings. Recordings were conducted at room temperature, with an extracellular (bath) solution containing the following (in mM): 125 NaCl, 5 KCl, 1 MgCl₂, 1 CaCl₂, 5 glucose, 10 HEPES, pH 7.4 (adjusted with NaOH), adjusted to ~300 mOsm with sucrose. Unless otherwise specified, the intracellular (pipette) solution contained (in mM): 100 K-aspartate, 40 KCl, 1 MgCl₂, 0.85 CaCl₂, 1 EGTA, 2 MgATP, 10 HEPES, pH 7.2 (adjusted with KOH), 280 mOsm. The internal free Ca²⁺ concentration was 1.1 μM. To obtain 10.9 μM free Ca²⁺, CaCl₂ was 9.85 mM and EGTA was 10 mM. WEB-MAXC Extended software (http://www.stanford.edu/~cpatton/webmaxc/webmaxcE.htm; Stanford University) was used to calculate free Ca²⁺ concentrations. A gravity-driven perfusion system flowing at 1.5–2 ml/min was used to exchange bath solutions. All recordings were made at room temperature. The
recording pipettes (8–12 MΩ) were pulled from thin-walled borosilicate glass (WPI, Sarasota, FL) using a Narishige puller (Narishige Scientific, Setagaya-Ku, Tokyo). Recordings were made with an Axopatch 200A amplifier (Molecular Devices, Sunnyvale, CA), digitized with a DigiDATA 1322A board, filtered at 5 kHz and sampled at 10 kHz. Junction potentials (reduced using agar bridges made with bath solution) were calculated with the pCLAMP utility, and after correction, all voltages were ~5 mV more negative than shown in the figures.

2.2.2. Inside-out patch configuration. Inside-out recordings were performed on HEK293 cells transfected with wild-type (wt) or S334A hKCa3.1. Using the same equipment and software as above, recordings were performed at room temperature, sampled at 5 kHz, and low-pass filtered at 1 kHz (−3 dB cut-off frequency). Recording pipettes (6–8 MΩ) pulled from thin-walled borosilicate glass were filled with an extracellular solution containing (in mM): 145 KCl, 1 MgCl₂, 1 CaCl₂, 5 HEPES; pH 7.4 (adjusted with KOH), adjusted to ~310 mOsm with sucrose. The bath solution contained (in mM): 145 KCl, 1 MgCl₂, 1.05 CaCl₂, 1 EGTA, 1 MgATP, 5 HEPES, 5 glucose; pH 7.2 (adjusted with KOH), 310 mOsm. The free Ca²⁺ concentration was 10 µM. NP₀, the product of the apparent number of active channels in the patch (N) and the KCa3.1 channel open probability (P₀) was calculated in pClamp by dividing the mean total current (I) by the single-channel current amplitude (i), where NP₀ = I/i. The single-channel current was determined from the best Gaussian fit to the single-channel event amplitude histogram. At the end of each recording, the single-channel currents were blocked by 1 µM TRAM-34.

2.3. Calmodulin affinity chromatography. Wild-type (wt) or S334A hKCa3.1 was subcloned into the expression vector, pCMV6-Entry which contains a C-terminal Myc-DDK tag (Origene).
HEK293 cells were transiently transfected (36 h) with the tagged wt or S334A hKCa3.1 using LipofectAMINE (as above), and then protein was harvested. To evaluate binding of hKCa3.1 protein to calmodulin-Sepharose 4B, I used a method modified from (Khanna et al., 1999). In brief, the dishes were washed twice in PBS containing Ca\textsuperscript{2+} and Mg\textsuperscript{2+}, and solubilization buffer was added (Bio-Rad, Berkeley, CA, USA), which contained 50 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, 1% Triton X-114 (pH 7.4). This buffer also contained a protease inhibitor cocktail (1:100 P8340; Sigma-Aldrich) with leupeptin, aprotinin, pepstatin A, bestatin, AEBSF, and E-64, and a phosphatase inhibitor cocktail (2 µg/mL PIC3; Sigma-Aldrich) that contained cantharidin, p-bromolevamisole oxalate, and calyculin A. Insoluble material was removed by centrifugation (300g, 10 min). For protein purification and CaM binding, the supernatant was warmed to 37°C for 3 min, and then centrifuged (300g, 5 min). The detergent phase (bottom) was diluted 9:1 in a binding buffer (Santa Cruz Biotechnology, Dallas, TX, USA) containing 50 mM Tris, 150 mM NaCl, 1 mM MgCl\textsubscript{2}, 0.5 mM CaCl\textsubscript{2}, pH 7.4. Total protein samples were first pre-cleared (1 h, 4°C) with 50 µL of Sepharose 4B (Sigma-Aldrich). The protein was then added to a 20% slurry of equilibrated calmodulin-conjugated agarose beads (Sigma-Aldrich) according to the Sepharose 4B protocol, and incubated overnight on a rotator. The beads were pelleted by centrifugation (10 s) and washed with binding buffer. Proteins were dissociated from the calmodulin-conjugated agarose beads by boiling in Laemmli buffer (Santa Cruz Biotechnology). Total protein and calmodulin-bound protein were analyzed by Western immunoblotting using an anti-DDK antibody (1:1000; Sigma-Aldrich) coupled to an avidin-horseradish peroxidase secondary antibody (1:3000; Cedarlane Labs, Burlington, ON, CA). As a loading control, GAPDH was analyzed using an anti-GAPDH antibody (1:5000; Cedarlane Labs). An interaction between GAPDH and CaM has been documented (Christova et al., 1996), and
GAPDH is present following CaM pull down (Wang et al., 2013).

2.4. Intracellular free Ca\(^{2+}\). The Fura-2 imaging methods were the same as previously described (Ferreira and Schlichter, 2013). Specifically, primary rat microglia growing on glass coverslips (~6\(\times\)10\(^4\) cells per 15 mm diameter coverslip) were incubated at room temperature with 3.5 µg/ml Fura-2AM (Invitrogen) for 40 min in the dark. For recording, a coverslip was mounted in a 300 µl volume perfusion chamber (Model RC-25, Warner Instruments, Hamden CT) that contained the same bath solution as for whole-cell recordings (see above). The effects of different treatments on UTP-evoked Ca\(^{2+}\) signals were assessed on different batches of cells from separate coverslips. Images were acquired at room temperature using a Nikon Diaphot inverted microscope, Retiga-EX camera (Q-Imaging, Burnaby, BC, Canada), and Northern Eclipse image acquisition software (Empix Imaging, Mississauga, ON, Canada). A Lambda DG-4 Ultra High Speed Wavelength Switcher (Sutter Instruments, Novato, CA) was used to alternately acquire images at 340 and 380 nm excitation wavelengths. Images were acquired every 4 s, and the excitation shutter was closed between acquisitions to prevent photobleaching. The intracellular free Ca\(^{2+}\) concentration was calculated from the standard equation (Gryniewicz et al., 1985).

2.5. Chemicals. The PKA activator, Sp-8-Br-cAMPS, and the PKA inhibitor, Rp-8-Br-cAMPS, were purchased from EMD Millipore (Billerica, MA). The adenosine A2 receptor agonist, CGS21680, was from R&D Systems (Minneapolis, MN). The catalytic subunit of human recombinant cAMP-dependent protein kinase (csPKA) was from Sigma-Aldrich (Ellisville, MO). All other chemicals, unless specified, were from Sigma-Aldrich.
2.6. Statistical analysis. Data are expressed as mean ± SEM. For multiple comparisons to assess treatment effects on currents, 1-way ANOVA and Tukey’s post hoc tests were used. Paired Student’s t tests were used to compare before and after drug application. Analyses were conducted using GraphPad Prism ver 6.01 (GraphPad Software, San Diego, CA), and statistical significance was determined at $p<0.05$. 
3. Results

3.1. PKA inhibits the native KCa3.1 current in the MLS-9 microglia cell line

First, I used the rat MLS-9 microglia cell line because it expresses a robust KCa3.1 current and it lacks the Kir2.1 and Kv1.3 currents (Ferreira and Schlichter, 2013; Liu et al., 2013) that make it more difficult to isolate KCa3.1 in primary rat microglia. However, MLS-9 cells have both KCa3.1 and KCa2.3 (SK3) currents that are readily activated (e.g., by riluzole; Liu et al., 2013) in whole-cell recordings with 1 µM intracellular Ca$^{2+}$. Therefore, I added 100 nM apamin to the bath to block SK3 for all recordings from MLS-9 cells. As the Schlichter lab previously showed (Ferreira and Schlichter, 2013; Liu et al., 2013), establishing a whole-cell recording with 1.1 µM free intracellular Ca$^{2+}$ was not sufficient to activate a KCa3.1 current in MLS-9 cells (Fig. 6A). However, in response to the positive gating modulator, 1-EBIO, a robust KCa3.1 current was activated. Like riluzole, 1-EBIO is considered to act by increasing the Ca$^{2+}$ sensitivity of the channel by as much as an order of magnitude (Pedersen et al., 1999; Syme et al., 2000; Pedarzani et al., 2001). I next quantified the KCa3.1 current amplitude as the component that was blocked by 1 µM TRAM-34; essentially no current remained after TRAM-34 addition. As expected for KCa3.1, the currents evoked by a step and ramp voltage protocol show voltage-independent gating and reversal at close to the Nernst potential for K$^+$ (−84 mV with the solutions used).

To test the effect of increasing PKA activity, MLS-9 cells were treated with the PKA activator, Sp-8-Br-cAMPS (‘Sp-cAMPS’), which is a membrane-permeant cyclic AMP analogue that is resistant to degradation by phosphodiesterases. The mechanism of action for this drug is identical to cAMP; however, it will remain whilst cAMP is broken down (see Fig. 5). Thus, it is expected that a high level of active catalytic subunits of PKA will be maintained in the cytoplasm. Conversely, effects of inhibiting PKA were examined using Rp-8-Br-cAMPS (‘Rp-
Figure 5. Mechanism of action of the PKA activator, Sp-cAMPS, and the PKA inhibitor, Rp-cAMPS. Schematic diagram depicting the activation of PKA by Sp-cAMPS, and inhibition of PKA by Rp-cAMPS. Both Sp- and Rp-cAMPS are hydrolysis-resistant membrane-permeant analogues of cAMP. They are distinguished by their absolute configurations, which differ at one chiral center.
cAMPS’). While this diastereomer also binds to the cAMP site on the regulatory subunit, it does not cause the requisite conformational change (see Fig. 5), and thus acts as a specific, competitive PKA inhibitor (Dostmann et al., 1990). Like Sp-cAMPS, Rp-cAMPS also has a long half-life. MLS-9 cells were pre-incubated (37°C; 30–45 min) with either 10 μM Sp-cAMPS or 10 μM Rp-cAMPS, and then patch-clamp recordings were carried out, as in Fig. 6A. Before adding the channel activator, 1-EBIO, no current was seen in cells treated with either Sp-cAMPS or Rp-cAMPS (Fig. 6B). Thus, PKA activation or inhibition alone did not activate a KCa3.1 current when free intracellular Ca\(^{2+}\) was 1.1 μM. As observed for untreated cells, 1-EBIO activated a KCa3.1 current in cells pre-treated with either Sp-cAMPS or Rp-cAMPS. In control cells, the mean current density of the TRAM-34-sensitive component (essentially all of the current) was 25.7±0.8 pA/pF at +80 mV (n=32) (Fig. 6C). Activating PKA with Sp-cAMPS reduced the current ~70% (to 7.3±0.7 pA/pF; n=14; p<0.0001); while inhibiting PKA with Rp-cAMPS increased it ~40% (to 36.5±0.9 pA/pF; n=19; p<0.0001). This potentiation by Rp-cAMPS implies a basal level of phosphorylation and activity of endogenous phosphatases, such that after inhibiting PKA, phosphorylation was reduced. From these data, I cannot say whether phosphorylation was on the channel itself (but see below).

I confirmed that 1-EBIO activated KCa3.1 by increasing the Ca\(^{2+}\) sensitivity about 10-fold. That is, with 10.9 μM intracellular free Ca\(^{2+}\) alone, the KCa3.1 current (TRAM-34-sensitive component) was 23.1±3.8 pA/pF (n=8), which is not different from the current activated by 1-EBIO with 1.1 μM Ca\(^{2+}\) (25.7±0.8 pA/pF; n=32; p=0.66). Furthermore, Sp-cAMPS similarly decreased the KCa3.1 current when it was activated by 10.9 μM Ca\(^{2+}\) alone: to 10.7±2.2 pA/pF (n=5; p<0.01). The observation that the KCa3.1 channels in microglia have a low intrinsic Ca\(^{2+}\) sensitivity is consistent with the Schlichter lab’s previous finding that the EC\(_{50}\) for Ca\(^{2+}\)-
Figure 6. Regulation of KCa3.1 by PKA in MLS-9 cells. The voltage protocol, which applies to all current traces for MLS-9 cells, was: holding potential, –70 mV; step to +50 mV; ramp from –100 to +80 mV. The KCa2.1–2.3 blocker, 100 nM apamin was present in all experiments. A. Representative current traces from a control cell after break in with 1.1 µM free Ca\(^{2+}\) (trace marked ‘ctrl’), followed by bath addition of the channel activator, 300 µM 1-EBIO (‘EBIO’), or 1-EBIO + 1 µM TRAM-34 (‘TRAM’). B. Representative current traces from separate control (ctrl) cells without 1-EBIO, and for treated cells in the presence of 1-EBIO; i.e., after 30–45 min incubation at 37°C with the PKA activator, 10 µM Sp-cAMPS (‘Sp’) or the PKA inhibitor, 10 µM Rp-cAMPS (‘Rp’). C. Summarized data from a population study with treatments as in panels A and B (first 4 bars). In each cell, 300 µM of 1-EBIO was used to activate the KCa3.1 current, and the amplitude was measured as the component blocked by the selective KCa3.1 blocker, 1 µM TRAM-34. For the two right-hand bars, 10.9 µM intracellular free Ca\(^{2+}\) alone was used to activate the KCa3.1 current and to compare the effect of 30–45 min incubation with 10 µM Sp-cAMPS. Data are expressed as mean±SEM for the number of cells indicated on each bar, and the three conditions were compared using 1-way ANOVA, with Tukey’s post-hoc test; ****p<0.0001, ††p<0.01
dependent activation of KCa3.1 in MLS-9 cells is 7.6±0.7 µM (Ferreira and Schlichter, 2013).
Because excessively high intracellular Ca\textsuperscript{2+} can have harmful effects; e.g., activation of Ca\textsuperscript{2+}-dependent proteases such as calpain (Castillo and Babson, 1998), for subsequent experiments on MLS-9 cells and primary microglia, I used 1 µM Ca\textsuperscript{2+} with 1-EBIO to activate the current.

3.2. The PKA inhibitor, PKI\textsubscript{14-22}, prevents PKA from reducing the current in MLS-9 cells
To confirm that the effects of Sp-cAMPS were mediated by PKA, and not a direct effect of cAMP, I added the highly selective, non-myristoylated version of the PKA inhibitor, PKI\textsubscript{14-22} to the pipette solution. This peptide binds directly to the catalytic subunit of PKA and prevents it from phosphorylating target molecules. Specifically, there are two residues (F239, Y235) on the catalytic subunit of PKA that are important for target docking before phosphorylation can occur. PKI\textsubscript{14-22} binds between these two residues, and ultimately prevents phosphorylation (Parang et al., 2001). Thus, its mechanism is distinct from PKA inhibition by Rp-cAMPS (see Fig. 7). As for Figure 6, the pipette solution contained 1.1 µM free Ca\textsuperscript{2+} and the KCa3.1 current was activated by 300 µM 1-EBIO. Figure 8 shows representative current traces (Fig. 8A, B), time courses (Fig. 8C), and the summarized current densities (Fig. 8D). In control recordings with normal pipette solution, 1-EBIO activated a robust KCa3.1 current that reached a plateau and then declined over several minutes after bath addition of the PKA activator, Sp-cAMPS. The delay and slow time course was not surprising, because Sp-cAMPS must enter the cell and then exert its actions; i.e., binding to PKA, followed by release of the catalytic subunit and target phosphorylation. The presence of PKI\textsubscript{14-22} with 1 µM Ca\textsuperscript{2+} in the pipette did not itself activate KCa3.1 current. Instead, PKI\textsubscript{14-22} prevented Sp-cAMPS from decreasing the current; thus, KCa3.1 inhibition by Sp-cAMPS depends on PKA.
Figure 7. Mechanism of action of the PKA inhibitor, PKI_{14-22}. Schematic diagram depicting the inhibition of PKA by PKI_{14-22} following PKA activation by Sp-cAMPS. PKI_{14-22} inhibits PKA activity after the catalytic subunits have been released. This differs from Rp-cAMPS, which inhibits PKA by preventing the release of the catalytic subunits.
3.3. Native KCa3.1 channels in primary rat microglia are similarly regulated by PKA

Because second-messenger signaling in cell lines can differ from primary cells, I next assessed whether primary rat microglia show the same KCa3.1 regulation by PKA as MLS-9 cells. The Schlichter lab discovered that KCa3.1 expression and current are dramatically increased in rat microglial cells when an anti-inflammatory, ‘alternative’ activation state is evoked by treatment with IL-4 (Ferreira et al., 2014). However, whereas the 1-EBIO-activated currents were stable in MLS-9 cells, the ones in primary microglia treated with IL-4 for 24hr quickly ran down (within 1 min; data not shown). Another student in the lab (Roger Ferreira) patched a primary microglial cell that had been stimulated by IL-4 for 7 days, and observed a large (≥1000 pA) stable KCa3.1 current. Thus, I tried the longer IL-4 stimulation and found that after 6 days, the KCa3.1 current readily activated in a stable manner, while current stability was not conferred after 24 or 48hr IL-4 stimulation. Therefore, I cultured rat microglia with 20 ng/mL of IL-4 for 6 days to induce alternative activation and increase the KCa3.1 current. Several precautions were used to isolate the KCa3.1 current: a holding potential of 0 mV to inactivate Kv1.3, 100 nM apamin to block any KCa2 channels, including KCa2.3 (Schlichter et al., 2010), and finally, TRAM-34 was added at the end of each recording to quantify the KCa3.1 current. In whole-cell recordings from microglia with 1.1 μM intracellular free Ca^{2+}, robust TRAM-34-sensitive KCa3.1 currents were activated by 1-EBIO. Representative traces are shown in Figure 8E, F, representative time courses illustrated in Figure 8G, and the current densities are summarized in Figure 8H. The KCa3.1 current in primary microglia was essentially identical to MLS-9 cells. It was time-independent during steps, had a reversal potential close to $E_K$ (Fig. 8E, F), and a similar amplitude (current density; Fig. 8H). Importantly, as for MLS-9 cells, a time-dependent inhibition by bath applied Sp-cAMPS was seen (Fig. 8G). At the plateau, the mean control current density was 27.9±2.9 pA/pF, and then Sp-cAMPS gradually reduced it 55% over a 5–10
Figure 8. KCa3.1 regulation in MLS-9 microglia and primary rat microglia is prevented by the PKA inhibitor, PKI14-22. For MLS-9 cells (A–D), the recording solutions and voltage protocols were the same as in Figure 1. For primary rat microglia (E–H), the holding potential was 0 mV to inactivate the Kv1.3 current. The KCa2.x blocker, 100 nM apamin was present in all experiments, and the selective KCa3.1 blocker, 1 mM TRAM-34, was used to verify that the current was KCa3.1. **A. MLS-9 microglial cell.** Representative KCa3.1 currents from the same MLS-9 cell show the control current after break-in with 1.1 µM free Ca2+ (trace marked ‘ctrl’), followed by bath addition of 300 µM 1-EBIO (‘EBIO’); 1-EBIO + 10 µM Sp-cAMPS (Sp+EBIO); or 1-EBIO + Sp-cAMPS + 1 µM TRAM-34 (‘TRAM’). **B.** Representative KCa3.1 currents from a different MLS-9 cell (labeled as in panel A), but with the PKA inhibitory peptide, 10 µM PKI14-22, in the pipette. **C.** The current was measured at +80 mV and used to examine the time course of effects on the KCa3.1 current for the two cells from panels A and B. The horizontal bars indicate bath perfusion and show rapid current activation by 300 µM 1-EBIO, followed by a slow but dramatic inhibition by 10 µM Sp-cAMPS in the cell containing normal saline but not in the cell containing the PKA inhibitor, PKI14-22. In both cells, the remaining current was fully blocked by 1 µM TRAM-34. **D.** Summarized data from a population study with treatments as in panels A, B and C. Data are expressed as mean±SEM for the number of cells indicated on each bar. For each treatment group, the current before and after bath addition of Sp-cAMPS was compared using a paired Student’s t-test; ****p<0.0001. Sp-cAMPS had no effect when PKI14-22 was in the pipette (p=0.91). **E. Primary microglial cell.** Representative KCa3.1 traces show the current before (‘ctrl’) and after bath addition of 300 µM 1-EBIO (‘EBIO’), followed by 10 µM Sp-cAMPS + 1-EBIO (‘Sp+EBIO’). The current in the presence of Sp-cAMPS + 1-EBIO was fully blocked by 1 µM TRAM-34 (‘TRAM’). **F.** KCa3.1 currents in a primary microglial cell with 1.1 µM free Ca2+ and the PKA inhibitory peptide, PKI14-22 (10 µM) in the pipette. The traces are labeled as in panel E. **G.** The current in primary microglia was measured at +80 mV and used to examine the time course of effects on the KCa3.1 current. There was rapid current activation by 300 µM 1-EBIO, followed by a slow inhibition by 10 µM Sp-cAMPS in the saline-containing cell but not in the cell containing PKI14-22. In both cells, the remaining current was fully blocked by 1 µM TRAM-34. **H.** Summarized data from a population study of primary microglia with treatments as in panels E, F and G. In each cell, 300 µM of 1-EBIO was used to activate the KCa3.1 current, and the amplitude of the TRAM-34-sensitive current was determined. Data are expressed as mean±SEM for the number of cells indicated on each bar. For each treatment group, the current before and after bath addition of Sp-cAMPS was compared using a paired Student’s t-test; **p<0.01. Sp-cAMPS had no effect when PKI14-22 was in the pipette (p=0.82).
min period (to 12.5±1.6 pA/pF; n=7; p<0.01). Again, I compared separate microglia with and without the PKA inhibitor, PKI₁₄₋₂₂ in the pipette. The control current was 25.8±4.9 pA/pF and, in cells containing PKI₁₄₋₂₂, it remained at 26.2±3.4 pA/pF (n=4; p=0.82) up to 10 min after adding Sp-cAMPS to the bath. Again, this shows that KCa3.1 inhibition by Sp-cAMPS depends on PKA.

3.4. In heterologously expressed human KCa3.1, mutating the sole PKA site in the CaMBD prevents regulation by PKA

The results above indicate that PKA activation led to a phosphorylation event that inhibited KCa3.1 channel activity, but did not identify whether phosphorylation was on the channel or a potential (unidentified) accessory molecule. I BLAST-searched the sequences of the known KCa3.1 accessory molecules: AMPK, MTMR6, NDPK-B, and PHPT-1 (Wulff and Castle, 2010; Balut et al., 2012), and did not find any PKA-specific sites. The KCa3.1 channel sequence has a single PKA site in the calmodulin-binding domain (CaMBD) at S332 in rat and mouse, and S334 in the human isoform. I first verified that the KCa3.1 sequence in primary rat microglia contained the PKA phosphorylation site at S332 (data not shown). This was done by sequencing the microglial KCNN4 gene from nucleotide 461 to the end of the C-terminus (Vector Core Facility, University Health Network): the sequence was identical to rKCa3.1 accession number NM023021.2 (BLAST, NCBI). To test the hypothesis that the single putative PKA site in KCa3.1 is responsible for the observed regulation of current, and to extend my findings from the rat to the human isoform, I mutated Ser334 to Ala (S334A) in human KCa3.1 (hKCa3.1). Then, the full-length wild-type or mutated hKCa3.1 construct was heterologously expressed in HEK293 cells, which have PKA regulatory molecules (Atwood et al., 2011) and lack endogenous KCa3.1 channels (Zagranichnaya et al., 2005). HEK293 cells have small,
endogenous Kv currents, which I inactivated using a holding potential of 0 mV (Fig. 9a). The lack of KCa3.1 current in non-transfected cells was verified using 1.1 µM free Ca\(^{2+}\) in the pipette solution and bath applying 300 µM 1-EBIO; no current was activated (Fig. 9b).

In HEK293 cells transfected with either a wild-type (wt hKCa3.1) or S334A mutant hKCa3.1 construct, a robust current activated spontaneously after establishing a whole-cell recording with 1.1 µM intracellular free Ca\(^{2+}\) (Fig. 9c). Unlike microglia, transfected HEK cells did not require a positive gating modulator, such as 1-EBIO. This is consistent with the Schlichter lab’s previous experience with wt hKCa3.1 expressed in CHO cells, which spontaneously activated with 1.1 µM intracellular free Ca\(^{2+}\) (Joiner et al., 2001). Both wt hKCa3.1 and the S334A mutant produced a large current that quickly reached a stable plateau, and was fully blocked by TRAM-34 (Fig. 9d).

Effects of the PKA activator, Sp-cAMPS, and the PKA inhibitor, Rp-cAMPS were compared on wt hKCa3.1 and S334A mutant channels. Representative currents are shown in Figure 10A and B, time courses in Figure 10C, and the summarized current densities in Figure 10D. Although no KCa current was seen in untreated transfected HEK293 cells, I added the KCa2.1–2.3 blocker, apamin, to the bath to be consistent with recordings from microglia and MLS-9 cells. As above, at the end of each recording, 1 µM TRAM-34 was added to the bath to confirm that the current was KCa3.1. For wt hKCa3.1, perfusing Sp-cAMPS into the bath evoked a time-dependent, 56% decline in the current (Fig. 10A, C) from 52.6±4.2 to 22.9±4.8 pA/pF (n=6; \(p<0.0001\)) (Fig. 10D). In stark contrast, Sp-cAMPS had no effect on the S334A mutant (Fig. 10B–D). Together, these results indicate that the PKA phosphorylation site (at S334 in hKCa3.1) is necessary and sufficient to confer PKA-dependent regulation, which is conserved in both the rat and human channels. In the converse experiment, the PKA inhibitor, Rp-cAMPS,
Figure 9. Characterization of the HEK 293 transient transfection system. A. Untransfected HEK 293 cells. Traces showing the presence of a voltage-dependent (Kv) current when the holding potential is –70 mV. This current is not present when the holding potential is 0 mV. Traces are from different cells. B. Non-transfected HEK 293 cells. Representative traces showing control after break-in with 1 µM free Ca\(^{2+}\) (1), addition of 1-EBIO [300 µM] to bath (2). Note the lack of current. Traces are from the same cell. C. HEK 293 cells transfected with wt hKCa3.1. Representative traces showing control after break-in with 1 µM free Ca2+ (1), addition of TRAM-34 [1 µM] to bath (2). Note the spontaneous activation of KCa3.1 current. Traces are from the same cell. D. HEK 293 cells transfected with S334A hKCa3.1. Representative traces showing control after break-in with 1 µM free Ca\(^{2+}\) (1), addition of TRAM-34 [1 µM] to bath (2). Note the spontaneous activation of KCa3.1 current. Traces are from the same cell. All recordings were done in whole cell configuration. Voltage protocol: +50 mV step, ramp from –100 to +80 mV, holding potential –70 mV.
slightly increased the \textit{wt} hKCa3.1 current but had no effect on the S334A mutant (Fig. 10E–H). Rp-cAMPS increased the current by about 8\%, from 53.9±4.1 to 58.3±3.8 pA/pF (n=13; \(p<0.05\)) (Fig. 10H); which was less than the \(~40\%\) increase in MLS-9 cells (Fig. 6C). Two possible explanations are that, in HEK293 cells, the initial level of channel phosphorylation is lower or less de-phosphorylation occurs during whole-cell recordings. I believe the former to be the case (see below).

Because I previously found that CaM binding during channel biogenesis is important for KCa3.1 channel assembly and trafficking to the cell surface (Joiner et al., 2001), I next addressed whether PKA affects channel activity or conductance, rather than trafficking. An effect on channel trafficking seemed unlikely because, in order for the current to decrease (PKA activation) and increase (PKA inhibition), both rapid channel removal and insertion would have to be triggered by the stimulus, occur in whole-cell recordings, at room temperature and, for Sp-cAMPS, be prevented by PKI\textsubscript{14-22}. When inside-out patches were excised from HEK cells expressing \textit{wt} hKCa3.1 or the S334A mutant, into a bath (intracellular) solution containing ATP and 10 \(\mu\)M Ca\(^{2+}\) to maximally activate the channels (Gerlach et al., 2000), I observed one or two channels in each patch (one channel in the example in Fig. 11A). In control recordings, the activity remained stable for many minutes, and the single-channel conductance was \(~33\) pS at a membrane potential of \(-100\) mV. Channel activity was then recorded for \(>10\) min, and the open probability was calculated from 2 min-long stretches, beginning 4–6 min after adding the active, catalytic subunit of PKA (csPKA) to the bath (intracellular solution). Thresholds were set for the closed level and single or double openings, and channel activity was quantified by dividing NP\(_{o}\) by N, which is the calculated number of active channels in the patch (see Methods). In control recordings from \textit{wt} hKCa3.1, \(P_o\) was 0.52±0.50 (n=3; data not shown), and following perfusion
Figure 10. Mutating the PKA site in the CaMBD prevents the PKA regulation of human KCa3.1 channels. All whole-cell recordings were made with 1.1 µM intracellular Ca²⁺, using a holding potential of 0 mV, followed by a step to +50 mV and a ramp from −100 to +80 mV. The bath contained 100 nM apamin (KCa2.1–2.3 blocker).

A. A recording of wild-type human KCa3.1 (wt hKCa3.1) in a transfected HEK293 cell, showing the current activated after break-in (‘ctrl’), ~7 min after bath addition of the PKA activator (10 µM Sp-cAMPS; ‘Sp’), and after adding 1 µM TRAM-34 + Sp-cAMPS (‘TRAM’). B. An HEK293 cell transfected with the S334A hKCa3.1 mutant, with the currents labeled as in panel A. C. Representative time-courses following bath addition of the PKA activator to cells transfected with either wt hKCa3.1 or the S334A mutant. At the end of each recording, 1 µM TRAM-34 was added to confirm that the current was KCa3.1. D. Summarized data showing the current densities before and 5–10 min after bath addition of the PKA activator, Sp-cAMPS. Values are expressed as mean±SEM for the number of cells indicated, and compared using paired Student’s t-tests. ****p<0.0001 for wt hKCa3.1; p=0.13 for the S334A mutant. E. A recording of wt hKCa3.1, shows the control current (‘ctrl’), ~8 min after bath addition of the PKA inhibitor (10 µM Rp-cAMPS; ‘Rp’), and after adding 1 µM TRAM-34 with Rp-cAMPS (‘TRAM’). F. A recording of the S334A hKCa3.1 mutant, with the currents labeled as in panel E. G. Representative time-courses following bath addition of the PKA inhibitor to cells transfected with either wt hKCa3.1 or the S334A mutant. TRAM-34 was added at the end of each recording. H. Summarized data showing the current densities before and 5–10 min after bath addition of the PKA inhibitor, Rp-cAMPS. Values (mean±SEM) were compared using paired Student’s t-tests. *p=0.027 for wt hKCa3.1; p=0.31 for the S334A mutant.
of csPKA into the bath, the $P_o$ gradually decreased and reached a steady-state in 4–6 min, after which the channel activity remained stable until 1 µM TRAM-34 was added to identify the current as KCa3.1. For wt hKCa3.1, csPKA reduced $P_o$ by 44.6%, from 0.56±0.02 to 0.31±0.03 (n=6; $p<0.001$; Fig. 11B), and the single-channel amplitude was not affected; i.e., it was 3.3±0.1 pA at −100 mV in controls and 3.4±0.1 after adding csPKA. Subsequent addition of TRAM-34 drastically reduced the $P_o$, to 0.03±0.01 (n=6; $p<0.001$; Fig. 11B). In contrast, with the S334A mutant, csPKA had no effect (Fig. 11C, D). $P_o$ was 0.53±0.02 in control bath and remained at 0.55±0.02 after adding csPKA (n=5; $p=0.46$). TRAM-34 then reduced $P_o$ to 0.04±0.01 ($p<0.001$).

3.5. By phosphorylating S334 in the CaMBD, PKA reduces CaM binding to KCa3.1

CaM binds to the CaM binding domain (CaMBD) of the KCa3.1 channel and this is essential for the channel to respond to Ca$^{2+}$ and open (see Introduction). I next tested the hypothesis that by phosphorylating S334, PKA reduced the current by interfering with the interaction between CaM and the CaMBD. CaM affinity chromatography was conducted on wt hKCa3.1 and the S334A mutant transfected into HEK293 cells, followed by Western immunoblotting. Rather than relying on a KCa3.1 antibody, I added a DDK tag to the C-terminus of the channels, and used an anti-DDK antibody to detect and quantify KCa3.1. The Schlichter lab and others have shown that the C-terminal DDK tag does not affect KCa3.1 expression or function (Joiner et al., 2001; Srivastava et al., 2008; Ashmole et al., 2012).

PKA-dependent phosphorylation was promoted by incubating the cells with the PKA activator, 10 µM Sp-cAMPS (40–60 min, 37°C) before harvesting protein. Total KCa3.1 protein expression in cell lysates was compared (Fig. 12A), and showed that channel expression was not affected by Sp-cAMPS treatment for either wt hKCa3.1 or the S334A mutant. This indicates that
Figure 11. Adding the catalytic subunit of PKA (csPKA) to the cytosolic face of isolated inside-out patches reduces hKCa3.1 channel activity. Channel activity was recorded with bath and pipette solutions containing the same K⁺ concentrations, with 10 µM free Ca²⁺ and 1 mM ATP in the bath, and the membrane potential was held at –100 mV. The dash beside each trace indicates the closed level, and channel openings are downward. 

A. Inside-out patches were excised from HEK293 cells transfected with wt hKCa3.1. The catalytic subunit of PKA (csPKA; 10 µM) was perfused into the bath, and at the end of the recording, 1 µM TRAM-34 was also added to the bath. 

B. Summarized data show the probability of opening, Pₒ (see Methods), for wt hKCa3.1 in control bath, 4–6 min after bath addition of csPKA, and after adding 1 µM TRAM-34. Values are expressed as mean±SEM (n=6), and compared using a one-way ANOVA with Tukey’s post-hoc test. ***p<0.001 compared with control recordings. 

C. Inside-out patches from HEK293 cells transfected with S334A hKCa3.1. Channel activity was recorded as in panel A. 

D. Summarized data shows Pₒ for the mutant S334A channel in control bath, 4–6 min after adding csPKA; 10 µM), and after adding 1 µM TRAM-34. Values are expressed as mean±SEM (n=5), and compared using a one-way ANOVA with Tukey’s post-hoc test. p=0.46 for csPKA compared with controls; ***p<0.001 for TRAM-34.
PKA-dependent phosphorylation of S334 is not necessary for KCa3.1 transcription or translation in HEK cells. CaM pull-down beads were used to assess whether the interaction between KCa3.1 and CaM was compromised by phosphorylation of S334 (Fig. 12B). In cells transfected with *wt* hKCa3.1, treatment with Sp-cAMPS dramatically reduced CaM-mediated channel pull-down; i.e., to 25±5% of the control level (n=3; *p*<0.05). The S334A mutation alone did not affect CaM binding to the channel (103±24% of the control level; n=3; *p*=0.84); however, the Sp-cAMPS-mediated decrease in binding was significantly reversed (n=3; *p*<0.05) and reached a level that was not significantly different from the control level (n=3; *p*=0.19). Less than complete reversal might have occurred because KCa3.1 has three potential sites for non-selective Ser/Thr phosphorylation in the CaMBD (Neylon et al., 2004) on which PKA might have acted. The similar control level of CaM-binding of the wild-type and S334A mutant channels suggests that basal KCa3.1 phosphorylation is low in HEK293 cells.

3.6. The PKA site (Ser334) is required for KCa3.1 inhibition by the adenosine A2a receptor

PKA can be activated through A2 adenosine receptors that are linked to G-proteins (Jacobson and Gao, 2006). Of the two A2 receptor subtypes, A2a signals only through the Gs subunit (Gao and Jacobson, 2007); whereas, A2b can also act on Gq leading to IP3 and DAG release, in addition to activating the PKA pathway (Ryzhov et al., 2006). Therefore, I used a selective A2a receptor agonist, CGS21680 (Jarvis et al., 1989), to ask whether activating PKA through this receptor-mediated pathway has the same effect as Sp-cAMPS on the KCa3.1 current. Rat microglia (Saura et al., 2005; Gomes et al., 2013) and HEK293 cells (Atwood et al., 2011) both express the A2a receptor, so for this experiment, I used primary rat microglia, and HEK293 cells transfected with either *wt* hKCa3.1 or the S334A hKCa3.1 mutant. When used, cells were pre-
Figure 12. Phosphorylation of S334 reduces calmodulin binding to KCa3.1. A. Expression of *wt* hKCa3.1 and the S334A hKCa3.1 mutant in HEK293 cells. Left: A representative Western blot shows hKCa3.1 protein in lysates from non-transfected HEK293 cells or 36 h after transfection with Myc-DDK-tagged *wt* hKCa3.1 or Myc-DDK-tagged S334A hKCa3.1. Cells were untreated or incubated with 10 µM Sp-cAMPS (40–60 min, 37°C). hKCa3.1 was detected with an anti-DDK antibody, and an anti-GAPDH antibody was used for the loading control. Right: Summary of KCa3.1 protein expression in cell lysates. Each group was first normalized to its respective GAPDH expression, and then normalized to control (untreated) *wt* hKCa3.1. Bars represent mean±SEM; n=3 each. B. Protein pulled down with CaM-agarose PD beads (see Methods). Left: Representative Western blot showing *wt* hKCa3.1 and S334A hKCa3.1 mutant. Cell treatment and protein detection were as in panel A. Right: Summary of amount of KCa3.1 protein pulled down. Data are expressed and normalized as in panel A. Bars represent mean±SEM; n=3 each. ANOVA with Tukey’s post-hoc test shows differences produced by Sp-cAMPS treatment for *wt* hKCa3.1 (*p<0.05), and between wild-type and mutant channels after Sp-cAMPS treatment († p<0.05).
treated with the A2a receptor agonist, CGS21680. The endogenous KCa3.1 current in rat microglia was activated using 1.1 µM intracellular free Ca\(^{2+}\) and bath addition of 300 µM 1-EBIO (as in Fig. 2). Figure 13A shows representative time courses for the KCa3.1 current in microglia without treatment, with CGS21680, or with CGS21680 + PKI\textsubscript{14-22} (PKA inhibitor). In all three cases, the current activated rapidly in response to 1-EBIO, and was then stable until TRAM-34 was added. The summarized data for microglia show that A2a receptor activation with CGS21680 reduced the current density by ~30%; from 35.0±3.8 (n=5) to 24.9±2.1 pA/pF (n=7; p<0.05); whereas, with CGS21680 + PKI\textsubscript{14-22} combined, the current density was not affected (37.9±5.2 pA/pF; n=4; p=0.87) (Fig. 13A). In HEK293 cells transfected with wt hKCa3.1, the current also decreased ~30% following A2a receptor stimulation; from 67.8±7.6 (n=4) to 44.3±5.4 pA/pF (n=6; p<0.05) (Fig. 13B). In contrast, A2a receptor stimulation had no effect in cells transfected with the S334A mutant (Fig. 13C). These results are all qualitatively consistent with effects of Sp-cAMPS, but there was less inhibition by CGS21680. It is possible that localized cAMP elevation and PKA activation through A2aR–G\(_s\) signaling was less extensive or shorter-lived than with the hydrolysis-resistant Sp-cAMPS.

3.7. PKA-mediated inhibition of KCa3.1 reduces Ca\(^{2+}\) influx in primary microglia

KCa3.1 activation is expected to maintain a negative membrane potential, which will increase Ca\(^{2+}\) influx through non-voltage gated Ca\(^{2+}\)-release-activated Ca\(^{2+}\) (CRAC) channels that are prevalent in rat microglia (Ohana et al., 2009; Ferreira and Schlichter, 2013). To examine the effect of PKA-mediated KCa3.1 regulation on Ca\(^{2+}\) entry through CRAC channels, metabotropic P\(_2\)Y\(_2\) purinergic receptors were activated with UTP, as before (Ferreira and Schlichter, 2013). Primary rat microglia were treated for 6 days with IL-4 (alternative activated), which increased the KCa3.1 current and its contribution to migration (Ferreira et al., 2014).
Figure 13. Activating PKA through the A2a receptor decreases the KCa3.1 current. All recordings were made in the whole-cell configuration with 1.1 µM intracellular free Ca\(^{2+}\), 100 nM apamin in the bath, and a holding potential of 0 mV to inactivate Kv channels. A voltage step to +50 mV was followed by a ramp from −100 to +80 mV. The current amplitude at +80 mV is illustrated in the left-hand panels and the current density (pA/pF; mean±SEM) is shown in the right-hand panels for the number of cells noted on each bar. At the end of each recording, 1 µM TRAM-34 was used to verify that the entire current was KCa3.1. When used, cells were pre-treated (40–60 min, 37°C) with the selective A2aR agonist, 300 nM CGS21680.

A. Primary rat microglia, in which endogenous KCa3.1 channels were activated with 300 µM 1-EBIO (as in Fig. 2). Left: Representative time course of the KCa3.1 current (at +80 mV) in a control cell; one that was pre-treated with CGS21680; and one that was pre-treated with PKI\(_{14-22}\) (10 µM, 15 min, 37°C) before adding CGS21680. Note that 1 µM TRAM-34 fully blocked the currents. Right: Summary from a population study of microglia that were untreated, CGS21680-treated, or treated with PKI\(_{14-22}\) + CGS21680. Results were compared with a one-way ANOVA, followed by Tukey’s test (*p<0.05).

B. In HEK293 cells transfected with wt hKCa3.1, the current spontaneously activated after break-in with 1.1 µM intracellular Ca\(^{2+}\), and was fully blocked by TRAM-34. Data were compared using paired Student’s t-tests (*p=0.024).

C. HEK293 cells were transfected with the S334A hKCa3.1 mutant, and the data were analyzed and presented as in panel B (p=0.58).
Figure 14A shows representative Fura-2 traces for each treatment, and Figure 14B shows the summarized data. As I previously showed for MLS-9 cells (Ferreira and Schlichter, 2013), under control conditions, bath application of 100 µM UTP evoked a short-lived peak in intracellular Ca\(^{2+}\) (release from stores), followed by a plateau phase (Ca\(^{2+}\) entry) and a return to the baseline. None of the treatments affected the initial brief spike. Thus, to reflect Ca\(^{2+}\) influx, the area under the curve (gray shaded) was integrated \((340/380 \text{ ratio} \times \text{time})\) and expressed in arbitrary units. First, I showed that this Ca\(^{2+}\) plateau component is strongly dependent on KCa3.1 activity. That is, compared to the area under the curve in response to UTP in untreated microglia \((905.3\pm231.4; n=35)\), 1 µM TRAM-34 reduced the signal by ~75% \((to 224.1\pm36.4; n=21; p<0.01)\). Activating PKA with Sp-cAMPS reduced the UTP-evoked signal by ~40% to 501.8\pm172.3 (n=19; p<0.05). The A2a receptor agonist, CGS21680 similarly reduced the signal, to 514.7\pm149.6 (n=8; p<0.05). If microglia were pre-treated with the membrane-permeant PKA inhibitor, myristoylated PKI\(_{14-22}\), these effects were abolished. The signal was not significantly different from the control value; i.e., with Sp-cAMPS the area under the curve was 1074.7\pm297.3 (n=9; p=0.81) and with CGS21680 it remained at 834.1\pm292.8; n=5; p=0.76). TRAM-34 reduced the Sp-cAMPS- and CGS21680-evoked signals to the same level as control cells with TRAM-34.
Figure 14. Involvement of KCa3.1 in UTP-induced intracellular Ca\(^{2+}\) increase in microglia. Primary rat microglia were stimulated for 6 days with 20 ng/mL IL-4 and then incubated with Fura-2 AM (3.5 µg/mL) at room temperature for 45 min in the dark. Images at 340 and 380 nm excitation wavelengths were alternatively acquired every 4 seconds. A. Representative Fura-2 recordings, in which 100 µM UTP was bath applied during the period marked by the horizontal bar. The area under the curve is shaded in gray. The treatments were: control bath, TRAM-34 (1 µM; 5 min; room temperature), Sp-cAMPS (10 µM; 30–45 min; 37°C), CGS21680 (300 nM; 30–45 min; 37°C), Sp-cAMPS + PKI14-22 (10 µM; 1 h; 37°C), CGS21680 + PKI14-22, Sp-cAMPS + TRAM-34, and CGS21680 + TRAM-34. B. Summarized data (mean±SEM) for the number of cells indicated on each bar. One symbol of any type represents \(p<0.05\); two, \(p<0.01\) (ANOVA with Tukey’s post-hoc test); where * compares treatments versus controls; ‡ compares each PKA activator with or without TRAM-34; and † compares each activator with and without PKI14-22.
4. Discussion

4.1. Past studies of KCa3.1 regulation by PKA

Studies addressing effects of PKA on the KCa3.1 current have had varied results. In principle, inconsistencies could reflect differing species, cell type or experimental approaches. PKA can act on separate mechanistic pathways, and there have been reports of different functional outcomes despite activating PKA in a similar manner. For example, the β-adrenergic receptor is a G-protein coupled receptor (GPCR) that up-regulates PKA (see below). In macrophages, β-adrenergic stimulation increases CD14 expression through PKA signaling (Muthu et al., 2010), whereas, β-adrenergic receptor activation prolongs cardiac action potentials by PKA regulation of Ca\(^{2+}\) channels (van der Heyden et al., 2005). In many epithelial cell types, PKA helps maintain cell polarity and establishes a signaling loop that modulates protrusion-retraction cycles (Edin et al., 2001; Lim et al., 2008; Tkachenko et al., 2011). However, in primary rat microglia, PKA activation (1 mM dbcAMP; 30 min incubation) reduced cell adhesion and migration by acting on the epidermal growth factor-like repeats domain of the extracellular matrix molecule, tenascin-R (Liao et al., 2005).

Interestingly, some results diverge even within studies using the same cloned channels and expression system, suggesting that differing experimental approaches are important. Some studies used methods that raise cAMP transiently (e.g., forskolin, cAMP), some also inhibited phosphodiesterase activity with IBMX or theophylline, some used cAMP analogues, and some directly applied PKA. Even the means of activating the channels varied from simply adding ATP without using elevated intracellular Ca\(^{2+}\), to high Ca\(^{2+}\) alone, or Ca\(^{2+}\) with the gating modulator, 1-EBIO.

Several studies used *Xenopus* oocytes but different recording configurations and means to
activate the current and PKA. Two studies applied the adenylate cyclase activator, forskolin, with the phosphodiesterase inhibitor, IBMX, to prolong the cAMP rise. Both showed an increase in the ionomycin-activated current: rKCa3.1 (von Hahn et al., 2001), hKCa3.1 (Gerlach et al., 2000). In the latter study, the increase was not reversed by mutating the PKA site (S334A), and this was also seen in excised inside-out patches, in which hKCa3.1 current was activated by adding cytoplasmic ATP and reduced by the PKA inhibitor, PKI5-24 (Gerlach et al., 2000). Differing results were seen in two other studies using inside-out patches from Xenopus oocytes. After activating rKCa3.1 with ATP and 1.2 µM Ca²⁺, adding the PKA catalytic subunit had no effect, nor did PKA inhibitors (PKI5-24, KT5270) or the S332A mutation (von Hahn et al., 2001). In contrast, after activating rKCa3.1 with 10 µM Ca²⁺, the current was reduced by adding PKA, and importantly, simultaneously mutating four sites in the CaMBD (S312A, T327A, S332A, T348A) abolished the effect of PKA, as did the S332A mutation alone (Neylon et al., 2004). The latter study also used a fusion protein of only the CaMBD, and showed that S332 was most strongly phosphorylated by PKA. By directly linking rKCa3.1 phosphorylation by PKA with current inhibition, the latter study is consistent with my results. In whole-cell and inside-out patch recordings from HEK293 cells expressing wt hKCa3.1 (but not the S334A mutant), I found that the current and the open probability were reduced by activating PKA. Two other studies of hKCa3.1 in HEK293 cells reported that PKI5-24 did not affect the theophylline-activated (Schroder et al., 2000) or ATP-activated current (Gerlach et al., 2000). Together, these observations suggest that differences can result from differing expression systems, and means of activating the current and assessing PKA effects.

Results of studies on native currents also vary, even though all channels described appear to be KCa3.1. I identified native KCa3.1 currents based on their biophysical and
pharmacological properties, including full block by 1 μM TRAM-34, which is KCa3.1-selective. PKA activation reduced the native KCa3.1 current, and this was prevented by a PKA inhibitor (PKI_{14-22}). Inhibiting PKA increased the current. This is consistent with a study on NIH-3T3 fibroblasts that showed KCa3.1 phosphorylation by PKA, and reduction of the 1-EBIO-activated, TRAM-34-sensitive current by forskolin, which was prevented by PKA inhibitors (PKI_{14-22}, H89) (Choi et al., 2012). Three studies used inside-out patches. In the T84 epithelial cell line, the ATP-activated channel activity was reduced by the PKA inhibitor, PKI_{5-24}. In human erythrocytes, the current that was activated by 2 μM free Ca^{2+} spontaneously ran down, was restored by a cocktail of ATP, cAMP and theophylline, and this was blocked by PKI_{5-24} (Pellegrino and Pellegrini, 1998). In rat acinar cells, charybdotoxin-sensitive channels were activated by 1 μM Ca^{2+}, and while this was unaffected by intracellular cAMP or forskolin, it was reduced by the PKA inhibitor, Rp-cAMPS (Hayashi et al., 2004). Surprisingly, when Ca^{2+} was reduced to 0.1 μM, both cAMP and forskolin increased the current.

4.2. Current study: Mechanism of PKA regulation on KCa3.1 elucidated

Through CaM-binding analysis, I demonstrated a mechanism for KCa3.1 current reduction by PKA—decreased CaM binding to the channel—which is expected to reduce the open probability and whole-cell conductance. As modeled in Figure 15, my results provided evidence that following PKA activation and phosphorylation of S334 on the channel, the integrity of the interaction between KCa3.1 and CaM was greatly reduced due to a potential conformation change of the KCa3.1 C terminus, and this decreased the current because CaM acts as the Ca^{2+}-dependent gate for KCa3.1 channels (see Introduction).

The Schlichter lab previously found that CaM binding to hKCa3.1 during channel biogenesis is important for channel assembly and trafficking to the cell surface (Joiner et al.,
Figure 15. Summary model.
Schematic diagram illustrating: 1) activation of PKA by Sp-cAMPS, or by endogenous cAMP (stimulated through adenosine A2a receptors); 2) inhibition of PKA by Rp-cAMPS or PKI_{14-22}; 3) the hypothesized conformation change of the C-terminus of KCa3.1 that prevents Ca^{2+}-CaM from binding and opening the channel when the PKA site (S334) is phosphorylated; 4) the opening of KCa3.1 by CaM when the PKA site is not, or cannot be, phosphorylated; and 5) the role for KCa3.1 in Ca^{2+}-signaling, where the channel generates negative membrane potentials (K^+ efflux) that maintain an electrical driving force for Ca^{2+}, leading to an increase in constitutive Ca^{2+} entry through CRAC channels. This is a positive feedback loop between KCa3.1 and CRAC channels, which would be compromised by a decrease in KCa3.1 channel activity due to PKA-dependent phosphorylation. S6 refers to the sixth transmembrane domain of the KCa3.1 channel.
Combining this with the fact that membrane hKCa3.1 is endocytosed within minutes when expressed in HEK293 cells (Balut et al., 2012), an alternative hypothesis was that after KCa3.1 gets recycled from the membrane surface, the channel cannot be trafficked back because PKA-dependent phosphorylation of S334 compromises its binding with CaM. As a result, KCa3.1 activity would be inhibited. For the other KCa channels, Clarysse et al. (2014) showed with proteinase K digestion experiments that KCa2.3 localization in the plasma membrane did not change with PKA activation. This is in contrast to KCa2.2, where phosphorylation by PKA of three residues (S568, S569, S570) decreased their plasma membrane localization (Ren et al., 2006). It is important to note that none of these residues are conserved in KCa3.1 or KCa2.3. In addition, to account for both the decrease in current by PKA activation and increase following PKA inhibition, channel removal and insertion would both have to rapidly occur during whole-cell recordings at room temperature. Moreover, these changes would have to be triggered by the stimulus, and for Sp-cAMPS, prevented by PKI1-4,22. In my study, the rapid decrease in open probability of wt hKCa3.1 (but not the S334A mutant) evoked by the PKA catalytic subunit in inside-out patches strongly support a direct effect on channel gating, rather than on trafficking.

While the pioneering studies of KCa2.2 suggested that CaM is constitutively and irreversibly bound to the CaMBD of the channel (Schumacher et al., 2001), the current study implies that CaM binds reversibly to the CaMBD of KCa3.1, which must occur for the channels to bind to the CaM-agarose beads. Earlier work in the Schlichter lab on T lymphocytes supports reversibility; the KCa3.1 current was reduced by CaM antagonists that act by preventing CaM from binding to its target (Khanna et al., 1999). In the crystal structure of the related hKCa2.2 channel, the corresponding residue (H446) lies between the two CaM-binding ends of the channel’s binding domain (Schumacher et al., 2001); thus, the S334A mutation might indirectly
affect CaM binding. Mechanistically, I hypothesized that phosphorylation of S334 changed the conformation of KCa3.1, thus compromising its interaction with CaM, and ultimately, gating was affected. That is, conformational change of the Ca\(^{2+}\)/CaM complex did not transfer to the pore opening of KCa3.1.

In KCa2.3, Clarysse et al. (2014) showed a PKA-dependent inhibition, and found that when expressed in breast cancer cells (MDA-MB-435), KCa2.3 formed a complex with Orai1, an essential subunit of CRAC channels. As mentioned above, Clarysse al. (2014) showed that PKA activation with the adenylyl cyclase activator, forskolin, did not change KCa2.3 localization in the plasma membrane; i.e., trafficking was not involved. However, KCa2.3 activity was decreased. Moreover, this study showed that constitutive Ca\(^{2+}\) entry (and consequently cancer cell migration) was reduced when PKA was activated with forskolin, and this effect was abolished with the PKA inhibitor, KT5720. From this, the authors suggested that PKA activation directly phosphorylated KCa2.3, which decreased channel activity, and lead to depolarization of the plasma membrane. They also suggested that this disrupted the positive feedback loop between KCa2.3 and CRAC, which in turn further decreased KCa2.3 activity. In microglia, CRAC is also coupled to KCa3.1 (Ferreira and Schlichter, 2013), so under physiological conditions, I hypothesize that a similar mechanism would be applicable following reduction of KCa3.1 current by PKA. Nevertheless, one important aspect to keep in mind is that PKA has many targets, and PKA-dependent phosphorylation can have broad implications on a wide variety of cellular processes (see below). Although there is no evidence of phosphorylation of CRAC channels, an alternative explanation is that cAMP/PKA can potentially inhibit Ca\(^{2+}\) influx by phosphorylating other molecules involved in capacitative Ca\(^{2+}\) entry. However, in the present literature, there is no support for this hypothesis.
In my work, primary microglia were alternatively activated by IL-4 stimulation, which has been shown to transiently increase cAMP intracellular levels in rat photoreceptor cells (Adao-Novaes et al., 2009), human B cells (Taieb et al., 1991; Vazquez et al., 1991), and endothelial cells (Galea et al., 1993). Therefore, one might suspect that IL-4 treatment would inhibit KCa3.1 currents. However, Ferreira et al. (2014) and the present data show otherwise. For primary microglia in the resting state, the KCa3.1 mRNA expression is low and the current is not readily activated. Treatment of primary cells with IL-4 (>24 h) activates a signaling pathway downstream of the IL-4 receptor, which eventually leads to a transcription factor that up-regulates KCa3.1 mRNA expression (Ferreira et al., 2014), and as a result, protein expression (unpublished observations from a lab mate, Roger Ferreira). Consequently, robust KCa3.1 currents can be activated by positive gating modulators (see above). And so, the mechanism by which IL-4 increases KCa3.1 current is a slow effect (>24 h), and acute application of IL-4 did not turn on the current in patch-clamp experiments (unpublished observations from a lab mate, Roger Ferreira). Nevertheless, the 1-EBIO activated current in IL-4 treated primary microglial cells had a comparable current density to MLS-9 cells, and application of a cAMP analogue (Sp-cAMPS) reduced the current similarly in both cell types. A possible explanation to account for the lack of KCa3.1 inhibition by IL-4 could be that the IL-4-induced cAMP elevation is rapidly reversed by phosphodiesterase activity. The true cellular half-life of cAMP was modeled to be 1 min 50 s in thyroid cells (van Sande et al., 1977), same half-life order in hepatocytes (Exton et al., 1971), and much shorter in tissue with high metabolic activity fluctuation, e.g., myocardium (Brooker, 1975). As sustained cAMP elevation is needed for PKA to remain active, cAMP degradation would reduce PKA activity (Schaap et al., 1993). Therefore, it is unlikely for PKA to be active six days after IL-4 treatment.
Interestingly, unlike microglia (present study) and MLS-9 cells (Liu et al., 2013; present study), 1-EBIO was not required to activate the current in transfected HEK293 cells. hKCa3.1 channels in HEK293 cells were activated by simply raising Ca\(^{2+}\) to 1 \(\mu\)M, as Dr. Schlichter’s laboratory previously observed for hKCa3.1 expressed in CHO cells (Joiner et al., 2001). The native channels in microglia also required a positive gating modulator (Liu et al., 2013; Ferreira et al., 2014). Such modulators increase the channel Ca\(^{2+}\) sensitivity (Pedersen et al., 1999; Syme et al., 2000; Pedarzani et al., 2001). 1-EBIO is thought to stabilize the interaction between CaM and the KCa3.1 channel, and to slow channel closing (Pedarzani et al., 2001). In the experiments using microglia, because the PKA activator Sp-cAMPS was always alongside 1-EBIO in the bath, an alternative explanation could be that cAMP elevation somehow interfered with the actions of 1-EBIO. However, this was not the case because, using HEK293 cells, I elevated cAMP in the absence of 1-EBIO. My results are consistent with a change in Ca\(^{2+}\) sensitivity by 1-EBIO but not by PKA. That is, the current was fully activated by 10.9 \(\mu\)M Ca\(^{2+}\) alone or by 1.1 \(\mu\)M Ca\(^{2+}\) plus 1-EBIO, while PKA activation decreased the current equally at 1.1 and 10.9 \(\mu\)M Ca\(^{2+}\). At present, it is not known why the current in microglia is less sensitive to Ca\(^{2+}\) than in other cell types but differential CaM binding is one possibility (see Introduction).

4.3. PKA and adenosine in the CNS (specifically in microglia)

KCa3.1 regulation by PKA and adenosine is of interest in the CNS. cAMP and adenosine are present in the healthy CNS but increase after injury (Latini and Pedata, 2001; Pearse et al., 2004), and this is thought to be neuroprotective (Cai et al., 2001; Qiu et al., 2002; Nikulina et al., 2004; Spencer and Filbin, 2004; Koga et al., 2009). The Schlichter lab previously reported that blocking KCa3.1 with TRAM-34 is neuroprotective \textit{in vitro} and \textit{in vivo} by reducing the pro-inflammatory, classical activation of microglia (Kaushal et al., 2007). In microglia, PKA is
activated in response to a wide range of agonists acting on GPCRs that are coupled to Gs proteins (Lattin et al., 2008), and numerous roles for PKA are relevant to microglial activation and CNS inflammation. It is difficult to predict overall outcome of activating PKA because it can be complex; e.g., PKA activation: 1) increased microglial phagocytosis of amyloid β peptide at normal cAMP levels but inhibited it when cAMP was elevated (Makranz et al., 2006); 2) increased CREB and NFκB binding to DNA in microglia activated with plasminogen or gangliosides (Min et al., 2004); 3) regulated the transcription of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which produces reactive oxygen species and contributes to oxidative stress (Savchenko, 2013); 4) reduced classical activation induced by toll-like receptor 2 and 4 agonists, amyloid β or lipoteichoic acid (Park et al., 2013); 5) reduced adhesion and migration (Liao et al., 2005); and 6) secreted brain-derived neurotrophic factor (BDNF), which plays a role in microglial proliferation following activation (Gomes et al., 2013).

Microglial cells express GPCRs that are known to stimulate cAMP/PKA (see below for mechanism) and are linked to CNS diseases; e.g., adenosine receptors type A2a and A2b (A2a is linked to Parkinson’s; Schwarzschild et al., 2006), adrenergic β2 receptors (Parkinson’s; Peterson et al., 2014), calcitonin-like receptors (bipolar disorder; Vik and Yatham, 1998), corticotrophin-releasing hormone receptors (Alzheimer's, Parkinson's, Huntington's; de Souza, 1995), glucagon receptors (Alzheimer’s; Talbot and Wang, 2014), histamine H2 receptors (multiple sclerosis; Emerson et al., 2002), luteinizing hormone receptors (Alzheimer’s; Webber et al., 2007), and secretin receptors (schizophrenia, autism; Alamy et al., 2004). Diseases have also been linked to GPCRs coupled to Gi proteins that inhibit the PKA pathway. The ones expressed in microglia include: adenosine A1 and A3 receptors (epilepsy; Boison, 2005), adrenergic α2A receptors (ADHD; Kieling et al., 2010), Ca^{2+}-sensing receptors (Alzheimer’s; Conley et al., 2009),
cannabinoid CB2 receptors (multiple sclerosis; Pryce et al., 2003), chemokine CXCR4 receptors (stroke; Cui et al., 2013), and histamine H3 and H4 receptors (multiple sclerosis; Jadidi-Niaragh and Mirshafiey, 2010). Certain receptors have multiple subtypes, with some linked to G_s while others are linked to G_i so that the same ligand can carry out different roles in the same cell. An example is the adrenergic receptor. Norepinephrine induced process retraction in non-activated microglia through adrenergic β2 receptors (PKA is stimulated), but this switched to adrenergic α2A receptors (PKA is inhibited) after microglia were classically activated by LPS (Gyoneva and Traynelis, 2013). This shows that depending on the activation state of microglia, norepinephrine stimulates microglial process retraction by activating and inhibiting cAMP/PKA, in both the non-activated and classically activated states, respectively.

The existence of multiple subtypes of adenosine receptors in microglia, neurons and astrocytes (Gebicke-Haerter et al., 1996; Haas and Selbach, 2000; Peakman and Hill, 1994), presence of adenosine in the extracellular space of the brain under resting conditions, and the up-regulation of its expression in response to CNS insults (e.g., hypoxia, ischemia, seizures) all suggest the physiological importance of adenosine in the brain (reviewed by Gomes et al., 2011). Although there are multiple potential mechanisms for maintaining a basal level of extracellular adenosine in the healthy brain; following cellular damage, ATP is released, and is rapidly broken down to adenosine (reviewed by Latini and Pedata, 2001). ATP has a T_1/2 of ~200 ms in the extracellular space in rat hippocampus, and its conversion to adenosine is by 5’-nucleotidase and the ectonucleotidase molecules, CD73 and CD39 (Dunwiddie et al., 1997). Although all subtypes of adenosine receptors have been confirmed in microglia (reviewed in Boison et al., 2010), to further address the physiological relevance of my finding that PKA inhibits human and rat KCa3.1, I focused on the G_s-coupled A2a receptor (A2aR). After the binding of adenosine to
A2aR, Gα is released and up-regulates the production of adenylyl cyclase, which catalyzes the formation of cAMP from ATP. cAMP can then bind with the regulatory subunits of the PKA homodimer and the catalytic subunits are released (Boison et al., 2010). Therefore, A2aR was of particular interest because it activates PKA and is present in both microglia (see below) and HEK293 cells (Atwood et al., 2011).

I found that activating PKA directly or through A2aR reduced the KCa3.1 current, and this was prevented by mutating S334. A2aR stimulation also has complex outcomes. It can increase production of BDNF, which should help with repair (Gomes et al., 2013) but also increases nitric oxide, which might be harmful (Saura et al., 2005), and it decreases microglial process retraction/extension, which is needed for surveillance (Orr et al., 2009). Further evidence that A2aR activation might be pro-inflammatory is that A2aR antagonists reduced p38 MAPK-dependent microglial activation after ischemia (Melani et al., 2006). Also, antagonizing A2aR prevents microglial activation induced by MPTP, which is the precursor of the Parkinsonian mimetic, MPP+ (Carta et al., 2009; Pierri et al., 2005). While my work was being completed, a study on activated human T lymphocytes showed that the A2a receptor reduced the native KCa3.1 current, and this was mediated by PKA (Chimote et al., 2013).

Further functional significance is that, by reducing the KCa3.1 current, I found that activating PKA directly or through the adenosine 2a receptor reduced the Ca2+ entry mediated by Ca2+-release-activated Ca2+ (CRAC) channels. Because microglia express many receptors that are coupled to this pathway, this work has broad implications. Any receptor that activates PKA will potentially inhibit KCa3.1 function by phosphorylating the PKA site (S334 in humans, S332 in rodents) and reducing the interaction between KCa3.1 and CaM. This, in turn, is expected to depolarize the cell and decrease Ca2+ influx through non-voltage-gated channels, and to have
broad consequences for cell functions. KCa3.1 is expressed in a wide array of cell types (see Introduction), and signaling pathways that activate or inhibit PKA are ubiquitous, including the large number of receptors linked to stimulatory (Gs) or inhibitory (Gi) G proteins. The ability of a specific receptor to ultimately result in KCa3.1 phosphorylation will depend on several factors. These include the degree and duration of cAMP elevation, which depends on adenylate cyclase and phosphodiesterase activity, and the proximity of active PKA to the channel, which likely depends on specific PKA kinase anchoring proteins.

4.4. In the grand scheme of things: Integrating my results into previous works from the Schlichter lab

A major topic of interest in Dr. Schlichter’s laboratory is stroke research, both intracerebral hemorrhage (ICH) and ischemic stroke (Wasserman and Schlichter, 2007; Wasserman et al., 2007; Wasserman and Schlichter, 2008; Moxon-Emre and Schlichter, 2010; Moxon-Emre and Schlichter, 2011; Lively et al., 2011; Lively and Schlichter, 2012a; Lively and Schlichter; 2012b; Hutchings and Schlichter, manuscript in preparation). From the Heart & Stroke Foundation data (2007–2008), the prevalence of stroke is expected to continue to rise, especially amongst the younger population. Unfortunately, there are no reliable treatments. The only drug approved for ischemic stroke, tPA, must be administered to the patient within an extremely narrow time frame after stroke onset, and so, not many can benefit from it (del Zoppo, 1998). For ICH, presently, there are no approved treatments. Studies have identified drugs to target the early neurotoxicity following stroke, but while these were effective in animal models, they failed in clinical trials (Ginsberg, 2009; Lakhan et al., 2009). Consequently, this shifted the focus of many basic science investigations to the secondary injury phase, which corresponds with a complex inflammatory response that is delayed for hours and can be prolonged for days (Emsley and Tyrrel, 2002; Jin et
There is insufficient understanding of beneficial versus harmful microglial functions after stroke. And so, a research goal of our lab is to diminish the detrimental and promote the favourable outcomes of inflammation with a microglial focus. One molecular target our lab is particularly interested in is the KCa3.1 channel.

From an immunology perspective, Dr. Schlichter’s laboratory has contributed extensively to the KCa3.1 literature, and my present results can have implications for each of these previous findings. Our lab discovered KCa3.1 in T lymphocytes, and that its activity controls proliferation and volume regulation in these cells (Mahaut-Smith and Schlichter, 1989; Khanna et al., 1999). cAMP and PKA are present in T lymphocytes (Ramstad et al., 2000), and these cells also express A2aRs (Himer et al., 2010). As a result, the cAMP/PKA pathway can be readily activated, which would subsequently inhibit KCa3.1 functions, and ultimately reduce proliferation. In alternatively activated microglia, our lab showed that KCa3.1 activity is involved in migration and invasion (Ferreira et al., 2014). Because migration/invasion of microglia to the injury site can be beneficial (see Introduction), KCa3.1 functions appear to contribute to neuroprotection when microglia are alternatively activated. By reducing KCa3.1 currents, PKA can interfere with these cellular roles, and thus elevation of cAMP/PKA can be considered pro-inflammatory (but see below). And so, tight regulation of cAMP/PKA levels, in addition to controlling intracellular free Ca^{2+}, can provide microglia with another manner to fine-tune its cellular functions through KCa3.1 activity.

The Schlichter lab also found that KCa3.1 assembly and trafficking are dependent on CaM binding, and, in contrast to the prevailing view that originated from the crystal structure of KCa2.2 (see above), suggested that CaM was not constitutively bound to the channel (Joiner et al., 2001). My present findings support this pioneering hypothesis of a non-permanent CaM-
KCa3.1 binding (see above). Additionally, I hypothesize that, during the biogenesis of KCa3.1, physiological processes in the CNS that elevate cAMP/PKA levels (see above) can lead to the phosphorylation of KCa3.1 at S334 before the channels are trafficked to the membrane. As a result, CaM binding with KCa3.1, and ultimately, trafficking of the channel to the membrane would be compromised. And so, chronic (e.g., >24 h) exposure to PKA can potentially interfere with KCa3.1 expression at the membrane.

Our lab was the first to report the expression of KCa3.1 in rat microglia, and that it regulates the respiratory burst (Khanna et al., 2001). In addition, our lab showed that in microglia, both in vitro and in vivo, KCa3.1 functions were responsible for LPS-mediated classical activation, p38 MAPK activation, NO production and neurotoxicity (Kaushal et al., 2007). Additionally, by inhibiting KCa3.1 activity, there was a reduction in neuron death and locomotor impairment after spinal cord injury (Bouhy et al., 2011). Because these findings suggest that KCa3.1 functions are pro-inflammatory, inhibiting channel activity appears to be neuroprotective. PKA phosphorylation of KCa3.1 at S334 represents one endogenous mechanism of reducing the channel’s activity. Consequently, PKA activation can attenuate the KCa3.1-mediated inflammation of classically activated microglia that our lab has previously discovered.

Unfortunately, as mentioned above, the inhibition of KCa3.1 functions by PKA can be pro-inflammatory when microglia are alternatively activated. And so, the timing of KCa3.1 activity inhibition determines whether a molecule is pro- or anti-inflammatory. Although the broad effects and ubiquity of PKA make it ill-suited as a therapeutic target, given the detrimental role of KCa3.1 in the secondary injury phase of stroke, my study suggests that the involvement of cAMP/PKA, within a certain time window, can contribute to the beneficial side of inflammation.
5. Future directions

In this thesis, I showed that PKA directly phosphorylated the KCa3.1 channel. And so, PKA is an example of a molecule that can interact with KCa3.1. However, I hypothesize that this interaction follows a kiss-and-run mechanism, i.e. a kinase phosphorylating its target serine site, rather than PKA being an accessory molecule of KCa3.1. Not much is known about this channel’s accessory molecules, and the available reports are unsatisfying, as they do not examine whether the molecules in question exert their effects on KCa3.1 as a true accessory molecule, like CaM, or as a modulator that transiently interacts with the channel, like PKA (see Introduction). Knowing whether there are accessory molecules associated with KCa3.1 is important because we still do not understand why the KCa3.1 channels in microglia are so insensitive to Ca$^{2+}$, and the presence of an inhibiting accessory molecule could help explain this (see Introduction). Here, I showed that, although inhibiting PKA potentiated the 1-EBIO-activated KCa3.1 current, PKA inhibition alone did not turn on the channel. This suggested that PKA did not play a role in the Ca$^{2+}$-insensitivity of microglial KCa3.1 channels.

As a tool for proteomic analysis, silver staining can detect proteins after electrophoretic separation on polyacrylamide gels with excellent sensitivity (in the low nanogram range) whilst using equipment and chemicals readily available to our lab (Chevallet et al., 2006). The silver stain technique can potentially be used to detect the presence of KCa3.1 accessory molecules; one potential experiment is as follows. First, MLS-9 cells or primary rat microglia can be used. However, I would have to be cautious with the interpretation of the results depending on the cell type. For instance, because they were created from chronic treatment of CSF-1 (see Introduction), data from MLS-9 cells might not be applicable in primary cells. On a similar note, because KCa3.1 expression is low in resting primary microglia, treatment of IL-4 (20 ng/ml; 24 h) is
necessary, and so, findings in alternatively activated microglia might not apply to microglia in general. Unfortunately, increasing KCa3.1 expression in primary microglia through gene transfections or viral-mediated infection is unreliable because these cells are extremely resistant to these techniques (Ohana et al., 2009). The next step would be protein harvest, and then immunoprecipitation to isolate KCa3.1 using a KCa3.1 antibody. One thing to note is that when I was doing the experiments for this thesis, our lab did not know of a reliable KCa3.1 antibody, so I had to attach a DDK-tag to KCa3.1 channels in order to detect the channels with an anti-DDK antibody. However, very recently, a lab mate, Roger Ferreira, has repeatedly used a KCa3.1 antibody with much success in both immunocytochemistry and Western immunoblotting. Using this antibody, I can examine native microglial KCa3.1 channels instead of heterologously expressed ones. Lastly, the isolated KCa3.1 protein would be silver stained. Because CaM is expected to be pulled down with KCa3.1, I would look for the presence of molecules with a molecular weight aside from 17 kDa (size of CaM; Babu et al., 1985). Transient modulators like PKA would not be precipitated with KCa3.1, so anything that does get pulled down is most likely an accessory molecule. The identity of these molecules can then be identified with mass spectrometry analysis.
6. References


de Souza EB. Corticotropin-releasing factor receptors: physiology, pharmacology, biochemistry and role in central nervous system and immune disorders. Psychoneuroendocrinology 20:789-819.


Kopil CM, Vais H, Cheung KH, Siebert AP, Mak DO, Foskett JK, Neumar RW. 2011. Calpain-cleaved type 1 inositol 1,4,5-trisphosphate receptor (InsP\textsubscript{3}R1) has InsP\textsubscript{3}-independent gating and disrupts intracellular Ca\textsuperscript{2+} homeostasis. J Biol Chem 286:35998-36010.


Melani A, Gianfriddo M, Vannucchi MG, Cipriani S, Baraldi PG, Giovannini MG, Pedata F. 2006. The selective A2A receptor antagonist SCH58261 protects from neurological
deficit, brain damage and activation of p38 MAPK in rat focal cerebral ischemia. Brain Res 1073:470-480.


Neylon CB, D'Souza T, Reinhart PH. 2004. Protein kinase A inhibits intermediate conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels expressed in Xenopus oocytes. Pflugers Arch 448:613-620.


Ohana L, Newell EW, Stanley EF, Schlichter LC. 2009. The Ca\textsuperscript{2+} release-activated Ca\textsuperscript{2+} current (I\textsubscript{CRAC}) mediates store-operated Ca\textsuperscript{2+} entry in rat microglia. Channels (Austin) 3:129-139.


Park SY, Bae YS, Ko MJ, Lee SJ, Choi YW. 2013. Comparison of anti-inflammatory potential of four different dibenzocyclooctadiene lignans in microglia; action via activation of


Ponomarev ED, Maresz K, Tan Y, Dittel BN. 2007. CNS-derived interleukin-4 is essential for the regulation of autoimmune inflammation and induces a state of alternative activation in microglial cells.


Blocking ion channel KCNN4 alleviates the symptoms of experimental autoimmune encephalomyelitis in mice. Eur J Immunol 35:1027-1036.


Schroder RL, Jensen BS, Strobaek D, Olesen SP, Christophersen P. 2000. Activation of the human, intermediate-conductance, \( Ca^{2+} \)-activated \( K^+ \) channel by methylxanthines. Pflugers Arch., EJP 440:809-818.


Stoneking CJ, Mason MJ. 2014. Mg\textsuperscript{2+} modulation of the single-channel properties of KCa3.1 in human erythroleukemia cells. Pflugers Arch 466:1529-1539.


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