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

Raymond Wong^{1,2} and Lyanne C. Schlichter^{1,2}

¹Genes and Development Division, Toronto Western Research Institute, University Health Network, Toronto, Ontario M5T 2S8, Canada and ²Department of Physiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada

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 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. Although cAMP protein kinase (PKA) signaling is important in many cells that use KCa3.1, reports of channel regulation by PKA are inconsistent. We 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 expressed KCa3.1 channels (but not S334A) 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+} entry following activation of metabotropic purinergic receptors in microglia.

Key words: adenosine A2a receptor; calmodulin binding; microglia ion channels; mutation analysis; protein kinase A; SK channel regulation

Introduction

The KCa3.1 channel was initially studied in red blood cells but is also expressed in immune cells (Lam and Wulff, 2011), some neurons (Vogalis et al., 2003), cardiac and smooth muscle myocytes (Bi et al., 2013; Weisbrod et al., 2013), and some cancer cells (Schwab et al., 2007). In the CNS, KCa3.1 has been detected in microglia (Eder et al., 1997; Khanna et al., 2001; Kaushal et al., 2007) and astrocytes and neurons (Kaushal et al., 2007; Bouhy et al., 2011; Engbers et al., 2011). KCa3.1 can regulate membrane potential, cell volume, proliferation, migration, and production of inflammatory mediators (Jensen et al., 2002; Edwards et al.,

2010; Köhler et al., 2010; Balut et al., 2012). KCa3.1 blockers have improved the outcome in animal models of trauma, spinal cord injury, ischemic stroke, multiple sclerosis, and Alzheimer's disease (Wulff and Zhorov, 2008; Maezawa et al., 2012). Because these disorders involve inflammation and microglial activation, KCa3.1 block might improve the outcome by altering the inflammatory state. We previously found that KCa3.1 blockers inhibit the proinflammatory "classical" activation of microglia, reducing production of reactive oxygen and nitrogen species and their ability to kill neurons *in vitro* and *in vivo* (Kaushal et al., 2007).

Given the broad contributions of KCa3.1 to cell functions, it is important to understand how the channel is regulated. KCa3.1 requires calmodulin (CaM) binding to a region of the C terminus, usually called the CaM-binding domain (CaMBD), which opens the channel (Fanger et al., 1999; Khanna et al., 1999), and facilitates channel assembly and trafficking to the plasma membrane (Joiner et al., 2001). Here, we focused on post-translational modulation, and on the CaMBD, which contains three putative phosphorylation sites that are conserved across mouse, rat, and human KCa3.1: a cAMP protein kinase (PKA) site (rat S332; human S334), a PKC site (T329), and a casein kinase II site (S365). PKA is especially interesting because it phosphorylated the S332 site of a CaMBD fusion protein (Neylon et al., 2004).

Received March 13, 2014; revised Aug. 25, 2014; accepted Aug. 27, 2014.

Author contributions: R.W. and L.C.S. designed research; R.W. performed research; R.W. and L.C.S. analyzed data; R.W. and L.C.S. wrote the paper.

This work was supported by Heart and Stroke Foundation Grant HSF 00493. We thank Roger Ferreira for crucial help with Fura-2 and single-channel recordings and analysis; Dr. Shuzo Sugita for very helpful advice and use of equipment for gene manipulation; Anna Han for help with mutagenesis; Doris Lam for help with Western immunoblotting; and Xiaoping Zhu for help preparing microglial cells each week.

The authors declare no competing financial interests.

Correspondence should be addressed to Dr. Lyanne C. Schlichter, Toronto Western Research Institute, Krembil Discovery Tower, Room 7KD-417, 60 Leonard Street, Toronto, Ontario M5T 2S8, Canada. E-mail: schlicht@uhnres.utoronto.ca

DOI:10.1523/JNEUROSCI.1008-14.2014

Copyright © 2014 the authors 0270-6474/14/3413371-13\$15.00/0

However, PKA regulation studies have provided conflicting results for both native and 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 (Schröder 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). Here, we compared PKA-dependent regulation of native rat KCa3.1 (in microglia) and cloned human KCa3.1 channels. We 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 Ca²⁺ signaling in alternative-activated microglia.

Materials and Methods

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

Primary rat microglia. Cultures of essentially pure microglia were prepared according to our standard protocols (Kaushal et al., 2007; Sivagnanam et al., 2010; Lively and Schlichter, 2013). In brief, the brains of 1- to 2-d-old Sprague Dawley pups of either sex (Charles River) were harvested, and the brains were minced in cold minimum essential medium (MEM) (Invitrogen) after removing the meninges.

The dissociated tissue was then centrifuged (300 × g, 10 min) and resuspended in MEM with 10% FBS (Wisent) and 0.05 mg/ml gentamycin (Invitrogen). Medium was replaced after 2 d of growth to remove cellular debris and nonadherent 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 nonadherent microglia was centrifuged (300 × g, 10 min), and the cells were resuspended in MEM with 2% FBS. Microglia were plated at 6 × 10⁴ cells/cover slip for 24 h and then exposed to 20 ng/ml rat recombinant interleukin-4 (IL-4; R&D Systems) for 6 d (37°C, 5% CO₂) before patch-clamp analysis. We have previously shown that this treatment shifts rat microglia from nonactivated (Sivagnanam et al., 2010) to an alternative-activated state (Liu et al., 2013; Lively and Schlichter, 2013).

MLS-9 cells. Many years ago, we 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. We have used MLS-9 cells extensively to study K⁺ and Cl[−] channels (Cayabyab et al., 2000, 2002; Cayabyab and Schlichter, 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 PBS with 0.25% trypsin and 1 mM EDTA, washed with MEM, centrifuged (300 × g, 10 min), and resuspended in MEM. Cells were plated at 4.5 × 10⁴ cells/cover slip for patch-clamp analysis.

HEK293 cells and transfection. HEK293 cells (human embryonic kidney cells of female origin) were grown for several days in DMEM (Invitrogen) with high glucose, 10% FBS, 100 mg/L penicillin-streptomycin (Invitrogen). They were harvested in PBS with 0.25% trypsin and 1 mM EDTA, washed with MEM, centrifuged (300 × g, 10 min), and resuspended in MEM. Cells were plated at 5.5 × 10⁴ cells/cover slip for patch-clamp analysis. The human KCa3.1 gene (hKCa3.1) was subcloned into the expression vector, pCMV6-XL5 (OriGene). The plasmids, pCMV6-XL5-hKCa3.1 and pEF-GFP, were cotransfected 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): forward primer, CATACTCGCAGGAAGGAGGCGCATGCTGCCCG-

CAGGCAT; reverse primer, CCTGCGGGCAGCATGCGCCTCC TTCCTGCGAGTATG. The italicized 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).

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.

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 the following (in mM): 100 K-aspartate, 40 KCl, 1 MgCl₂, 0.85 CaCl₂, 0.1 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) using a Narishige puller (Narishige Scientific). Recordings were made with an Axopatch 200A amplifier (Molecular Devices), 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.

Single-channel recordings. 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 cutoff frequency). Recording pipettes (6–8 MΩ) pulled from thin-walled borosilicate glass were filled with an extracellular solution containing the following (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 the following (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. NP_o, the product of the apparent number of active channels in the patch (*N*) and the KCa3.1 channel open probability (*P*_o), was calculated in pClamp by dividing the mean total current (*I*) by the single-channel current amplitude (*i*), where NP_o = *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.

Calmodulin affinity chromatography. *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, we used a method modified from Khanna et al. (1999). In brief, the dishes were washed twice in PBS containing Ca²⁺ and Mg²⁺, and solubilization buffer was added (Bio-Rad), which contained 50 mM Tris-HCl, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1% Triton X-114, pH 7.4. This buffer also contained a protease inhibitor mixture (1:100 P8340; Sigma-Aldrich) with leupeptin, aprotinin, pepstatin A, bestatin, AEBSF, and E-64, and a phosphatase inhibitor mixture (2 μg/ml PIC3; Sigma-Aldrich) that contained cantharidin, p-bromolevamisole oxalate, and calyculin A. Insoluble material was removed by centrifugation (300 × g, 10 min). For protein purification and CaM binding, the supernatant was warmed to 37°C for 3 min and then centrifuged (300 × g, 5 min). The detergent phase (bottom) was diluted 9:1 in a binding buffer (Santa Cruz Biotechnology) containing 50 mM Tris, 150 mM NaCl, 1 mM MgCl₂, 0.5 mM CaCl₂, pH 7.4. Total protein samples were first precleared (1 h, 4°C)

with 50 μ l 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 Laboratories). As a loading control, GAPDH was analyzed using an anti-GAPDH antibody (1:5000; Cedarlane Laboratories). 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).

Intracellular free Ca²⁺. The fura-2 imaging methods were the same as previously described (Ferreira and Schlichter, 2013). Specifically, primary rat microglia growing on glass coverslips ($\sim 6 \times 10^4$ cells per 15 mm diameter coverslip) were incubated at room temperature with 3.5 μ g/ml fura-2 AM (Invitrogen) for 40 min in the dark. For recording, a coverslip was mounted in a 300 μ l volume perfusion chamber (model RC-25, Warner Instruments) that contained the same bath solution as for whole-cell recordings (see above). The effects of different treatments on UTP-evoked Ca²⁺ 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), and Northern Eclipse image acquisition software (Empix Imaging). A Lambda DG-4 Ultra High Speed Wavelength Switcher (Sutter Instruments) 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 Ca²⁺ concentration was calculated from the standard equation (Grynkiewicz et al., 1985).

Chemicals. The PKA activator Sp-8-Br-cAMPS and the PKA inhibitor Rp-8-Br-cAMPS were purchased from EMD Millipore. The adenosine A2 receptor agonist CGS21680 was from R&D Systems. The catalytic subunit of human recombinant cAMP-dependent protein kinase (cPKA) was from Sigma-Aldrich. All other chemicals, unless specified, were from Sigma-Aldrich.

Statistical analysis. Data are expressed as mean $\pm$ SEM. For multiple comparisons to assess treatment effects on currents, one-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 version 6.01 (GraphPad Software), and statistical significance was determined at $p < 0.05$.

Results

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

First, we 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 μ M intracellular Ca²⁺. Therefore, we added 100 nM apamin to the bath to block SK3 for all recordings from MLS-9 cells. As we previously showed (Ferreira and Schlichter, 2013; Liu et al., 2013), establishing a whole-cell recording with 1.1 μ M free intracellular Ca²⁺ was not sufficient to activate a KCa3.1 current in MLS-9 cells (Fig. 1A). 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 Ca²⁺ sensitivity of the channel by as much as an order of magnitude (Pedersen et al., 1999; Syme et al., 2000; Pedarzani et al., 2001). We next quantified the KCa3.1 current amplitude as the component that was blocked by 1 μ M TRAM-34; essentially, no current remained after TRAM-34 addition. As

Figure 1. Regulation of KCa3.1 by PKA in MLS-9 cells. The voltage protocol, which applies to all current traces for MLS-9 cells, was as follows: 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 μ M free Ca²⁺ (trace marked ctrl), followed by bath addition of the channel activator, 300 μ M 1-EBIO (EBIO), or 1-EBIO + 1 μ M 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: after 30–45 min incubation at 37°C with the PKA activator, 10 μ M Sp-cAMPS (Sp) or the PKA inhibitor, 10 μ M Rp-cAMPS (Rp). **C**, Summarized data from a population study with treatments as in **A**, **B** (first 4 bars). In each cell, 300 μ M 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 μ M TRAM-34. For the two right-hand bars, 10.9 μ M intracellular free Ca²⁺ alone was used to activate the KCa3.1 current and to compare the effect of 30–45 min incubation with 10 μ M Sp-cAMPS. Data are mean $\pm$ SEM for the number of cells indicated on each bar, and the three conditions were compared. **** $p < 0.0001$ (one-way ANOVA, with Tukey's *post hoc* test). †† $p < 0.01$ (one-way ANOVA, with Tukey's *post hoc* test).

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 cAMP analog that is resistant to degradation by phosphodiesterases. Conversely, effects of inhibiting PKA were examined using Rp-8-Br-cAMPS (Rp-cAMPS). Although this diastereomer also binds to the cAMP site on the regulatory subunit, it does not cause the requisite conformational change, and thus acts as a specific, competitive PKA inhibitor (Dostmann et al., 1990). MLS-9 cells were preincubated (37°C; 30–45 min) with either 10 μM Sp-cAMPS or 10 μM Rp-cAMPS, and then patch-clamp recordings were performed, as in Figure 1A. Before adding the channel activator 1-EBIO, no current was seen in cells treated with either Sp-cAMPS or Rp-cAMPS (Fig. 1B). 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 pretreated 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. 1C). Activating PKA with Sp-cAMPS reduced the current $\sim 70\%$ (to 7.3 ± 0.7 pA/pF; $n = 14$; $p < 0.0001$), whereas inhibiting PKA with Rp-cAMPS increased it $\sim 40\%$ (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, we cannot say whether phosphorylation was on the channel itself (but see below).

We confirmed that 1-EBIO activated KCa3.1 by increasing the Ca^{2+} sensitivity approximately 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 our previous finding that the EC_{50} for Ca^{2+} -dependent activation of KCa3.1 in MLS-9 cells is 7.6 ± 0.7 μM (Ferreira and Schlichter, 2013). Because excessively high intracellular Ca^{2+} can have harmful effects (e.g., activation of Ca^{2+} -dependent proteases such as calpain) (Castillo and Babson, 1998), for subsequent experiments on MLS-9 cells and primary microglia, we used 1 μM Ca^{2+} with 1-EBIO to activate the current.

The PKA inhibitor, PKI_{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, we added the highly selective, nonmyristoylated version of the PKA inhibitor PKI_{14–22} to the pipette solution. This peptide binds directly to the catalytic subunit of PKA and prevents it from phosphorylating target molecules (Parang et al., 2001). Thus, its mechanism is distinct from PKA inhibition by Rp-cAMPS. As for Figure 1, the pipette solution contained 1.1 μM free Ca^{2+} and the KCa3.1 current was activated by 300 μM 1-EBIO. Figure 2 shows representative current traces (Fig. 2A,B), time courses (Fig. 2C), and the summarized current densities (Fig. 2D). 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 were 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_{14–22} with 1 μM Ca^{2+} in the pipette did not itself activate KCa3.1 current. Instead, PKI_{14–22} prevented Sp-cAMPS from decreasing the current; thus, KCa3.1 inhibition by Sp-cAMPS depends on PKA.

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, we next assessed whether primary rat microglia show the same KCa3.1 regulation by PKA as MLS-9 cells. We recently 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). Therefore, we cultured rat microglia with 20 ng/ml of IL-4 for 6 d 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 2E, F, representative time courses illustrated in Figure 2G, and the current densities are summarized in Figure 2H. 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. 2E,F), and a similar amplitude (current density; Fig. 2H). Importantly, as for MLS-9 cells, a time-dependent inhibition by bath applied Sp-cAMPS was seen (Fig. 2G). 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 min period (to 12.5 ± 1.6 pA/pF; $n = 7$; $p < 0.01$). Again, we compared separate microglia with and without the PKA inhibitor PKI_{14–22} in the pipette. The control current was 25.8 ± 4.9 pA/pF; and in cells containing PKI_{14–22}, 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.

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. We 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 CaMBD at S332 in rat and mouse and S334 in the human isoform. We 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 our findings from the rat to the human isoform, we 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

Figure 2. 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 Ca^{2+} (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 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 A, B. 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 A–C. Data are mean $\pm$ 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 Ca^{2+} and the PKA inhibitory peptide PKI₁₄₋₂₂ (10 μM) in the pipette. The traces are labeled as in 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 E–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 mean $\pm$ 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).

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 we inactivated using a holding potential of 0 mV. 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 (data not shown).

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+} . Unlike microglia, transfected HEK cells did not require a positive gating modulator, such as 1-EBIO. This is consistent with our 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 (data not shown).

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 3A, B, time courses in Figure 3C, and the summarized current densities in Figure 3D. Although no KCa current was seen in untreated transfected HEK293 cells, we 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. 3A, C) from 52.6 ± 4.2 to 22.9 ± 4.8 pA/pF (*n* = 6; *p* < 0.0001) (Fig. 3D). In stark contrast, Sp-cAMPS had no effect on the S334A mutant (Fig. 3B–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, slightly increased the *wt* hKCa3.1 current but had no effect on the S334A mutant (Fig. 3E–H). Rp-cAMPS increased the current by ~8%, from 53.9 ± 4.1 to 58.3 ± 3.8 pA/pF (*n* = 13; *p* < 0.05) (Fig. 3H), which was less than the ~40% increase in MLS-9 cells (Fig. 1C). Two possible explanations are that,

in HEK293 cells, the initial level of channel phosphorylation is lower or less dephosphorylation occurs during whole-cell recordings. We believe the former to be the case (see below).

Because we 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), we 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_{14–22}. When inside-out patches were excised from HEK cells expressing *wt* hKCa3.1 or the S334A mutant, into a bath (intracellular) solution containing ATP and 10 μ M Ca²⁺ to maximally activate the channels (Gerlach et al., 2000), we observed one or two channels in each patch (one channel in the example in Fig. 4A). 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, 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 Materials and Methods). In control recordings from *wt* hKCa3.1, P_o was 0.52 ± 0.50 ($n = 3$; data not shown), and channel activity remained stable for at least 10 min following excision if no treatment was added. For *wt* hKCa3.1, after perfusing 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 μ M 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. 4B), and the single-channel amplitude was not affected: that is, 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. 4B). In contrast, with the S334A mutant csPKA had no effect (Fig. 4C,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 ($p < 0.001$).

Figure 3. 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 μ M 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 μ M Sp-cAMPS; Sp), and after adding 1 μ M TRAM-34 + Sp-cAMPS (TRAM). **B**, An HEK293 cell transfected with the S334A hKCa3.1 mutant, with the currents labeled as in **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 μ M 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 mean $\pm$ SEM for the number of cells indicated. **** $p < 0.0001$ for *wt* hKCa3.1 (paired Student's *t* test). $p = 0.13$ for the S334A mutant (paired Student's *t* test). **E**, A recording of *wt* hKCa3.1 shows the control current (ctrl), ~ 8 min after bath addition of the PKA inhibitor (10 μ M Rp-cAMPS; Rp), and after adding 1 μ M TRAM-34 with Rp-cAMPS (TRAM). **F**, A recording of the S334A hKCa3.1 mutant, with the currents labeled as in **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 are mean $\pm$ SEM. * $p = 0.027$ for *wt* hKCa3.1 (paired Student's *t* test). $p = 0.31$ for the S334A mutant (paired Student's *t* test).

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

CaM binds to the CaMBD of the KCa3.1 channel, and this is essential for the channel to respond to Ca²⁺ and open (see Introduction). We 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

Figure 4. PKA reduces single-channel activity in inside-out patches for wt hKCa3.1, but not the S334A mutant. Inside-out patches were excised from HEK293 cells transfected with wt hKCa3.1 or the S334A mutant. Channel activity was recorded with bath and pipette solutions containing the same K⁺ concentrations, with 10 μ M 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**, wt hKCa3.1. The control current (top) was recorded, and then the csPKA (10 μ M) was perfused into the bath (middle). At the end of the recording, 1 μ M TRAM-34 was also perfused into the bath (bottom). **B**, Summarized data show the probability of opening P_o (see Materials and Methods) for wt hKCa3.1 in control bath 4–6 min after bath addition of csPKA, and after adding 1 μ M TRAM-34. Values are mean $\pm$ SEM ($n = 6$). *** $p < 0.001$ compared with control recordings (one-way ANOVA with Tukey's *post hoc* test). **C**, S334A hKCa3.1. Channel activity was recorded as in **A**. **D**, Summarized data show P_o for the S334A mutant in control bath 4–6 min after adding csPKA (10 μ M), and after adding 1 μ M TRAM-34. Values are mean $\pm$ SEM ($n = 5$). $p = 0.46$ for csPKA compared with controls (one-way ANOVA with Tukey's *post hoc* test). *** $p < 0.001$ for TRAM-34 (one-way ANOVA with Tukey's *post hoc* test).

into HEK293 cells, followed by Western immunoblotting. Rather than relying on a KCa3.1 antibody, we added a DDK tag to the C terminus of the channels and used an anti-DDK antibody to detect and quantify KCa3.1. We 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 μ M Sp-cAMPS (40–60 min, 37°C) before harvesting protein. Total KCa3.1 protein expression in cell lysates was compared (Fig. 5A), and showed that channel expression was not affected by Sp-cAMPS treatment for either wt hKCa3.1 or the S334A mutant. This indicates that 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. 5B). In cells transfected with wt hKCa3.1, treatment with Sp-cAMPS dramatically reduced CaM-mediated channel pull-down: that is, to $25 \pm 5\%$ of the control level ($n = 3$; $p < 0.05$). The S334A mutation alone did not affect CaM binding to the channel ($103 \pm 24\%$ 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.

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 G_s subunit (Gao and Jacobson, 2007), whereas A2b can also act on G_q leading to IP₃ and DAG release, in addition to activating the PKA pathway (Ryzhov et al., 2006). Therefore, we 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, we used primary rat microglia, and HEK293 cells transfected with either wt hKCa3.1 or the S334A hKCa3.1 mutant. When used, cells were pretreated with the A2a receptor agonist, CGS21680. The endogenous KCa3.1 current in rat microglia

was activated using 1.1 μ M intracellular free Ca²⁺ and bath addition of 300 μ M 1-EBIO (as in Fig. 2). Figure 6A shows representative time courses for the KCa3.1 current in microglia without treatment, with CGS21680, or with CGS21680 + PKI_{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 $\sim 30\%$; from 35.0 ± 3.8 ($n = 5$) to 24.9 ± 2.1 pA/pF ($n = 7$; $p < 0.05$), whereas with CGS21680 + PKI_{14–22} combined, the current density was not affected (37.9 ± 5.2 pA/pF; $n = 4$; $p = 0.87$) (Fig. 6A). In HEK293 cells transfected with wt hKCa3.1, the current also decreased $\sim 30\%$ following A2a receptor stimulation from 67.8 ± 7.6 ($n = 4$) to 44.3 ± 5.4 pA/pF ($n = 6$; $p < 0.05$) (Fig. 6B). In contrast, A2a receptor stimulation had no effect in cells transfected with the S334A mutant (Fig. 6C). 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.

Figure 5. 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 nontransfected 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 μ M 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. Error bars indicate mean $\pm$ SEM; $n = 3$ each. **B**, Protein pulled down with CaM-agarose PD beads (see Materials and Methods). Left, Representative Western blot showing wt hKCa3.1 and S334A hKCa3.1 mutant. Cell treatment and protein detection were as in **A**. Right, Summary of amount of KCa3.1 protein pulled down. Data are expressed and normalized as in **A**. Error bars indicate mean $\pm$ SEM; $n = 3$ each. * $p < 0.05$, differences produced by Sp-cAMPS treatment for wt hKCa3.1 (ANOVA with Tukey's *post hoc* test). † $p < 0.05$, between wild-type and mutant channels after Sp-cAMPS treatment (ANOVA with Tukey's *post hoc* test).

PKA-mediated inhibition of KCa3.1 reduces Ca²⁺ influx in primary microglia

KCa3.1 activation is expected to maintain a negative membrane potential, which will increase Ca²⁺ influx through nonvoltage gated Ca²⁺-release-activated Ca²⁺ (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 Ca²⁺ entry through CRAC channels, metabotropic P₂Y₂ purinergic receptors were activated with UTP, as described previously (Ferreira and Schlichter, 2013). Primary rat microglia were treated for 6 d with IL-4 (alternative activated), which increased the KCa3.1 current and its contribution to migration (Ferreira et al., 2014).

Figure 7A shows representative fura-2 traces for each treatment, and Figure 7B shows the summarized data. As we previously showed for MLS-9 cells (Ferreira and Schlichter, 2013), under control conditions, bath application of 100 μ M UTP evoked a short-lived peak in intracellular Ca²⁺ (release from stores), followed by a plateau phase (Ca²⁺ entry) and a return to the baseline. None of the treatments affected the initial brief spike. Thus, to reflect Ca²⁺ influx, the area under the curve (gray shaded) was integrated (340/380 ratio $\times$ time) and expressed in arbitrary units. First, we show that this Ca²⁺ plateau component is strongly dependent on KCa3.1 activity. That is, compared with the area under the curve in response to UTP in untreated microglia (905.3 ± 231.4 ; $n = 35$), 1 μ M TRAM-34 reduced the signal by $\sim 75\%$ (to 224.1 ± 36.4 ; $n = 21$; $p < 0.01$). Activating PKA with Sp-cAMPS reduced the UTP-evoked signal by $\sim 40\%$ to 501.8 ± 172.3 ($n = 19$; $p < 0.05$). The A2a receptor agonist

CGS21680 similarly reduced the signal to 514.7 ± 149.6 ($n = 8$; $p < 0.05$). If microglia were pretreated with the membrane-permeant PKA inhibitor, myristoylated PKI_{14–22}, these effects were abolished. The signal was not significantly different from the control value; that is, with Sp-cAMPS, the area under the curve was 1074.7 ± 297.3 ($n = 9$; $p = 0.81$) and with CGS21680 it remained at 834.1 ± 292.8 ; $n = 5$; $p = 0.76$). TRAM-34 reduced the Sp-cAMPS- and CGS21680-evoked signals to the same level as control cells with TRAM-34.

Discussion

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. 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 analogs, and some directly applied PKA. Even the means of activating the channels varied from simply adding ATP without using elevated intracellular Ca²⁺, to high Ca²⁺ alone, or Ca²⁺ 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).

Figure 6. Activating PKA through the A2a receptor decreases the KCa3.1 current. All recordings were made in the whole-cell configuration with $1.1 \mu\text{M}$ 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 $\pm$ SEM) is shown in the right-hand panels for the number of cells noted on each bar. At the end of each recording, $1 \mu\text{M}$ TRAM-34 was used to verify that the entire current was KCa3.1. When used, cells were pretreated (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 $300 \mu\text{M}$ 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 pretreated with CGS21680, and one that was pretreated with PKI₁₄₋₂₂ ($10 \mu\text{M}$, 15 min , 37°C) before adding CGS21680. At $1 \mu\text{M}$, TRAM-34 fully blocked the currents. Right, Summary from a population study of microglia that were untreated, CGS21680-treated, or treated with PKI₁₄₋₂₂ + 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 $1.1 \mu\text{M}$ 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 **B** ($p = 0.58$).

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 $1.2 \mu\text{M}$ Ca^{2+} , 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 $10 \mu\text{M}$ Ca^{2+} , 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 our results. In whole-cell and inside-out patch recordings from HEK293 cells expressing wt hKCa3.1 (but not the S334A mutant), we 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 (Schröder 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. We identified native KCa3.1 currents based on their biophysical and pharmacological properties, including full block by $1 \mu\text{M}$ TRAM-34, which is KCa3.1-selective. PKA activation reduced the native KCa3.1 current, and this was prevented by a PKA inhibitor (PKI₁₄₋₂₂). 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 (PKI₁₄₋₂₂, 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, PKI₅₋₂₄. In human erythrocytes, the current that was activated by $2 \mu\text{M}$ free Ca^{2+} spontaneously ran down was restored by a mixture of ATP, cAMP, and theophylline, and this was blocked by PKI₅₋₂₄ (Pellegrino and Pellegrini, 1998). In rat acinar cells, charybdotoxin-sensitive channels were

activated by $1 \mu\text{M}$ Ca^{2+} ; and although 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 $0.1 \mu\text{M}$, both cAMP and forskolin increased the current.

Through CaM-binding analysis, we demonstrate 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. The PKA-evoked decrease in open probability of wt hKCa3.1 channels in inside-out

Figure 7. PKA-mediated inhibition of KCa3.1 reduces Ca²⁺ influx in primary microglia. Primary rat microglia were stimulated for 6 d with 20 ng/ml IL-4 and then incubated with fura-2 AM (3.5 μ M) at room temperature for 45 min in the dark. Images were alternately acquired every 4 s at 340 and 380 nm excitation wavelengths. **A**, Representative recordings, in which 100 μ M UTP was bath applied during the period marked by the horizontal bar. The area under the curve is shaded in gray. The treatments were as follows: control bath; TRAM-34 (1 μ M, 5 min, room temperature); Sp-cAMPS (10 μ M, 30–45 min, 37°C); CGS21680 (300 nM, 30–45 min, 37°C); Sp-cAMPS + PKI_{14–22} (10 μ M, 1 h, 37°C); CGS21680 + PKI_{14–22}; Sp-cAMPS + TRAM-34; and CGS21680 + TRAM-34. **B**, Summarized data (mean $\pm$ SEM) for the number of cells indicated on each bar. One symbol indicates $p < 0.05$; two symbols indicate $p < 0.01$ (ANOVA with Tukey's *post hoc* test): *Treatments versus controls. #Each PKA activator with or without TRAM-34. †Each activator with and without PKI_{14–22}.

patches (but not the S334A mutant) strongly supports a direct effect on channel gating, rather than on trafficking. These results also imply that CaM binds reversibly to KCa3.1, which must occur for the channels to bind to the CaM-agarose beads. Our earlier work 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). This reversibility should be considered in comparing the crystal structure of the related hKCa2.2 channel, where the corresponding residue

(H446) lies very close to the Ca²⁺-independent CaM-binding sequence (Schumacher et al., 2001). In KCa3.1, S334 phosphorylation might affect CaM binding indirectly, through a local conformation change.

hKCa3.1 channels expressed in HEK cells were activated by simply raising Ca²⁺ to 1 μ M, as we previously observed for hKCa3.1 expressed in CHO cells (Joiner et al., 2001), whereas the native channels in microglia also required a gating modulator (Liu et al., 2013; Ferreira et al., 2014). Such modulators increase

the channel Ca²⁺ 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). Our results are consistent with a change in Ca²⁺ sensitivity by 1-EBIO but not by PKA. That is, the current was fully activated by 10.9 μ M Ca²⁺ alone or by 1.1 μ M Ca²⁺ plus 1-EBIO, whereas PKA activation decreased the current equally at 1.1 and 10.9 μ M Ca²⁺. We do not know why the current in microglia is less sensitive to Ca²⁺ than in other cell types, but differential CaM binding is one possibility.

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; Pearce 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). We previously reported that blocking KCa3.1 with TRAM-34 is neuroprotective *in vitro* and *in vivo* by reducing the proinflammatory, classical activation of microglia (Kaushal et al., 2007). In microglia, PKA is activated in response to a wide range of agonists acting on receptors that are coupled to G_s 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 increased microglial phagocytosis of amyloid β peptide at normal cAMP levels but inhibited it when cAMP was elevated) (Makranz et al., 2006). In microglia, PKA activation can increase CREB and NF κ B binding to DNA (Min et al., 2004), regulate transcription of NADPH oxidase, which produces reactive oxygen species (Savchenko, 2013), reduce TLR2/4 agonist-induced classical activation (Park et al., 2013), and reduce adhesion and migration (Liao et al., 2005). To further address the physiological relevance of our finding that PKA inhibits human and rat KCa3.1, we focused on the G_s-coupled A2a receptor (A2aR) because it activates PKA and is present in both microglia (see below) and HEK293 cells (Atwood et al., 2011). We 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 brain-derived neurotrophic factor, 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 proinflammatory is that A2aR antagonists reduced p38 MAPK-dependent microglial activation after ischemia (Melani et al., 2006). While our 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, we found that activating PKA directly or through the adenosine 2a receptor reduced the Ca²⁺ entry mediated by Ca²⁺-release-activated Ca²⁺ channels. Because microglial cells 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 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 (G_s) or inhibitory (G_i) 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.

References

- Ashmole I, Duffy SM, Leyland ML, Morrison VS, Begg M, Bradding P (2012) CRACM/Orai ion channel expression and function in human lung mast cells. *J Allergy Clin Immunol* 129:1628–1635. [CrossRef Medline](#)
- Atwood BK, Lopez J, Wager-Miller J, Mackie K, Straiker A (2011) Expression of G-protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics* 12:1–14. [CrossRef Medline](#)
- Balut CM, Hamilton KL, Devor DC (2012) Trafficking of intermediate (KCa3.1) and small (KCa2.x) conductance, Ca²⁺-activated K⁺ channels: a novel target for medicinal chemistry efforts? *Chem Med Chem* 7:1741–1755. [CrossRef Medline](#)
- Bi D, Toyama K, Lemaître V, Takai J, Fan F, Jenkins DP, Wulff H, Gutterman DD, Park F, Miura H (2013) The intermediate conductance calcium-activated potassium channel KCa3.1 regulates vascular smooth muscle cell proliferation via controlling calcium-dependent signaling. *J Biol Chem* 288:15843–15853. [CrossRef Medline](#)
- Bouhy D, Ghasemlou N, Lively S, Redensek A, Rathore KI, Schlichter LC, David S (2011) Inhibition of the Ca²⁺-dependent K⁺ channel, KCNN4/KCa3.1, improves tissue protection and locomotor recovery after spinal cord injury. *J Neurosci* 31:16298–16308. [CrossRef Medline](#)
- Cai D, Qiu J, Cao Z, McAtee M, Bregman BS, Filbin MT (2001) Neuronal cyclic AMP controls the developmental loss in ability of axons to regenerate. *J Neurosci* 21:4731–4739. [Medline](#)
- Castillo MR, Babson JR (1998) Ca²⁺-dependent mechanisms of cell injury in cultured cortical neurons. *Neuroscience* 86:1133–1144. [CrossRef Medline](#)
- Cayabyab FS, Schlichter LC (2002) Regulation of an ERG K⁺ current by Src tyrosine kinase. *J Biol Chem* 277:13673–13681. [CrossRef Medline](#)
- Cayabyab FS, Khanna R, Jones OT, Schlichter LC (2000) Suppression of the rat microglia Kv1.3 current by src-family tyrosine kinases and oxygen/glucose deprivation. *Eur J Neurosci* 12:1949–1960. [CrossRef Medline](#)
- Cayabyab FS, Tsui FW, Schlichter LC (2002) Modulation of the ERG K⁺ current by the tyrosine phosphatase, SHP-1. *J Biol Chem* 277:48130–48138. [CrossRef Medline](#)
- Chimote AA, Hajdu P, Kucher V, Boiko N, Kuras Z, Szilagyi O, Yun YH, Conforti L (2013) Selective inhibition of KCa3.1 channels mediates adenosine regulation of the motility of human T cells. *J Immunol* 191:6273–6280. [CrossRef Medline](#)
- Choi S, Kim MY, Joo KY, Park S, Kim JA, Jung JC, Oh S, Suh SH (2012) Modafinil inhibits KCa3.1 currents and muscle contraction via a cAMP-dependent mechanism. *Pharmacol Res* 66:51–59. [CrossRef Medline](#)
- Christova TY, Orosz F, Ovádi J (1996) Interaction between D-glyceraldehyde-3-phosphate dehydrogenase and calmodulin. *Biochem Biophys Res Commun* 228:272–277. [CrossRef Medline](#)
- Dostmann WR, Taylor SS, Genieser HG, Jastorff B, Døskeland SO, Ogrëid D (1990) Probing the cyclic nucleotide binding sites of cAMP-dependent protein kinases I and II with analogs of adenosine 3',5'-cyclic phosphorothioates. *J Biol Chem* 265:10484–10491. [Medline](#)
- Ducharme G, Newell EW, Pinto C, Schlichter LC (2007) Small-conductance Cl[−] channels contribute to volume regulation and phagocytosis in microglia. *Eur J Neurosci* 26:2119–2130. [CrossRef Medline](#)
- Eder C, Klee R, Heinemann U (1997) Pharmacological properties of Ca²⁺-activated K⁺ currents of ramified murine brain macrophages. *Naunyn Schmiedeberg Arch Pharmacol* 356:233–239. [CrossRef Medline](#)
- Edwards G, Félétou M, Weston AH (2010) Endothelium-derived hyperpolarising factors and associated pathways: a synopsis. *Pflugers Arch* 459:863–879. [CrossRef Medline](#)
- Engbers JD, Anderson D, Tadayonnejad R, Mehaffey WH, Molineux ML, Turner RW (2011) Distinct roles for I_T and I_H in controlling the frequency and timing of rebound spike responses. *J Physiol* 589:5391–5413. [CrossRef Medline](#)
- Fanger CM, Ghanshani S, Logsdon NJ, Rauer H, Kalman K, Zhou J, Beckingham K, Chandy KG, Cahalan MD, Aiyar J (1999) Calmodulin mediates calcium-dependent activation of the intermediate conductance KCa channel, IKCa1. *J Biol Chem* 274:5746–5754. [CrossRef Medline](#)

- Ferreira R, Lively S, Schlichter LC (2014) IL-4 type 1 receptor signaling up-regulates KCNN4 expression, and increases the KCa3.1 current and its contribution to migration of alternative-activated microglia. *Front Cell Neurosci* 8:183. [CrossRef Medline](#)
- Ferreira R, Schlichter LC (2013) Selective activation of KCa3.1 and CRAC channels by P₂Y₂ receptors promotes Ca²⁺ signaling, store refilling and migration of rat microglial cells. *PLoS One* 8:1–12. [CrossRef Medline](#)
- Gao ZG, Jacobson KA (2007) Emerging adenosine receptor agonists. *Expert Opin Emerging Drugs* 12:479–492. [CrossRef Medline](#)
- Gerlach AC, Gangopadhyay NN, Devor DC (2000) Kinase-dependent regulation of the intermediate conductance, calcium-dependent potassium channel, hIK1. *J Biol Chem* 275:585–598. [CrossRef Medline](#)
- Gomes C, Ferreira R, George J, Sanches R, Rodrigues DI, Gonçalves N, Cunha RA (2013) Activation of microglial cells triggers a release of brain-derived neurotrophic factor (BDNF) inducing their proliferation in an adenosine A2A receptor-dependent manner: A2A receptor blockade prevents BDNF release and proliferation of microglia. *J Neuroinflammation* 10:16. [CrossRef Medline](#)
- Gryniewicz G, Poenie M, Tsien RY (1985) A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440–3450. [Medline](#)
- Hayashi M, Kunii C, Takahata T, Ishikawa T (2004) ATP-dependent regulation of SK4/IK1-like currents in rat submandibular acinar cells: possible role of cAMP-dependent protein kinase. *Am J Physiol Cell Physiol* 286:C635–C646. [CrossRef Medline](#)
- Jacobson KA, Gao ZG (2006) Adenosine receptors as therapeutic targets. *Nat Rev Drug Discov* 5:247–264. [CrossRef Medline](#)
- Jarvis MF, Schulz R, Hutchison AJ, Do UH, Sills MA, Williams M (1989) [³H]CGS21680, a selective A2 adenosine receptor agonist directly labels A2 receptors in rat brain. *J Pharmacol Exp Ther* 251:888–893. [Medline](#)
- Jensen BS, Hertz M, Christophersen P, Madsen LS (2002) The Ca²⁺-activated K⁺ channel of intermediate conductance: a possible target for immune suppression. *Expert Opin Ther Targets* 6:623–636. [CrossRef Medline](#)
- Joiner WJ, Khanna R, Schlichter LC, Kaczmarek LK (2001) Calmodulin regulates assembly and trafficking of SK4/IK1 Ca²⁺-activated K⁺ channels. *J Biol Chem* 276:37980–37985. [CrossRef Medline](#)
- Kaushal V, Koeberle PD, Wang Y, Schlichter LC (2007) The Ca²⁺-activated K⁺ channel KCNN4/KCa3.1 contributes to microglia activation and nitric oxide-dependent neurodegeneration. *J Neurosci* 27:234–244. [CrossRef Medline](#)
- Khanna R, Chang MC, Joiner WJ, Kaczmarek LK, Schlichter LC (1999) hSK4/hIK1, a calmodulin-binding KCa channel in human T lymphocytes: roles in proliferation and volume regulation. *J Biol Chem* 274:14838–14849. [CrossRef Medline](#)
- Khanna R, Roy L, Zhu X, Schlichter LC (2001) K⁺ channels and the microglial respiratory burst. *Am J Physiol Cell Physiol* 290:C796–C806. [Medline](#)
- Köhler R, Kaistha BP, Wulff H (2010) Vascular KCa-channels as therapeutic targets in hypertension and restenosis disease. *Expert Opin Ther Targets* 14:143–155. [CrossRef Medline](#)
- Lam J, Wulff H (2011) The lymphocyte potassium channels Kv1.3 and KCa3.1 as targets for immunosuppression. *Drug Dev Res* 72:573–584. [CrossRef Medline](#)
- Latini S, Pedata F (2001) Adenosine in the central nervous system: release mechanisms and extracellular concentrations. *J Neurochem* 79:463–484. [CrossRef Medline](#)
- Lattin JE, Schröder K, Su AI, Walker JR, Zhang J, Wiltshire T, Saijo K, Glass CK, Hume DA, Kellie S, Sweet MJ (2008) Expression analysis of G protein-coupled receptors in mouse macrophages. *Immunome Res* 4:1–13. [CrossRef Medline](#)
- Liao H, Bu WY, Wang TH, Ahmed S, Xiao ZC (2005) Tenascin-R plays a role in neuroprotection via its distinct domains that coordinate to modulate the microglia function. *J Biol Chem* 280:8316–8323. [CrossRef Medline](#)
- Liu BS, Ferreira R, Lively S, Schlichter LC (2013) Microglial SK3 and SK4 currents and activation state are modulated by the neuroprotective drug, riluzole. *J Neuroimmune Pharmacol* 8:227–237. [CrossRef Medline](#)
- Lively S, Schlichter LC (2013) The microglial activation state regulates migration and roles of matrix-dissolving enzymes for invasion. *J Neuroinflammation* 10:1–14. [CrossRef Medline](#)
- Maezawa I, Jenkins DP, Jin BE, Wulff H (2012) Microglial KCa3.1 channels as a potential therapeutic target for Alzheimer's disease. *Int J Alzheimers Dis* 2012:1–8. [CrossRef Medline](#)
- Makranz C, Cohen G, Reichert F, Kodama T, Rotshenker S (2006) cAMP cascade (PKA, Epac, adenylyl cyclase, G_i, and phosphodiesterases) regulates myelin phagocytosis mediated by complement receptor-3 and scavenger receptor-AI/II in microglia and macrophages. *Glia* 53:441–448. [CrossRef Medline](#)
- 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. [CrossRef Medline](#)
- Min KJ, Yang MS, Jou I, Joe EH (2004) Protein kinase A mediates microglial activation induced by plasminogen and gangliosides. *Exp Mol Med* 36:461–467. [CrossRef Medline](#)
- Neylon CB, D'Souza T, Reinhart PH (2004) Protein kinase A inhibits intermediate conductance Ca²⁺-activated K⁺ channels expressed in *Xenopus* oocytes. *Pflugers Arch* 448:613–620. [CrossRef Medline](#)
- Nikulina E, Tidwell JL, Dai HN, Bregman BS, Filbin MT (2004) The phosphodiesterase inhibitor rolipram delivered after a spinal cord lesion promotes axonal regeneration and functional recovery. *Proc Natl Acad Sci U S A* 101:8786–8790. [CrossRef Medline](#)
- Ohana L, Newell EW, Stanley EF, Schlichter LC (2009) The Ca²⁺ release-activated Ca²⁺ current (I_{CRAC}) mediates store-operated Ca²⁺ entry in rat microglia. *Channels (Austin)* 3:129–139. [CrossRef Medline](#)
- Orr AG, Orr AL, Li XJ, Gross RE, Traynelis SF (2009) Adenosine A2A receptor mediates microglial process retraction. *Nat Neurosci* 12:872–878. [CrossRef Medline](#)
- Parang K, Till JH, Ablooglu AJ, Kohanski RA, Hubbard SR, Cole PA (2001) Mechanism-based design of a protein kinase inhibitor. *Nat Struct Biol* 8:37–41. [CrossRef Medline](#)
- 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 PKA and Nrf-2 signaling and inhibition of MAPK/STAT/NF-kappaB pathways. *Mol Nutr Food Res* 58:738–748. [CrossRef Medline](#)
- Pearse DD, Pereira FC, Marcillo AE, Bates ML, Berrocal YA, Filbin MT, Bunge MB (2004) cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. *Nat Med* 10:610–616. [CrossRef Medline](#)
- Pedarzani P, Mosbacher J, Rivard A, Cingolani LA, Oliver D, Stocker M, Adelman JP, Fakler B (2001) Control of electrical activity in central neurons by modulating the gating of small conductance Ca²⁺-activated K⁺ channels. *J Biol Chem* 276:9762–9769. [CrossRef Medline](#)
- Pedersen KA, Schröder RL, Skaaning-Jensen B, Strøbaek D, Olesen SP, Christophersen P (1999) Activation of the human intermediate-conductance Ca²⁺-activated K⁺ channel by 1-ethyl-2-benzimidazolinone is strongly Ca²⁺-dependent. *Biochim Biophys Acta* 1420:231–240. [CrossRef Medline](#)
- Pellegrino M, Pellegrini M (1998) Modulation of Ca²⁺-activated K⁺ channels of human erythrocytes by endogenous cAMP-dependent protein kinase. *Pflugers Arch* 436:749–756. [CrossRef Medline](#)
- Qiu J, Cai D, Dai H, McAtee M, Hoffman PN, Bregman BS, Filbin MT (2002) Spinal axon regeneration induced by elevation of cyclic AMP. *Neuron* 34:895–903. [CrossRef Medline](#)
- Ryzhov S, Goldstein AE, Biaggioni I, Feoktistov I (2006) Cross-talk between G_s- and G_q-coupled pathways in regulation of interleukin-4 by A(2B) adenosine receptors in human mast cells. *Mol Pharmacol* 70:727–735. [CrossRef Medline](#)
- Saura J, Angulo E, Ejarque A, Casadó V, Tusell JM, Moratalla R, Chen JF, Schwarzschild MA, Lluís C, Franco R, Serratos J (2005) Adenosine A2A receptor stimulation potentiates nitric oxide release by activated microglia. *J Neurochem* 95:919–929. [CrossRef Medline](#)
- Savchenko VL (2013) Regulation of NADPH oxidase gene expression with PKA and cytokine IL-4 in neurons and microglia. *Neurotox Res* 23:201–213. [CrossRef Medline](#)
- Schlichter LC, Kaushal V, Moxon-Emre I, Sivagnanam V, Vincent C (2010) The Ca²⁺ activated SK3 channel is expressed in microglia in the rat striatum and contributes to microglia-mediated neurotoxicity in vitro. *J Neuroinflammation* 7:1–15. [CrossRef Medline](#)
- Schröder RL, Jensen BS, Strøbaek D, Olesen SP, Christophersen P (2000) Activation of the human, intermediate-conductance, Ca²⁺-activated K⁺

- channel by methylxanthines. *Pflugers Arch* 440:809–818. [CrossRef Medline](#)
- Schumacher MA, Rivard AF, Bächinger HP, Adelman JP (2001) Structure of the gating domain of a Ca²⁺-activated K⁺ channel complexed with Ca²⁺/calmodulin. *Nature* 410:1120–1124. [CrossRef Medline](#)
- Schwab A, Nechiporuk-Zloy V, Fabian A, Stock C (2007) Cells move when ions and water flow. *Pflugers Arch* 453:421–432. [CrossRef Medline](#)
- Sivagnanam V, Zhu X, Schlichter LC (2010) Dominance of *E. coli* phagocytosis over LPS in the inflammatory response of microglia. *J Neuroimmunol* 227:111–119. [CrossRef Medline](#)
- Spencer T, Filbin MT (2004) A role for cAMP in regeneration of the adult mammalian CNS. *J Anat* 204:49–55. [CrossRef Medline](#)
- Srivastava S, Zhdanova O, Di L, Li Z, Albaqumi M, Wulff H, Skolnik EY (2008) Protein histidine phosphatase 1 negatively regulates CD4 T cells by inhibiting the K⁺ channel KCa3.1. *Proc Natl Acad Sci U S A* 105:14442–14446. [CrossRef Medline](#)
- Syme CA, Gerlach AC, Singh AK, Devor DC (2000) Pharmacological activation of cloned intermediate- and small-conductance Ca²⁺-activated K⁺ channels. *Am J Physiol Cell Physiol* 278:C570–C581. [Medline](#)
- Vogalis F, Harvey JR, Furness JB (2003) PKA-mediated inhibition of a novel K⁺ channel underlies the slow after-hyperpolarization in enteric AH neurons. *J Physiol* 548:801–814. [CrossRef Medline](#)
- von Hahn T, Thiele I, Zingaro L, Hamm K, Garcia-Alzamora M, Köttgen M, Bleich M, Warth R (2001) Characterisation of the rat SK4/IK1 K⁺ channel. *Cell Physiol Biochem* 11:219–230. [CrossRef Medline](#)
- Wang H, Gao X, Yang JJ, Liu ZR (2013) Interaction between p68 RNA helicase and Ca²⁺-calmodulin promotes cell migration and metastasis. *Nat Commun* 4:1354. [CrossRef Medline](#)
- Weisbrod D, Peretz A, Ziskind A, Menaker N, Oz S, Barad L, Eliyahu S, Itskovitz-Eldor J, Dascal N, Khananshvili D, Binah O, Attali B (2013) SK4 Ca²⁺-activated K⁺ channel is a critical player in cardiac pacemaker derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* 110:E1685–E1694. [CrossRef Medline](#)
- Wulff H, Castle NA (2010) Therapeutic potential of KCa3.1 blockers: recent advances and promising trends. *Expert Rev Clin Pharmacol* 3:385–396. [CrossRef Medline](#)
- Wulff H, Zhorov BS (2008) K⁺ channel modulators for the treatment of neurological disorders and autoimmune diseases. *Chem Rev* 108:1744–1773. [CrossRef Medline](#)
- Zagranichnaya TK, Wu X, Danos AM, Villereal ML (2005) Gene expression profiles in HEK-293 cells with low or high store-operated calcium entry: can regulatory as well as regulated genes be identified? *Physiol Genomics* 21:14–33. [CrossRef Medline](#)