A role for TREK1 in generating the slow afterhyperpolarization in developing starburst amacrine cells

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ABSTRACT

Myriad neuronal types exhibit slow afterhyperpolarizations (sAHPs). These sAHPs play an important role in establishing the firing pattern of neurons that in turn influence network activity. They are mediated by calcium-activated potassium channels. However, the molecular identity of these channels and the mechanism linking calcium entry to their activation are still unknown. Here we present several lines of evidence suggesting that the sAHPs in developing starburst amacrine cells are mediated by two-pore potassium channels. First, we use whole cell and perforated patch voltage clamp recordings to characterize the sAHP conductance under different pharmacological conditions. We find that this conductance is calcium dependent, reversed at E_K, blocked by barium, insensitive to apamin and TEA, and activated by arachidonic acid. In addition, pharmacological inhibition of calcium-activated phosphodiesterase reduces the sAHP. Second, we perform gene profiling on isolated SACs and find that they show strong preferential expression of the two-pore channel gene kcnk2 that encodes TREK1. Third, we demonstrate that TREK1 knockout animals exhibit an altered frequency of retinal waves, a frequency that is set by the sAHPs in starburst amacrine cells. With these results we propose a model in which depolarization-induced decreases in cAMP lead to disinhibition of the two-pore potassium channels and in which the kinetics of this biochemical pathway dictate the slow activation and deactivation of the sAHP conductance. Our model offers a novel pathway for the activation of a conductance that is physiologically important.
INTRODUCTION

Several types of neurons throughout the central and peripheral nervous system exhibit prolonged hyperpolarizations following bursts of action potentials (Hirst et al., 1985; Lancaster and Nicoll, 1987; Schwindt et al., 1988). These slow afterhyperpolarizations (sAHPs) underlie oscillatory burst firing in cholinergic striatal neurons and gonadotropin-releasing hormone neurons (Goldberg et al., 2009; Lee et al., 2010), and they give rise to spike frequency adaptation in the principal cells of the cortex, hippocampus, and amygdala (Lancaster and Adams, 1986; Lorenzon and Foehring, 1992; Faber and Sah, 2002). During development, retinal interneurons called starburst amacrine cells exhibit sAHPs (Zheng et al., 2006; Ford et al., 2012). These sAHPs play an important functional role within the developing retina by setting the frequency of spontaneous retinal waves, which play an instructive role in the eye-specific and retinotopic organization of retinofugal projections (Zheng et al., 2006; Godfrey and Swindale, 2007; Hennig et al., 2009; Ford et al., 2012).

Despite the prevalence and functional importance of sAHPs, the channels that give rise to them are unknown. It is generally accepted that sAHPs are mediated by potassium channels that are activated upon calcium influx through a variety of voltage gated calcium channels (Sah and Louise Faber, 2002). No specific antagonists of sAHPs have been found, but several intracellular signaling pathways modulate the sAHP, including PKA, PKC, and PIP2 pathways (Lancaster and Nicoll, 1987; Sah and Isaacson, 1995; Vogalis et al., 2002; Lancaster et al., 2006; Villalobos et al., 2011). The slow kinetics of the sAHP have led to speculation that sAHP channels are not directly activated by calcium entry, but rather are indirectly opened by a signaling cascade, possibly involving phosphorylation of the channel (Abel et al., 2004). Implicated in this activation are the calcium-activated phosphatase calcineurin (Vogalis et al., 2004) and the calcium sensors hippocalcin (Tzingounis et al., 2007) and neurocalcin (Villalobos and Andrade, 2010). However the pathway leading from calcium entry to the channel’s activation is still unknown.

Two-pore potassium (K2P) channels produce the hyperpolarized resting membrane potential in most neurons (Enyedi and Czirjak, 2010), and they exhibit properties that are similar to those of channels underlying sAHPs. K2P channels are insensitive to most potassium channel antagonists but are modulated by second messenger cascades (Mathie, 2007). Several K2P channel family members can be distinguished by their current rectification, modulation by protein kinases, and activation by heat, stretch, and lipids (Lesage and Lazdunski, 2000; Enyedi and Czirjak, 2010). While no family member is directly activated by calcium, their channel openings are gated by activation of calcium-dependent signaling cascades (Czirjak et al., 2004).
Here we combine perforated patch recordings with pharmacology in order to implicate a specific K2P channel, TREK1, in the generation of sAHPs in developing starburst amacrine cells. Second, we perform calcium imaging to investigate how modulating sAHP conductance affects circuit function. Last, we used multielectrode array recordings from mice lacking TREK1 to implicate this channel in the normal patterning of retinal waves.

METHODS

Animals. All experiments were performed on acutely isolated mouse retinas. Male and female C57Bl/6 mice obtained from Harlan were used for all WT recordings. mGluR2-GFP mice contained a transgene insertion of interleukin-2 receptor fused GFP under control of the mGluR2 promoter (Watanabe et al., 1998). ChAT-Cre/TdTom mice were generated by crossing a mouse in which an IRES-Cre recombinase was knocked in downstream of the endogenous choline acetyl transferase gene (Ivanova et al., 2010) with a separate tdTomato driver line (B6.129S6-ChAT<sup>tm1(cre)low</sup>/J x B6.129S6-Gt(Rosa)26Sor<sup>tm1(CAG-tdTomato)Hze</sup>/J, Jackson Labs). TREK1<sup>-/-</sup> mice (Namiranian et al., 2010) were used for assessing the role of TREK1 (gene name KCNK2) in retinal waves. Age-matched non-littermate C57BL/6 WT mice were used as controls. Genotypes were determined by genomic PCR using primer sequences described previously (Namiranian et al., 2010). All animal procedures were approved by the University of California, Berkeley and the Baylor School of Medicine, and they conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the Public Health Service Policy, and the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research.

Whole-Mount Retinal Preparation. P4-P7 mice were anesthetized with isoflurane and decapitated. Retinas were isolated in cold artificial cerebrospinal fluid (ACSF) (in mM: 119 NaCl, 26.2 NaHCO<sub>3</sub>, 11 glucose, 2.5 KCl, 1 K<sub>2</sub>HPO<sub>4</sub>, 2.5 CaCl<sub>2</sub>, 1.3 MgCl<sub>2</sub>) and mounted ganglion cell side up on filter paper. Retinas were incubated at room temperature in oxygenated ACSF until transfer to the recording chamber, where they were continuously superfused with oxygenated ACSF at 30-34 C.

Electrophysiology. Perforated patch and whole cell recordings were performed on whole-mount retinas from mice aged P4-P7. The inner limiting membrane was removed using a glass recording pipette, and SACs were identified using fluorescence and targeted using a Sutter micromanipulator. Voltage clamp recordings were sampled at 2.5 kHz and filtered at 1 kHz. Current clamp recordings were sampled at 5 kHz and filtered at 2 kHz. Analysis was performed using custom MATLAB (Mathworks) scripts. All reported voltages were corrected for liquid junction potential. Statistical significance was accessed using paired t-tests.
Perforated patch voltage and current clamp recordings were performed in the presence of DHβE (4 µM) and gabazine (5 µM) to block nAChR and GABA-A receptor mediated synaptic conductances, and in the presence of tolbutamide (100 µM) to block an ATP sensitive potassium conductance that develops during prolonged recordings (Figure 2). During recordings of conductance (Figure 1E) and current-voltage relationships (Figure 1C and D), TTX (200 nM), 4-AP (1 mM), TEA (1 mM), and cesium chloride (2 mM) were included in the bath solution to block voltage activated conductances. A gluconate-based perforated patch internal solution (in mM: 122 KGluconate, 20 HEPES, 0.5 EGTA, 2 NaCl, pH 7.2, liquid junction potential: 14 mV) was front-filled into electrodes and then back-filled with internal solution containing 750 µg/ml Amphotericin B made fresh hourly. Seals were formed and then access resistance was monitored continuously. Recordings were performed when access resistance was stable and less than 5% of the input resistance of the cell (typically, $R_a = 30-80$ MOhm, $R_{in} = 1-2$ GOhm). While access resistance prevented efficient voltage clamp of voltage-gated calcium and potassium currents during depolarizing steps, the small current (~5 pA) underlying the sAHP is unlikely to induce errors in voltage clamp during measurement of the reversal potential. Furthermore, maximum amplitudes and kinetics of the slow AHP were not correlated with the access resistance.

Whole cell recordings from SACs were made using potassium phosphate based internal solution (in mM: 110 KH$_2$PO$_4$, 6 MgCl$_2$, 1 EGTA, 4 adenosine 5′-triphosphate magnesium salt, 0.3 guanosine 5′-triphosphate trisodium salt, 10 HEPES and 10 phosphocreatine disodium salt, pH 7.2, liquid junction potential: 14 mV).

**Calcium Imaging.** Retinas from mice aged P2- P6 were bulk loaded with the calcium indicator Oregon Green Bapta-1 AM (OGB-1 AM) using the multicell bolus loading technique (Stosiek et al., 2003; Blankenship et al., 2009). Epifluorescence imaging and analysis were performed as described earlier (Blankenship et al., 2009). Significance was accessed using paired t-tests.

**MEA recordings.** TREK1 -/- and WT mice were dissected and placed ganglion cell side down onto a 60 electrode array (Multi-Channel Systems). The array electrodes were 10 µm in diameter and were arranged in an 8 x 8 grid (minus 4 corners) with 100 µm interelectrode spacing. The retina was held in place on the array with a weighted piece of dialysis membrane and was superfused continuously with oxygenated ACSF. The voltage trace on each electrode was sampled at 20 kHz and stored for offline analysis. The traces were then pass filtered between 120 and 2000 Hz. Spikes that crossed a threshold of three times the root mean square of the noise on each electrode were sorted according to the two principal components of their voltage waveforms. A valley seeking algorithm was then used to sort spike clusters into individual units. To verify that each unit identified by this algorithm
corresponded to a single cell, we inspected units manually. Furthermore, units that lacked a refractory
period in their autocorrelation function were considered contaminated by other neurons and were
excluded from the analysis. The mean spike rate, \( r \), was calculated by dividing the total number of
spikes for each unit by the recording duration. Units whose mean spike rate was \( \frac{1}{10} \) of the mean
firing rate of all cells were excluded from additional analysis to reduce contamination from low spiking
cells. After this cut, 20 – 45 units remained. Using the sorted spikes, waves were defined as events
where the average firing rate increased over a selected threshold (values varied from 0.3 to 2.1
standard deviations above the mean) and were separated by more than 10 seconds. The threshold
was adjusted for each retina such that the number of defined waves was consistent with the number of
waves identified by eye in the raster plots. Inter-wave intervals where pooled, and TREK-/- and WT
waves were compared using two-tailed t-test with a 99% confidence interval.

**Microarray analysis.** A database of gene expression in 13 retinal neuron subtypes was generated
using Affymetrix Mouse Genome 430 2.0 microarrays as described (Kay et al., 2012a, Kay et al.,
2012b). The data was collected at P6, a time of strong retinal wave activity. To determine the
expression profile of K2P-family channels, we began by identifying Affymetrix probesets corresponding
to each of the 13 genes in the mouse Kcnk gene family, which encode the K2P channels (Talley et al.,
2003). This was done by searching the Affymetrix NetAffx online database and curating probesets by
hand, using the Ensembl mouse genome viewer. To ensure completeness we identified all probesets
present on the 430 2.0 arrays that represent Kcnk family genes; some genes were represented on the
array as multiple independent sequences, whereas others were represented by only one sequence.
Next we queried our microarray database to determine the expression values of each of the Kcnk
gene sequences across the 13 cell types. Probesets that were not expressed in any of the cell types
were excluded from further analysis, which left 16 probesets (corresponding to the 13 Kcnk genes).
Finally, we used a hierarchical clustering algorithm (dChip microarray data analysis software) to
generate a heat map showing expression of these 16 sequences (Figure 3B). Sequences that have
similar expression patterns are clustered together. Because preliminary analysis suggested that
Kcnk2, which encodes TREK1, was specifically expressed in SACs, we included in the clustering
analysis two known SAC-specific genes, encoding the vesicular acetylcholine transporter and choline
acetyltransferase.

The Affymetrix probesets used for clustering (and the genes to which they correspond) were:

- 1455896_a_at (Kcnk1)
- 1448690_at (Kcnk1)
- 1449158_at (Kcnk2)
- 1445929_at (Kcnk2)
- 1425341_at (Kcnk3)
- 1421419_at (Kcnk4)
- 1421852_at (Kcnk5)
- 1435342_at (Kcnk6)
- 1425437_a_at (Kcnk7)
- 1445309_at (Kcnk9)
- 1431613_a_at (Kcnk10)
- 1441280_at (Kcnk12)
- 1447645_x_at (Kcnk13)
- 1424125_at (Kcnk13)
- 1447972_at (Kcnk15)
- 1429913_at (Kcnk16)
- 1440070_at (Chat, encoding
RESULTS

Slow AHPs in starburst amacrine cells exhibit properties similar to those of K2P channels

Following spontaneous depolarization during waves or evoked depolarization via current injection, starburst amacrine cells (SACs) in developing mouse retina exhibit sAHPs (Figure 1A) (Zheng et al., 2006; Ford et al., 2012). To determine the current underlying these sAHPs, we performed perforated patch voltage clamp recordings of SACs. We blocked synaptic input with cholinergic and GABAergic antagonists (dihydro-β-erthryoidine + Gabazine) to isolate the cell-intrinsic conductances. At this stage of development, glutamatergic inputs are immature and do not shape spontaneous activity patterns (Bansal et al., 2000). We found that, as previously described, depolarizing steps (of 500 ms duration from -64 mV to -14 mV) evoked a slow outward current at -64 mV (Figure 1B) (Ford et al., 2012). This current has a slow rise and decay similar to sAHPs measured in current clamp (Figure 1B). Thus, henceforth we refer to this current as $I_{sAHP}$.

Several lines of evidence indicate that $I_{sAHP}$ is mediated by potassium channels and that it requires calcium entry for activation. First, channel activation was associated with an increase in conductance (Figure 1E, n = 3). This implies the opening of channels rather than the activation of a transporter, a potential alternative source of the slow outward current (Pulver and Griffith, 2010). Second, $I_{sAHP}$ reversed at the reversal potential for potassium ($E_K$, Figure 1C). At physiological external potassium concentration (4.5 mM), the current exhibited outward rectification. Third, as external potassium increased, the reversal potential for $I_{sAHP}$ shifted, consistent with the Nernst equation prediction for a potassium conductance (Figure 1D). Fourth, $I_{sAHP}$ was reversibly blocked when calcium was removed from the bath solution (Figure 1F, n = 4). Thus, our data indicate that $I_{sAHP}$ is mediated by a calcium-activated potassium channel.

To further characterize this channel, we conducted pharmacological experiments. Since calcium-activated potassium channels include the BK and SK families (Sah, 1996), we investigated their involvement. BK channels mediate rapid repolarization during action potentials, while SK channels underlie the medium length afterhyperpolarization that follows individual action potentials. We blocked BK channels (Yellen, 1984), as well as other voltage gated channels, with 1 mM tetraethylammonium chloride (TEA) and found that the amplitude of $I_{sAHP}$ was unaffected (Figure 1F, n = 5). We blocked SK
channels with its specific antagonist, apamin (Sah and Louise Faber, 2002), and found that the amplitude of $I_{\text{saHP}}$ was again unaffected (Figure 1F, $n = 5$). Hence, neither BK nor SK channels contribute to the generation of $I_{\text{saHP}}$.

We next tested the involvement of ATP-dependent potassium channels ($K_{\text{atp}}$). These channels are activated by depolarization, contribute to a slow afterhyperpolarization in hippocampal pyramidal cells (Tanner et al., 2011), and are thought to be activated by a decrease in the cell's ATP levels. We found these channels in the SACs. After gaining whole cell access to a SAC, we observed a rapidly developing conductance that produced a dramatic drop in input resistance (Figure 2A). This conductance reversed at $E_K$ (data not shown), indicating the opening of potassium channels. Subsequent depolarizing steps activated a transient outward tail current (Figure 2A-B). Both this transient tail current and the whole cell activated conductance were blocked by the $K_{\text{atp}}$ channel antagonist tolbutamide (100 µM; $R_{in}$ for control = 0.33 ± 0.15 GOhm and for tolbutamide = 1.74 ± 0.22 GOhm; $p = 0.0004$, $n = 5$, Figure 2B), indicating that these currents are mediated by $K_{\text{atp}}$ channels. To determine if these channels mediate $I_{\text{saHP}}$, we blocked with tolbutamide while using the perforated patch configuration and found that $I_{\text{saHP}}$ did not change (Figure 2C, $n = 5$). Thus, $K_{\text{atp}}$ channels in SACs are activated by the reduction of intracellular ATP caused by intracellular dialysis during whole cell recordings. However, when the intracellular milieu is left intact, these channels do not contribute to the sAHPs.

To determine if K2P channels mediate $I_{\text{saHP}}$, we first approached the family as a whole. The K2P family consists of 13 members, including the TWIK, TASK, TREK, TALK, THIK, and TRESK subfamilies (Talley et al., 2003). These subfamilies are insensitive to several potassium channel antagonists, including TEA, 4-aminopyridine (4-AP), and cesium (Lesage, 2003). Consistent with this, a combination of 1 mM TEA, 1 mM 4-AP, and 2 mM cesium did not block the $I_{\text{saHP}}$ in SACs ($n = 4$, data not shown, see Methods). However, $I_{\text{saHP}}$ was blocked by barium (2 mM, $n=7$, Figure 3A), a blocker of the K2P channel subfamilies TASK, TREK, TWIK, and TRESK (Deng et al., 2009). These data, along with our finding that $I_{\text{saHP}}$ is outward rectifying (Figure 1C), suggest that $I_{\text{saHP}}$ in SACs is mediated by one of the K2P subfamilies, which include TREK, TASK, and TRESK channels.

To distinguish between these subfamilies, we first conducted a microarray analysis of mRNA isolated from SACs (Kay et al., 2011; Kay et al., 2012). We found that $kcnk2$, the mRNA that encodes the K2P channel TREK1, is highly expressed in SACs during the first postnatal week (Figure 3B). In fact, within the subset of cell types analyzed, it is specifically expressed only in SACs. Moreover, it is expressed with a degree of specificity that is similar to that seen for other markers of SACs, such as
choline acetyl transferase and Megf10 (Kay et al., 2012). These results suggest that TREK1 channels likely mediate the slow potassium conductance underlying sAHPs.

We next conducted pharmacological experiments to ask whether other K2P subfamilies might also contribute to the sAHP. TRESK channels as well as TASK-3 and TASK-9 channels are inhibited by extract derived from sanshool chili peppers (Bautista et al., 2008). Bath application of 0.02% sanshool extract did not block IsAHP or increase the input resistance (Figure 3A, n = 4), indicating that SACs do not express these K2P channel types. TREK and TRAAK channels exhibit potentiated currents with arachidonic acid (AA) (Lesage and Lazdunski, 2000). Bath application of 10μM AA led to a significant increase in the holding current at -60mV and a corresponding decrease in the input resistance (in control, -1.0 ± 2.5 pA, 1.52 ± 0.24 GOhm; in AA, 10.0 ± 3.6 pA, 1.12 ± 0.23 GOhm; p = 0.0059 for holding current at -64 mV, p = 0.0117 for input resistance, n = 7). Moreover, IsAHP was increased in amplitude (Figure 3A, n = 7). Thus, our results support the hypothesis that IsAHP in SACs is mediated by TREK, but not TASK or TRESK channels.

However, IsAHP requires calcium influx for activation, while TREK channels are not activated by changes in intracellular calcium (Fink et al., 1996). How might these channels be activated to generate the sAHP in SACs? Previously, we along with others showed that IsAHP is inhibited by the elevation of cAMP with forskolin (Zheng et al., 2006; Ford et al., 2012). Recently, TREK1 was shown to be activated by decreases in cAMP following the activation of metabotropic GABA receptors (Sandoz et al., 2012). Thus, we hypothesize that calcium influx into SACs activates calcium-activated PDEs leading to a decrease in cAMP level that then activates the TREK1 channels. We tested if PDE-1C, the calcium-activated phosphodiesterase that is expressed in SACs (Santone, 2006), plays a role in generating IsAHP. We applied 8-Methoxymethyl-3-isobutyl-1-methyl xanthine (MMPX, 100 μM), a specific PDE-1C inhibitor and found that it inhibited IsAHP (Figure 3A, n = 8). Though at this concentration, MMPX may inhibit multiple PDEs, it exhibited specificity in pancreatic β-cells (Tian et al, 2012). Also, in a previous study based on imaging of PKA activity, we found that high concentrations of MMPX did not lead to a tonic increase in basal cAMP levels in retinal neurons as observed in the presence of the broad spectrum inhibitor IBMX (Dunn et al., 2009). In addition, these data are consistent with previous studies showing forskolin, an adenylate cyclase activator, decreases IsAHP (Zheng et al., 2006; Ford et al., 2012). All together, our findings indicate that IsAHP may be generated by activation of TREK channels via a calcium-dependent decrease in cAMP.

Changing the kinetics of sAHPs alters the frequency of retinal waves
To determine the effect of sAHPs on network function in the developing retina, we used calcium imaging to investigate how changing sAHPs altered retinal waves (Ford et al., 2012). The timing of spontaneous retinal waves is critical for normal visual system development. We know that SACs control wave timing, but the mechanism is unclear. The sAHPs are well positioned to regulate the frequency of spontaneous retinal waves by generating a refractory period following the depolarization caused by a wave. If sAHPs really do set wave frequency, then blocking them should increase the frequency of waves. To test this hypothesis, we manipulated sAHPs via the cAMP-PDE pathway described above. We used MMPX to block calcium-dependent PDE activity, increasing cAMP levels and thereby inhibiting sAHPs. As predicted, this increased the frequency of waves, as we have previously shown using the broad-spectrum PDE blocker IBMX and direct elevation of cAMP levels using forskolin (Ford et al., 2012)(Figure 4A-B, p = 0.001). By contrast, potassium channel antagonists, cesium and tolbutamide, that had no effect on $I_{sAHP}$, also failed to influence the interwave interval (Figure 4B, p = 0.42 and p = 0.29 for cesium and tolbutamide, respectively). These results support the notion that sAHP currents in SACs, and the calcium-regulated cAMP pathway that regulates them, are critical for controlling the timing of retinal waves. Thus, sAHPs appear to play an important role in the control of circuit function in the retina.

**TREK1 knockout mice exhibit altered retinal waves**

Our molecular data suggest that the key channel mediating sAHPs in SACs is TREK1. If so, then loss of TREK1 function should also increase the frequency of waves. We were not able to import mice to use calcium imaging, so to investigate the functional role of TREK1 in retinal waves, we conducted multi-electrode array (MEA) recordings in P1-P3 WT and TREK1-/- mice (Namiranian et al., 2010). MEA recordings and calcium imaging lead to the same results in terms of assaying the frequency of waves (Torborg and Feller, 2005). When compared to WT animals, TREK1-/- mice exhibited interwave intervals that were approximately half the length (WT = 85.23 ± 15.96 s, TREK1-/- = 45.41 ± 15.51 s, t-test p<0.001, Figure 5), similar in scale to the effect of inhibiting the sAHP with MMPX (Figure 4). Thus TREK1 plays an important role in setting the pace for retinal waves. These data are consistent with the idea that TREK1 is a major contributor to the sAHP in SACs.

**DISCUSSION**

We have demonstrated that sAHPs in developing starburst amacrine cells are mediated by a potassium channel that shows pharmacological and rectification properties consistent with the K2P channel, TREK1. TREK1 is highly and specifically expressed in starburst amacrine cells. The channel underlying sAHPs is inhibited by tonically elevating cAMP and is blocked by preventing the
calcium-dependent degradation of cAMP by PDEs, consistent with TREK1. In addition, we have shown that altering sAHPs pharmacologically and in mice lacking TREK1 changes the frequency of retinal waves, indicating a functional role for TREK1 in circuit function. Below we discuss the role of the K2P channel TREK1 in generating $I_{sAHP}$, and we propose a model for its activation by the regulation of cAMP levels.

**TREK1 as a candidate $I_{sAHP}$ channel**

It has become increasingly evident that K2P channels play a role in generating slow currents. Metabotropic GABAB receptors use cAMP-dependent disinhibition of TREK2 channels to generate second-long hyperpolarizations (Deng et al., 2009; Sandoz et al., 2012). Similarly, serotonin activates TWIK-1 via a decrease in cAMP (Deng et al., 2007) or TASK-1 (Talley et al., 2000) channels and produces a decrease in neuronal excitability. Conversely, metabotropic glutamate receptors activate phospholipase C and inhibit TREK and TASK channels, generating slow depolarizations in neurons (Chemin et al., 2003). Thus the slow modulation of second messengers, including cAMP and IP3, seems to be read out by K2P channels so they can generate slow hyperpolarizations and depolarizations.

In agreement, our results implicate the K2P channel TREK1 in the generation of $I_{sAHP}$ in retinal starburst amacrine cells using several lines of evidence. First, during development, SACs are enriched for *kcnk2* transcripts, the mRNA that encodes the TREK1 channel (Figure 3B). Second, $I_{sAHP}$ was insensitive to a variety of K-channel antagonists that are also known not to block K2P channels. In particular, TEA (1 mM), 4-AP (1 mM), cesium (2 mM), apamin (1 µM), and tolbutamide (100 µM) do not inhibit $I_{sAHP}$. (Figures 1 and 2). Thus, we can rule out the involvement of voltage gated, A-type, SK, BK, or ATP-dependent potassium channels and $I_h$. Third, the $I_{sAHP}$ was blocked by 2 mM barium, a blocker of the K2P channel subfamilies TASK, TREK, TWIK, and TRESK (Figure 3A). Fourth, application of the fatty acid arachidonic acid increased $I_{sAHP}$ (Figure 3A), consistent with the observation that some K2P channels are activated by manipulating pressure, pH, and lipids (Lesage and Lazdunski, 2000; Enyedi and Czirjak, 2010). Fifth, at physiological potassium concentrations, $I_{sAHP}$ were outward rectified (Figure 1), consistent with the outward rectification of K2P channels (Goldstein et al., 2005). Furthermore, this outward rectification excludes the possibility that $I_{sAHP}$ is mediated by inwardly rectified channels that are sensitive to barium, such as $K_{atp}$. Sixth, $I_{sAHP}$ is inhibited by blockade of the calcium-dependent phosphodiesterase PDE1C (Figure 3A), consistent with the known inhibition of TREK channels by PKA phosphorylation. Seventh, mice lacking TREK1 display an increase in wave frequency (Figure 5), consistent with the frequency increase seen with decreased $I_{sAHP}$ (Figure 4).
A cAMP model for the activation of $I_{sAHP}$

A hallmark of both TREK channels and the channels underlying the sAHPs in other neurons is their regulation by intracellular signaling cascades. PKA phosphorylation inhibits TREK channels (Honore et al., 2002) and inhibits the channels underlying the sAHPs in hippocampal pyramidal neurons (Lancaster and Nicoll, 1987; Sah and Isaacson, 1995; Lancaster et al., 2006). In developing retinal SACs, forskolin, which elevates cAMP levels, inhibits $I_{sAHP}$ (Ford et al., 2012), indicating that $I_{sAHP}$ is sensitive to cAMP levels.

This finding could explain how TREK channels generate sAHPs in SACs even though $I_{sAHP}$ requires calcium influx for activation while TREK channels are not activated by changes in intracellular calcium (Fink et al., 1996). The activation of $I_{sAHP}$ occurs over several seconds, suggesting that channel opening following depolarization is mediated by a signaling cascade, rather than the direct activation of calcium. Calcium influx can activate adenylyl cyclases (ACs) and phosphodiesterases (PDEs) and thus can regulate levels of cAMP. Decreases in cAMP have been shown to activate TREK channels with the activation of metabotropic GABA (Deng et al., 2009; Sandoz et al., 2012) and glutamate receptors (Lesage et al., 2000; Chemin et al., 2003). Thus, calcium entry could regulate cAMP levels that would then control the TREK channel that produces $I_{sAHP}$.

We propose a model for this mechanism, though it is important to note that this study does not specifically address the mechanism underlying calcium-activation of the conductance. During depolarization, calcium influx through voltage gated calcium channels (Zheng et al., 2006) may activate calcium-dependent PDE1C and cause a decrease in cAMP. We note that other pathways may also contribute to this calcium-dependent decrease in cAMP. Some adenylate cyclases, including AC5, AC6 and AC9, are inhibited by intracellular calcium signaling (Halls and Cooper, 2011). Of these, AC5 and AC9 are expressed within the retina early in development (Nicol et al., 2006), though their specific localization is unknown. The decrease in cAMP caused by these potential pathways and by calcium influx may lead to activation of the channel underlying the sAHP. The corresponding decrease in PKA activity would then produce a decrease in phosphorylation that disinhibits the channel. In support of this idea, TREK1 channels in neurons associate with PKA anchoring proteins (AKAPs) that allow for rapid regulation by PKA (Sandoz et al., 2006). Furthermore, $I_{sAHP}$ in hippocampal CA1 neurons is modulated by both PKA and phosphatases (Pedrazzani et al., 1998), suggesting that a balance between kinase and phosphatase activity actively regulates the channel phosphorylation state.
The role of $I_{sAHP}$ in retinal waves

Retinal waves during the first postnatal week in mice critically depend upon cholinergic transmission from SACs but not upon other neurotransmitters (Bansal et al., 2000; Stacy et al., 2005; Wang et al., 2007). SACs are spontaneously active (Zheng et al., 2006; Ford et al., 2012), and stimulation of a single SAC is sufficient to initiate a wave (Ford et al., 2012). Thus a network of SACs is both necessary and sufficient for generating retinal waves.

The intrinsic conductances in SACs dictate the spatio-temporal properties of retinal waves. During development of the visual system, the distinct spatio-temporal features convey information to target regions (Penn et al., 1994; Bansal et al., 2000; Xu et al., 2011; Ackman et al., 2012). Several computational models have been developed to investigate how the intrinsic properties of SACs give rise to the frequency, propagation speed, and spatial coverage of waves (Godfrey and Swindale, 2007; Hennig et al., 2009; Ford et al., 2012). Each of these models relies on the spontaneous depolarization of SACs to initiate waves, the local excitatory connections to allow propagation, and a slow afterhyperpolarization to limit propagation of waves into recently active regions. Recent experimental evidence has indicated that decreasing the sAHP by elevating cAMP levels increases wave frequency (Zheng et al., 2006; Ford et al., 2012). In accordance, here we have shown that inhibiting calcium-activated PDE1C decreases $I_{sAHP}$ and wave frequency. This is consistent with predictions from modeling studies (Godfrey and Swindale, 2007; Hennig et al., 2009; Ford et al., 2012). Furthermore, we have shown that animals lacking TREK1 exhibit waves with double the frequency of wild type waves. Thus, our data supports a role for TREK1 in generating the sAHP in SACs that limits wave frequency.

Why are waves not more frequent than 2 to 3 waves per minute when the sAHP is blocked or reduced? The sAHP prevents the propagation of waves into recently active regions but is not the only mechanism that sets wave frequency. The frequency of retinal waves is determined by the spontaneous depolarization rate of SACs. In mice, SACs are spontaneously active only about once every 10 minutes when neurotransmitters are blocked (but see in rabbit Zheng et al., 2006; Ford et al., 2012). This slow intrinsic depolarization rate may set an upper limit to the wave frequency when the sAHP is reduced. Alternatively, a reduction in sAHP might be compensated for with additional cellular mechanisms. During initial whole cell recordings, we found a $K_{atp}$-mediated conductance following depolarizing voltage steps (Figure 2). While this conductance was not activated during less invasive perforated patch recordings, a $K_{atp}$-mediated sAHP may occur when sAHP is inhibited and causes excessive depolarization that depletes energy levels.
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**FIGURE CAPTIONS**

**Figure 1.** Slow afterhyperpolarization (sAHP) in starburst amacrine cells (SACs) is mediated by a two-pore potassium channel

A. Current clamp perforated patch recording of SAC shows sAHP evoked by 500 ms 100 pA current injection. Inset shows expanded version of initial spike (Scale: 10 mV, 100 ms).

B. Voltage clamp perforated patch recording of SAC shows IsAHP evoked by 500 ms voltage step to -14 mV from a holding potential of -64 mV. Trace shows average from n = 32 cells with gray indicating ± SD.

C. Left: Voltage protocol used to determine current voltage relationship of IsAHP. Average IsAHP is shown above for reference. A series of 50 ms voltage steps to different holding potentials were given before and at the peak of IsAHP. Scale: 2 pA, 10 s. Right: Inset: Example current traces from voltage steps before (1) and after (2) depolarizing step to activate IsAHP. Scale: 10 pA, 10 ms. Graph shows average current voltage relationship at peak of IsAHP. n = 4, Mean ± SD.

D. Reversal potential as a function of external potassium concentration. Dots represent individual cells; boxes indicate mean. Line is fit to prediction from Nernst equation for a potassium conductance.

E. Example conductance (top) and current (bottom) measurements taken from a baseline of -64 mV following a 500 ms voltage step to -14 mV. Conductance was determined from trains of 50 ms 10 mV hyperpolarizing steps. Inset: Voltage protocol used to determine conductance.

F. Top: Average current evoked by 500 ms voltage step to -14 mV, as in B-D, in the presence (black) and absence (gray) of external calcium (n = 4), apamin (1 µM, n = 5), and TEA (1 mM, n = 5). Bottom: Peak amplitude of evoked currents for each cell before (Pre) and after (Post) application. Boxes represent the mean; error bars indicate SEM. Scale: 2 pA, 10 s

**Figure 2.** Whole cell recordings from SACs reveal a K$_{atp}$ conductance

A. Whole cell voltage clamp recording from SAC. Cell is voltage clamped at -64 mV. Arrows indicate 500 ms voltage step to -14 mV followed by return to -64 mV. Dotted lines indicate changes in holding current at -60 mV in control, tolbutamide (100 µM), and rinse.

B. Peak amplitude of transient outward current following 500 ms depolarizing voltage step in whole cell configuration in the absence and presence tolbutamide application (n = 5).

C. Left: Example current clamp perforated patch recordings of sAHPS evoked by 500 ms, 100 pA current injection before and during tolbutamide bath application. Middle: Example voltage clamp perforated patch recording of IsAHP evoked by 500 ms step from -64 mV to -14 mV, before and during tolbutamide bath application. Right: Peak amplitude of IsAHP following 500 ms depolarizing voltage step in perforated patch configuration before, during, and after tolbutamide application (n = 5).
**Figure 3.** *I*$_{\text{AHP}}$ has the pharmacological characteristics of K2P channels

A. Top: Peak amplitude of *I*$_{\text{AHP}}$ before (pre) and during (post) bath application of barium (2 mM as BaCl$_2$, $n = 7$), sanshool extract (0.02%, $n = 4$), arachidonic acid (10 µM, $n = 7$), or MMPX (100 µM, $n = 8$). Bottom: Average current evoked by 500 ms voltage step from -64 mV to -14 mV, in control (black) and during application of each drug (gray). Scale: 2 pA, 10 s.

B. Expression of the Kcnk gene family, which encodes K2P channels, was assessed in mouse retinal neurons using microarrays. Heatmap shows expression of the Kcnk genes across 13 different retinal neuron subtypes, which include subtypes of retinal ganglion cells (RGCs), amacrine cells (ACs), and bipolar cells (BCs). Color (red, high; blue, low; see bottom for scale) indicates gene expression level in each cell type relative to mean level for that gene. Unsupervised hierarchical clustering was used to determine the X-axis order in which the genes are listed, such that genes with similar expression patterns across the 13 cell types are found next to each other. All 13 members of the Kcnk family are represented at least once (some are represented twice because they appeared twice on the microarray). Expression of Kcnk2, the gene encoding TREK1, is specific to SACs, as shown both by the heatmap and by the fact that it clustered with two known SAC-specific genes, Chat and Vacht.

**Figure 4.** Modulation of sAHP alters the frequency of spontaneous retinal waves

A. Calcium imaging of retinal waves. Left: Fluorescence image of a retina that is bolus loaded with OGB-1AM. Scale: 100 µm. Right: Pseudo-colored images show time progression of wave fronts. Each panel represents the activity during 4 consecutive 20 s time periods. Wave fronts are colored according to their location during the 20 s period, with darker colors occurring earlier in the period (see color scale). Black indicates the absence of a wave. Top shows a control retina, and bottom shows the same retina during application of 100 µM MMPX.

B. Mean interwave interval (relative to control) measured by calcium imaging in the presence of MMPX (100 µM), tolbutamide (100 µM) and cesium (2 mM as CsCl). Number of retinas per manipulation is shown on each bar. Mean ± SD. *p = 0.001.

**Figure 5.** TREK1 -/- mice exhibit more frequent cholinergic retinal waves

A. Raster plots of single-unit spike trains over a 20 minute period recorded from retinas of P1 WT (top) and TREK1 -/- (bottom) mice.

B. Summary histograms show distribution of inter-wave intervals from WT (top, $n = 4$) and TREK1 -/- (bottom, $n = 4$) retinas.

C. Average inter-wave intervals for WT ($n = 52$ waves from 4 retinas) and TREK1 -/- ($n = 83$ waves from 4 retinas) mice. Error bars indicate SD; *p<0.001.
Ford et al. Figure 1

**p = 0.0027

Peak Current (pA)

**p = 0.0027

[0 Calcium ]

Apamin (1 µM)

TEA 1 mM

**p = 0.0027

p = 0.74602

p = 0.28442

**p = 0.0027

p = 0.74602

p = 0.28442

**p = 0.0027

p = 0.74602

p = 0.28442
A

Barium 2 mM

* p = 0.038754

Sanshool Extract 0.02%

p = 0.3686

Arachidonic Acid 10 μM

*p = 0.023066

MMPX 100 μM

** p = 0.0065

Peak Current (pA)

B

Kcnk gene:

TREK-1

ChAT

Vacht

J-RGC

BD

W3

W7

Isl2

Cad3

SACs

PaxCre

nGnG

J-AC

AII

MP-BC

ON BC

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Figure 3