Hippocalcin and KCNQ Channels Contribute to the Kinetics of the Slow Afterhyperpolarization

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ABSTRACT The calcium-activated slow afterhyperpolarization (sAHP) is a potassium conductance implicated in many physiological functions of the brain including memory, aging, and epilepsy. In large part, the sAHP's importance stems from its exceedingly long-lasting time-course, which integrates action potential-induced calcium signals and allows the sAHP to control neuronal excitability and prevent runaway firing. Despite its role in neuronal physiology, the molecular mechanisms that give rise to its unique kinetics are, to our knowledge, still unknown. Recently, we identified KCNQ channels as a candidate potassium channel family that can contribute to the sAHP. Here, we test whether KCNQ channels shape the sAHP rise and decay kinetics in wild-type mice and mice lacking Hippocalcin, the putative sAHP calcium sensor. Application of retigabine to speed KCNQ channel activation accelerated the rise of the CA3 pyramidal neuron sAHP current in both wild-type and *Hippocalcin* knockout mice, indicating that the gating of KCNQ channels limits the sAHP activation. Interestingly, we found that the decay of the sAHP was prolonged in *Hippocalcin* knockout mice, and that the decay was sensitive to retigabine modulation, unlike in wild-type mice. Together, our results demonstrate that sAHP activation in CA3 pyramidal neurons is critically dependent on KCNQ channel kinetics whereas the identity of the sAHP calcium sensor determines whether KCNQ channel kinetics also limit the sAHP decay.

INTRODUCTION

In the brain, calcium-dependent potassium channels tune neuronal excitability and prevent aberrant activity. Hippocampal pyramidal neurons express members of three different calcium-activated potassium channel families: large-conductance calcium-dependent potassium channels (BK channels), small conductance calcium-activated potassium channels (SK channels), and calcium-activated slow afterhyperpolarization potassium channels (sAHP) (1–3). Although all three families play important roles in brain physiology, the sAHP in particular has also been implicated in learning and memory, aging, Alzheimer's disease, and epilepsy (4–6). Activated in response to high-frequency neuronal firing or paroxysmal depolarization, the sAHP acts as a feedback mechanism to prevent runaway neuronal activity. Unlike the BK and SK channels, the molecular underpinnings of the sAHP, its mechanism of activation, and the basis of its unique biophysical properties have remained controversial since its characterization over 30 years ago (7).

The hallmark of the sAHP is its exceptionally slow activation and decay kinetics (1,3) that are on the order of hundreds of milliseconds (rise) and seconds (decay). Several studies have suggested that this slow time-course is critical for the role of the sAHP in synaptic plasticity, spike frequency adaptation, and network synchronization (8–11). Recent studies have suggested that the sAHP time-course might be due to the properties of the sAHP diffusible calcium sensor whereas earlier modeling work had proposed that the sAHP time-course is a function of slow channel-gating kinetics (12–16). However, these proposals have not been tested directly because the molecular components of the sAHP were unknown. The recent demonstration that KCNQ potassium channels contribute to the sAHP current (IsAHP) in hippocampal CA3 pyramidal neurons (17,18), and the identification of Hippocalcin, a diffusible neuronal calcium sensor of the VILIP family, as a sAHP calcium sensor in pyramidal neurons, have made it possible to test these models (14,19).

In this work, we make use of these two molecular targets to investigate the mechanisms underlying the slow sAHP time-course in CA3 pyramidal neurons. We used KCNQ-specific pharmacology to test the role of KCNQ channels in both wild-type and Hippocalcin null CA3 pyramidal neurons. Our results show that the IsAHP rise is limited by the KCNQ channel kinetics in both wild-type and *Hippocalcin* knockout mice. Unexpectedly, we also find that the identity of the IsAHP calcium sensor determines the contribution of KCNQ channel kinetics to the IsAHP decay because application of retigabine, a KCNQ channel activator, accelerated the IsAHP decay in Hippocalcin null neurons, but not in wild-type neurons. Thus, the extent to which the KCNQ channel kinetics contributes to the sAHP time-course is dependent upon the identity of the calcium sensor that gates the sAHP.

MATERIALS AND METHODS

Animals

Experiments were carried out according to the guidelines of the University of Connecticut-Storrs Institutional Animal Care and Use Committee. Both

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male and female mice were used. *Hippocalcin* knockout mice were maintained by either breeding $HC^{-/-}$ males with $HC^{-/-}$ females or breeding $HC^{+/-}$ males with $HC^{+/-}$ females. The $HC^{-/-}$ mice have been backcrossed to a C57Bl/6 background for 11–12 generations.

PCR genotyping

PCR genotyping of the mouse tail prep DNA was performed as described previously in Tzingounis et al. (14).

Slice preparation and electrophysiology

Transverse hippocampal slices (300 μm) were prepared from 12 to 22 day-old mice. After animals were deeply anesthetized with isoflurane, they were rapidly decapitated, and the hippocampus was dissected out. The hippocampus was then secured with glue to the stage of a micron vibroslicer in ice-cold cutting solution containing 26 mM $NaHCO_3$, 210 mM sucrose, 10 mM glucose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$, and 7 mM $MgCl_2$. After slicing of the hippocampus, slices were placed in a storage chamber containing artificial cerebrospinal fluid (ACSF): 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, 10 mM glucose, equilibrated with 95% O_2 and 5% CO_2. Slices were maintained at 35°C (30–40 min) and then at room temperature in a storage chamber that was continuously perfused with ACSF for at least 1.5 h before recordings. For all recordings, we perfused slices in the storage ACSF with the addition of 2–10 μM CNQX (IC_{50}: 40 nM) to block fast synaptic transmission. With the exception of SK channel experiments seen later in Fig. 4, the recording ACSF also included 100-nM apamin to block SK channels (IC_{50}: 10–100 pM). In a subset of experiments, 100-μM picrotoxin was also used to inhibit $GABA_A$ receptors, but we did not see any differences between cells recorded with or without picrotoxin. Therefore, the majority of our recordings took place in the absence of picrotoxin.

Whole-cell recordings were made with infrared differential interference contrast optics using a 40× water-immersion objective on an upright microscope (model No. BX51W; Olympus, Melville, NY). Pipettes with resistances of 3–5 MΩ were filled with an intracellular pipette solution containing 130 mM potassium methylsulfate, 10 mM KCl, 10 mM HEPES, 4 mM NaCl, 4 mM Mg_2ATP, 0.4 mM Na_4GTP, and 20 mM myo-inositol (20). Osmolarity was adjusted to 295–300 mOsm and pH to 7.25–7.35 with KOH. All recordings were from pyramidal neurons located in the CA3b area of the hippocampus (21) with an initial resting membrane potential <-55 mV. Voltage or current responses were collected with a Multiclamp 700B (Molecular Devices, Sunnyvale, CA), filtered at 2 kHz, digitized at 10–50 kHz and analyzed online using the softwares IgorPro (WaveMetrics, Lake Oswego, OR), Clampfit (Molecular Devices), or AxoGraph X (AxoGraph Scientific, Queensland, Australia).

In voltage-clamp recordings, the series resistance was measured by the instantaneous current response to a 4-mV (100-ms) step with only the pipette capacitance canceled. The series resistance was measured continuously with recordings being discarded if it changed >20%. Recordings were not adjusted for the junction potential (−8.6 mV), with the exception of data presented in Table S1 in the Supporting Material. For all voltage-clamp recordings, the membrane potential was kept at −60 mV by applying a small DC current as needed. For all voltage-clamp recordings, the membrane potential was clamped to −55 mV.

Drugs and reagents

Chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO) with the exception of retigabine (Santa Cruz Biotechnologies, Santa Cruz, CA), ICA-27243 (gift from ICAgen, Durham, NC), tetrapotassium-BAPTA (Molecular Probes, Eugene, OR), and EGTA (Molecular Probes).

Data analysis and statistics

The built-in algorithms of KaleidaGraph 4 (Synergy Software, http://www.synergy.com/) were used for Student's *t*-test statistics (paired or unpaired). Single-exponential fits to determine the activation and decay times were carried out with the built-in algorithms of Clampfit (Molecular Devices). For the weighted decay time-constants in Fig. 1 and seen later in Fig. 5, the peak IsAHP amplitude was measured and divided by the sAHP charge integrated across 18 s from the peak IsAHP amplitude.

RESULTS

KCNQ channel kinetics govern the sAHP current activation

If KCNQ gating kinetics contribute to the prolonged kinetics of the IsAHP, a compound that alters the kinetics of KCNQ channels should alter the IsAHP time-course. Retigabine is an allosteric KCNQ channel activator that increases the KCNQ channel open probability and shifts the KCNQ channel voltage-dependence toward more hyperpolarized membrane potentials. Importantly, work in heterologous cells shows that retigabine speeds the activation kinetics of KCNQ channels (22,23). If KCNQ gating kinetics determine the IsAHP time-course, retigabine will alter the IsAHP time-course. We recorded the IsAHP in CA3b pyramidal neurons from wild-type mice in the presence of 100 nM apamin to block SK channels (21). To induce the IsAHP we held cells at −55 mV and gave a brief depolarization step (100 ms, +45 mV) to open voltage-activated calcium channels. Upon return to −55 mV, a slow activating and decaying sAHP current developed. Bath application of 40 μM retigabine, a saturating concentration, significantly accelerated the rise time of the IsAHP (τ_{rise} = 191 ± 9 ms; $\tau_{rise\text{-}ret}$ = 153 ± 11 ms, $n = 7$; $p < 0.01$; see Fig. 1 *a*), suggesting that KCNQ channel kinetics limit the time-course of the sAHP current activation. In contrast, we found that retigabine had no effect on the IsAHP decay time-course ($\tau_{w\text{-}decay}$ = 6.6 ± 0.3 s; $\tau_{w\text{-}decay\text{-}ret}$ = 6.2 ± 0.5 s, $n = 7$; see Fig. 1 *a*), suggesting KCNQ channel kinetics are not a determinant of the IsAHP decay.

To further test the idea that KCNQ channel kinetics limit IsAHP rise kinetics, we repeated the retigabine application experiments in the presence of the calcium chelator BAPTA (0.5 mM) (Fig. 1 *b*). In the presence of 0.5 mM BAPTA, the rise of the IsAHP is significantly prolonged, presumably due to the slow calcium exchange between BAPTA and the IsAHP diffusible calcium sensors (τ_{rise} = 197 ± 16 ms, $n = 6$; $\tau_{rise\text{-}BAPTA}$ = 531 ± 34 ms, $n = 6$; $p < 0.01$). BAPTA binds incoming calcium quickly (K_{on} ~10^8 M^{-1} s^{-1}) and thus competes effectively with endogenous calcium sensors. As incoming calcium ions equilibrate, calcium is transferred to the sAHP sensors with BAPTA acting as the calcium source (13,24,25). This slow exchange of calcium from BAPTA should limit the IsAHP activation in BAPTA-loaded neurons, and thus diminish any role of KCNQ channels kinetics. Consistent with this hypothesis, application of

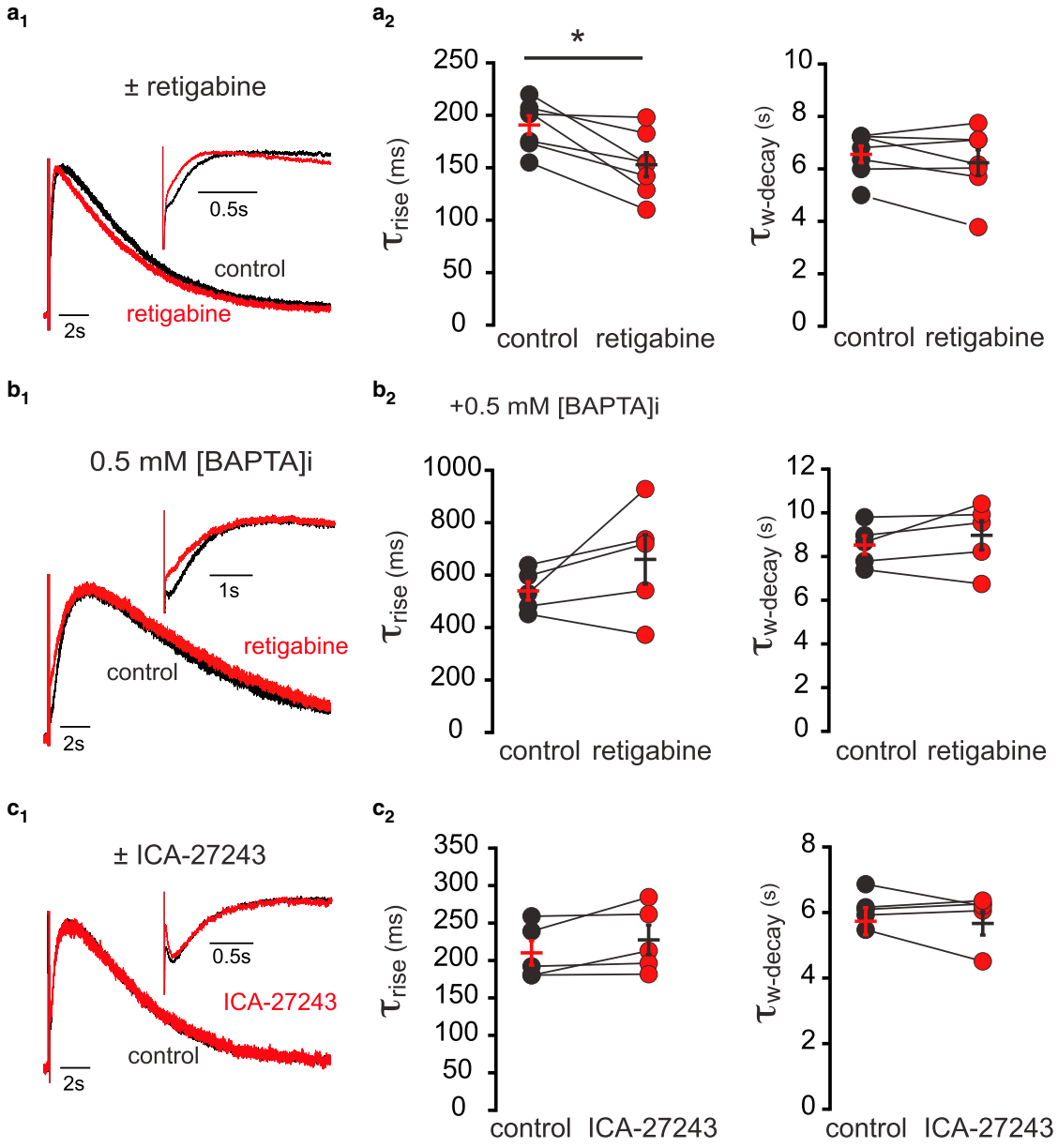

FIGURE 1 The IsAHP activation depends on KCNQ channel kinetics. (a_1) Superimposed normalized IsAHP traces before (*black*) and after (*red*) application of 40 μM retigabine. (a_2) Summarized data showing the effect of retigabine on the IsAHP activation and decay time constants ($n = 6$, $^*p < 0.01$ Student's *t*-test). (b_1) Superimposed normalized IsAHP traces before (*black*) and after (*red*) application of 40 μM retigabine in neurons loaded with 0.5 mM BAPTA. (b_2) Summarized data showing the effect of retigabine on the IsAHP activation and decay time-constants in neurons loaded with 0.5 mM BAPTA ($n = 5$). (c_1) Superimposed normalized IsAHP traces before (*black*) and after (*red*) application of 20 μM ICA27243. (c_2) Summary showing the effect of ICA27243 on the activation and decay time-constants ($n = 5$). All recordings were in the presence of 100-nM apamin.

retigabine to BAPTA-loaded neurons no longer accelerated the IsAHP activation kinetics ($\tau_{rise} = 540 \pm 35$ ms; $\tau_{rise\text{-}ret} = 660 \pm 94$ ms, $n = 5$; $p = 0.2$; see Fig. 1 *b*).

The lack of effect of retigabine in BAPTA-loaded neurons was not due to failure of retigabine to activate KCNQ channels. This is because retigabine application induced a holding current change in BAPTA-loaded neurons of a similar magnitude to that in BAPTA-free conditions (0 mM BAPTA, $I_{ret\text{-}holding}$: 173 ± 18 pA, $n = 7$; 0.5 mM BAPTA, $I_{ret\text{-}holding}$: 168 ± 23 pA, $n = 5$; $p = 0.9$).

We also tested the effects of a different KCNQ channel allosteric activator, ICA-27243 (20 μM; see Fig. 1 *c*, and see (26,27)). Similar to retigabine, ICA-27243 increases KCNQ channels' open probability and induces a leftward shift in their voltage-dependence. However, work in heterologous cells has shown that, unlike retigabine, ICA-27243 does not interfere with the KCNQ2/3 or KCNQ5 channel activation time-course (26,27). Bath application of ICA-27243 increased the holding current due to KCNQ channel opening ($I_{ICA\text{-}holding}$: 101 ± 11 pA, $n = 6$) similar to

retigabine, but differed in that it had no significant effect on the IsAHP activation kinetics ($\tau_{rise} = 210 \pm 16$ ms; $\tau_{rise\text{-}ica} = 227 \pm 20$ ms, $n = 5$; $p = 0.13$; see Fig. 1 *c*). Together, these data strongly suggest that the IsAHP activation is limited by the KCNQ channel kinetics in CA3 pyramidal neurons.

Hippocalcin is the primary sAHP calcium sensor in CA3 pyramidal neurons

The lack of retigabine's effect on the sAHP decay suggested that other factors are responsible for this part of the IsAHP time-course. A likely candidate is the sAHP calcium sensor, and consequently, we tested the role of Hippocalcin in the IsAHP time-course. As there are no pharmacological blockers for Hippocalcin, we used *Hippocalcin* knockout mice (28).

We first determined whether Hippocalcin acts as the principal sAHP calcium sensor in CA3 pyramidal neurons. To do this, we compared the magnitude of both the sAHP and IsAHP between wild-type and *Hippocalcin* knockout mice. We recorded the sAHP in current-clamp by rapidly depolarizing CA3 pyramidal neurons with a 1-nA current injection for 100 ms to activate voltage-gated calcium channels. Using this protocol, an sAHP developed after the termination of the pulse in both $HC^{+/+}$ and $HC^{-/-}$ mice (Fig. 2 *a*). There was a significant reduction of the sAHP peak response in $HC^{-/-}$ mice when compared to wild-type ($HC^{+/+}$) mice ($HC^{+/+}$: 14.7 ± 0.6 mV, $n = 22$; $HC^{-/-}$: 4.8 ± 0.4 mV, $n = 22$; $p < 0.001$; see Fig. 2 *c*). In the same neurons, we also measured the sAHP current under voltage-clamp after a 100-ms depolarization to +45 mV. Similar to the sAHP measures, the IsAHP amplitude was also dependent on Hippocalcin (IsAHP in $HC^{+/+}$: 248 ± 13 pA, $n = 22$; $HC^{-/-}$: 109 ± 9 pA, $n = 22$; $p < 0.001$; see Fig. 2, *b* and *c*).

To increase the sensitivity of our sAHP measurements, we also compared the responses of $HC^{+/+}$ and $HC^{-/-}$ mice to 1 s current injections whose amplitude ranged from 0 pA to 300 pA. In both $HC^{+/+}$ and $HC^{-/-}$ pyramidal neurons, a sAHP developed whose magnitude depended on the injected current (see Fig. S1 in the Supporting Material). We found a reduction of the sAHP across all current injections in neurons lacking Hippocalcin (see Fig. S1). Similar measurements were made in neurons whose excitability was increased by blocking the D-type potassium current (see Fig. S1), a conductance that limits the number and frequency of somatic action potentials in CA3 pyramidal neurons (29). In the presence of D-type potassium current blockers, current injections elicited a greater number of action potentials and a correspondingly larger sAHP in both wild-type and *Hippocalcin* knockout mice. Nonetheless, the sAHP was still smaller in *Hippocalcin* knockout mice compared to wild-type mice (see Fig. S1). The reduction of the sAHP in *Hippocalcin* knockout mice was not due to a reduction of the intrinsic excitability of CA3 pyramidal neurons (see Fig. S1 and Table S1), as current injections in *Hippocalcin* knockout mice elicited more action potentials than in wild-type controls. Thus, we conclude Hippocalcin acts as a (I)sAHP calcium sensor in CA3 pyramidal neurons similarly to CA1 and L5 pyramidal neurons.

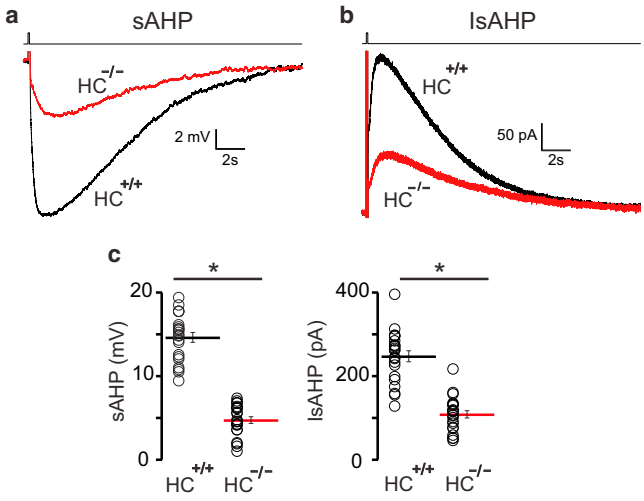

FIGURE 2 Hippocalcin is an sAHP calcium sensor in CA3 pyramidal neurons. (*a*) Representative sAHP traces from $HC^{+/+}$ (*black*) and $HC^{-/-}$ null neurons (*red*). (*b*) Representative IsAHP traces from $HC^{+/+}$ (*black*) and $HC^{-/-}$ null neurons (*red*). (*c*) Summarized results showing that the sAHP and sAHP current is significantly decreased in $HC^{-/-}$ mice ($n = 22$ each, $*p < 0.001$ Student's *t*-test). All recordings were in the presence of 100-nM apamin.

Hippocalcin determines the role of KCNQ channels in the IsAHP time-course

We also assessed the role of Hippocalcin in the (I)sAHP kinetics (Fig. 3). After the termination of a brief stimulation pulse (100 ms, +45 mV), $HC^{+/+}$ pyramidal neurons developed an sAHP that activated and decayed with time constants of 223 ± 9 ms ($n = 22$) and 4.6 ± 0.1 s ($n = 22$), respectively. In $HC^{-/-}$ neurons, the same protocol induced a smaller amplitude sAHP with significantly slower activation (τ_{rise}: 486 ± 32 ms, $n = 22$) and decay kinetics (τ_{decay}: 6.4 ± 0.25 s, $n = 22$; see Fig. 3, *a* and *b*). We found similar prolonged kinetics for the IsAHP generated in voltage-clamp (τ_{rise}: $HC^{+/+}$: 223 ± 9.7 ms, $n = 22$; $HC^{-/-}$: 391 ± 24 ms, $n = 22$, $p < 0.001$; τ_{decay}: $HC^{+/+}$: 5.04 ± 0.1 s, $n = 22$; $HC^{-/-}$: 7.2 ± 0.2 s, $n = 22$, $p < 0.001$; see Fig. 3, *c* and *d*). Thus, loss of Hippocalcin leads to slower (I)sAHP activation and decay kinetics, suggesting that Hippocalcin is critical for the IsAHP time-course.

We considered whether loss of Hippocalcin, a small cytosolic calcium-binding protein, slows the (I)sAHP kinetics by altering the neurons' calcium-handling in such a way as to prolong the calcium signal. To test this possibility, we made use of calcium-activated SK currents as

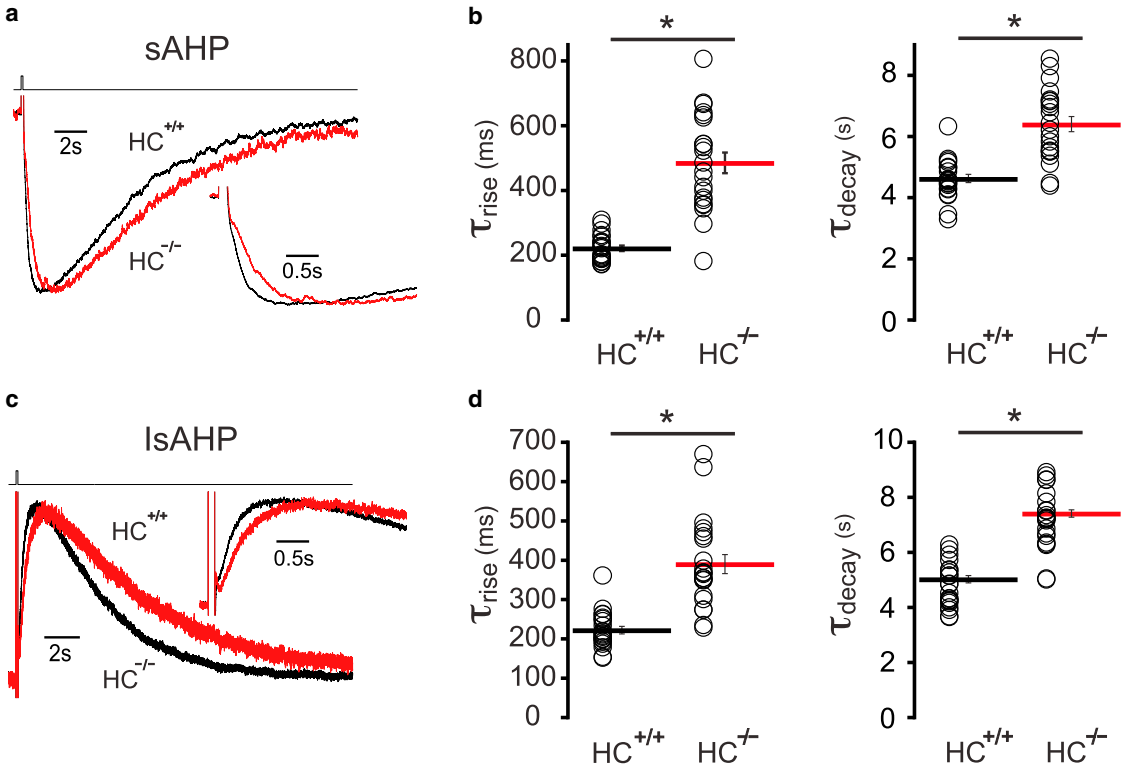

FIGURE 3 Loss of Hippocalcin slows the (I)sAHP time-course. (*a*) Superimposed normalized sAHP traces from $HC^{+/+}$ (*black*) and $HC^{-/-}$ null neurons (*red*). (*Inset*) sAHP activation on an expanded timescale. (*b*) Summarized results showing that the sAHP τ_{rise} and τ_{decay} are slower in $HC^{-/-}$ than $HC^{+/+}$ pyramidal neurons ($n = 22$ each, $*p < 0.001$ Student's *t*-test). (*c*) Superimposed normalized IsAHP traces from $HC^{+/+}$ (*black*) and $HC^{-/-}$ null neurons (*red*). (*Inset*) IsAHP activation on an expanded timescale. (*d*) Summarized data depicting that the IsAHP τ_{rise} and τ_{decay} are slower in $HC^{-/-}$ than $HC^{+/+}$ pyramidal neurons ($n = 22$ each, $*p < 0.001$ Student's *t*-test). All recordings were in the presence of 100-nM apamin.

a reporter for calcium-handling, as has been done previously in Sah (30). Although the peak of the I-SK current was similar in wild-type and *Hippocalcin* knockout mice ($HC^{+/+}$ I-SK = 162 ± 10 pA, $n = 23$; $HC^{-/-}$ I-SK = 184 ± 14 pA, $n = 23$; $p = 0.2$) we found that $HC^{-/-}$ neurons have a larger SK channel charge transfer ($HC^{+/+}$ Q-SK = 24 ± 2 pC, $n = 23$; $HC^{-/-}$ Q-SK = 40 ± 4 pC, $n = 23$; $p < 0.001$) due to their significantly slowed decay kinetics ($HC^{+/+}$ I-SK τ_{decay} = 146 ± 7 ms, $n = 23$; $HC^{-/-}$ I-SK τ_{decay} = 214 ± 8 ms, $n = 23$; $p < 0.001$; see Fig. 4). This experiment indicated that CA3 pyramidal neurons from *Hippocalcin* knockout mice undergo a slower calcium time-course than control cells, and suggested that this might explain some or all of their slowed IsAHP kinetics.

The considerably slower IsAHP time-course in *Hippocalcin* knockout mice raised the possibility that KCNQ activation has a diminished contribution to the rise kinetics in these mice. However, retigabine application to $HC^{-/-}$ neurons accelerated the IsAHP activation, just as it did in $HC^{+/+}$ mice ($HC^{-/-}$ τ_{rise}: 262 ± 19 ms, $n = 9$; $\tau_{rise\text{-}ret}$: 135 ± 20 ms, $n = 9$; $p < 0.001$; see Fig. 5 *a*). Similar to wild-type mice, ICA-27243 had no effect on the IsAHP rise kinetics ($HC^{-/-}$ τ_{rise}: 298 ± 10 ms, $n = 4$; $\tau_{rise\text{-}ica}$: 326 ± 16 ms, $n = 4$; $p = 0.2$; see Fig. 5 *b*). Surprisingly, retigabine greatly accelerated the IsAHP decay time-course in $HC^{-/-}$ neurons ($HC^{-/-}$ $\tau_{w\text{-}decay}$: 6.8 ± 0.4 s, $n = 9$; $\tau_{w\text{-}decay\text{-}ret}$: 4.4 ± 0.6 s, $n = 9$; $p < 0.001$; see Fig. 5 *a*), despite having no effect in $HC^{+/+}$ mice. More striking was that in the absence of Hippocalcin, retigabine accelerated the IsAHP decay to an even faster time-course than was seen in the $HC^{+/+}$ IsAHP ($HC^{+/+}$ $\tau_{w\text{-}decay}$: 6.2 ± 0.5 s, $n = 7$; $HC^{-/-}$ $\tau_{w\text{-}decay\text{-}ret}$: 4.4 ± 0.6 s, $n = 9$; $p < 0.05$). Collectively, our data suggest that KCNQ channels contribute to the slow IsAHP activation time-course, but whether they limit the IsAHP decay unexpectedly depends on the identity of the calcium sensor.

DISCUSSION

KCNQ channel kinetics and the (I)sAHP time-course

Although the slow time-course of the (I)sAHP has been noted for decades, its origin has remained enigmatic. In early work, it was assumed that calcium interacts directly with the (I)sAHP potassium channel, and contemporaneous modeling studies suggested that slow potassium-channel gating was responsible for the protracted

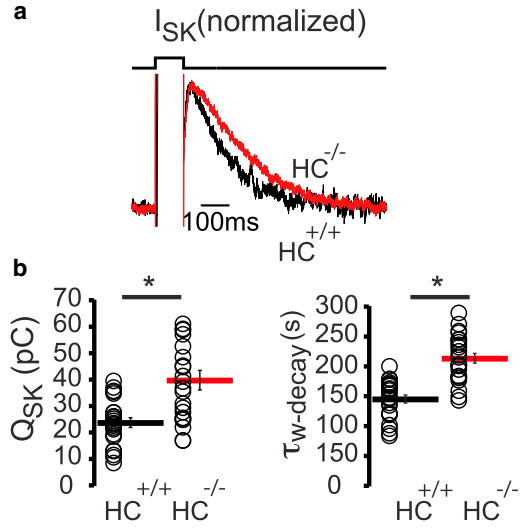

FIGURE 4 Increased SK channel activity in Hippocalcin null neurons. To isolate the apamin-sensitive potassium current (I-SK), 100-nM apamin was bath-applied to produce a complete block of SK channels. Currents in the presence of apamin were subtracted from those without apamin to determine the I-SK. (*a*) Superimposed normalized I-SK from $HC^{+/+}$ (*black*) and $HC^{-/-}$ (*red*) neurons. (*b*) Summarized results showing the apamin-sensitive charge (Q) and decay time constant from $HC^{+/+}$ and $HC^{-/-}$ neurons ($n = 23$ each, $*p < 0.001$ Student's *t*-test).

kinetics (12,16,31). Recently, we and others have suggested that diffusible calcium sensors such as Hippocalcin bind cytosolic calcium, translocate to the plasma membrane, and trigger the opening of potassium channels to elicit the (I)sAHP (13–15,19). This suggested that the calcium sensors might limit the (I)sAHP time-course. Therefore, the primary goal of this study was to test whether channel kinetics can also contribute to the (I)sAHP time-course.

Previously, we have shown that KCNQ channels partly mediate the IsAHP in CA3 pyramidal neurons (17,18). Consequently, we assessed the role of KCNQ channels in the IsAHP kinetics by using the allosteric KCNQ channel-activator retigabine, which is known to speed KCNQ channel activation kinetics in heterologous cells (23). We found that application of retigabine speeded the IsAHP activation in wild-type neurons, suggesting that KCNQ channel activation is limiting the IsAHP activation process (Fig. 1). We further found that the effect of retigabine was more robust in $HC^{-/-}$ neurons; despite their slower IsAHP time-course, application of retigabine accelerated the IsAHP in $HC^{-/-}$ mice to an even faster time-course than the time-course it induced in wild-type mice (compare Figs. 1 and 5). Thus, the simplest explanation to account for these observations is that KCNQ channels kinetics contribute to the slow (I)sAHP activation in both wild-type and $HC^{-/-}$ CA3 pyramidal neurons, whereas the identity of the calcium sensor dictates whether KCNQ channels are critical for the IsAHP decay.

Our finding that retigabine accelerates the IsAHP decay exclusively in the *Hippocalcin* knockout mice suggested the unexpected possibility that the identity of the (I)sAHP calcium sensor determines the composition of the (I)sAHP channels. CA3 pyramidal neurons express KCNQ2, KCNQ3, and KCNQ5 channels, all of which could potentially contribute to the (I)sAHP (17,18,32). Considering that KCNQ3 and KCNQ5 are most susceptible to retigabine modulation (23,33), it is likely that in $HC^{-/-}$ null neurons KCNQ3 and/or KCNQ5 channels play a larger role in the (I)sAHP time-course than in wild-type mice, thus leading to the more robust retigabine effect. Currently, we cannot determine whether such shift in the (I)sAHP channel KCNQ subunit composition is due to homeostatic changes in response to loss of Hippocalcin, or if the remaining (I)sAHP calcium sensor, whose identity is unknown, specifically targets a different subset of KCNQ channels.

Although our results clearly demonstrate a role for KCNQ channels in the sAHP time-course, the contribution of Hippocalcin is less clear. The (I)sAHP in *Hippocalcin* knockout mice has significantly slower rise and decay kinetics, but it appears from our experiments on SK channels that calcium handling is altered; this most likely contributes to the slowed time-course. It is also possible that the altered (I)sAHP channel composition plays a role in the slower kinetics as different KCNQ channel subunits have different activation and deactivation rates (23).

Nonetheless, we cannot rule out the possibility that the properties of the remaining (I)sAHP calcium sensor also slow down the (I)sAHP kinetics in *Hippocalcin* knockout mice. Indeed, although application of retigabine speeds the IsAHP kinetics, the IsAHP is still slow to activate in its presence compared with other potassium channels in both wild-type and $HC^{-/-}$ neurons. This might be because retigabine cannot speed the KCNQ channels any further due to a ceiling effect, or else because Hippocalcin and other (I)sAHP calcium sensors bind calcium slowly, contributing to the prolonged sAHP activation (13). Supporting this latter suggestion, we found that inclusion of the slow calcium-binding buffer EGTA in the intracellular solution led to a slower IsAHP activation time constant in both wild-type and $HC^{-/-}$ mice ($HC^{+/+}$ τ_{rise}: 197 ± 15 ms, $n = 6$; $\tau_{rise\text{-}EGTA}$: 394 ± 31 ms, $n = 6$, $p < 0.001$; $HC^{-/-}$ τ_{rise}: 358 ± 24 ms, $n = 6$; $\tau_{rise\text{-}EGTA}$: 557 ± 39 ms, $n = 6$, $p < 0.01$; see Fig. S2).

For EGTA to delay the IsAHP activation, it must bind incoming calcium before Hippocalcin or any other IsAHP calcium sensor. An IsAHP delay will then develop as calcium is slowly released by EGTA and then bound by the IsAHP calcium sensors (25). Thus we would predict that the (I)sAHP calcium sensors should have a calcium-binding on-rate no greater than the EGTA binding on-rate (~10^6 M^{-1} s^{-1}; see also Goldberg et al. (13)) and are a slow step in the (I)sAHP activation mechanism. Together, our data suggest that there may be no single rate-limiting step that generates the exceedingly slow time-course of the (I)sAHP, but rather that the (I)sAHP kinetics are most likely a function of both slow calcium-binding to the IsAHP

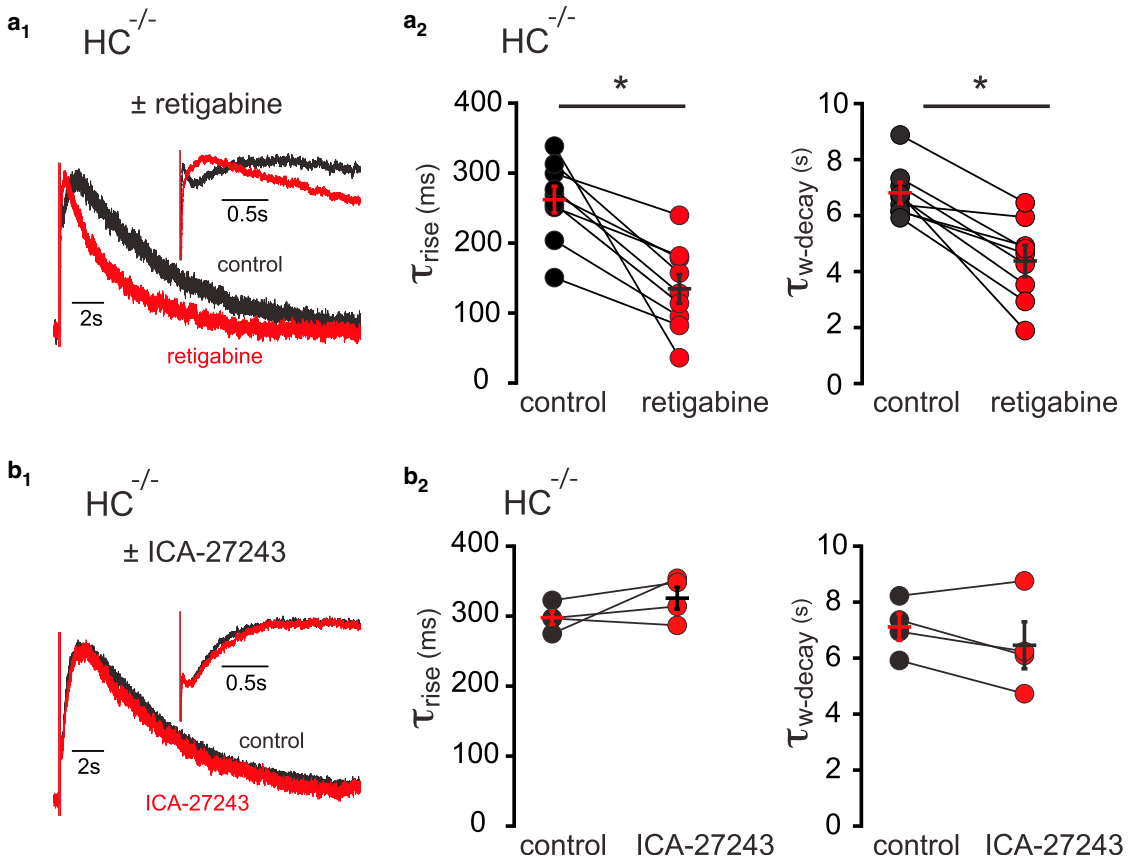

FIGURE 5 KCNQ channel kinetics determine the IsAHP time-course in *Hippocalcin* knockout mice. (a_1) Superimposed normalized IsAHP traces before (*black*) and after (*red*) application of 40 μM retigabine. (a_2) Summary data showing the effect of retigabine on the activation and decay time constants in $HC^{-/-}$ neurons ($n = 9$, $*p < 0.001$ Student's *t*-test). (b_1) Superimposed normalized IsAHP traces before (*black*) and after (*gray*) application of 20 μM ICA27243. (b_2) Summarized results showing the effect of ICA27243 on the activation and decay time-constant in $HC^{-/-}$ neurons ($n = 4$). All recordings were in the presence of 100-nM apamin.

calcium sensors as well as slow activation kinetics of KCNQ channels.

Molecular diversity of calcium-activated sAHPs

A slow afterhyperpolarization develops following a train of action potentials in multiple cell types throughout the brain. Depending on the neuron, membrane potential, frequency, and number of action potentials, this slow afterhyperpolarization is mediated by Ih channels, K-ATP channels, sodium-activated potassium channels, and/or calcium-activated sAHP channels (34–37). Traditionally, the calcium-activated sAHP has been considered as one molecular entity. Support for this model has come from the observation that, independent of the cell type, the calcium-activated sAHP has a similar time-course; is inhibited by norepinephrine through the downstream activation of protein kinase A; and is insensitive to traditional potassium channel blockers and toxins (1,31).

However, the recent observation that multiple KCNQ channels and high-affinity neuronal calcium sensors can mediate the sAHP suggests that the calcium-activated sAHP is more molecularly diverse than originally thought. Further support for this idea stems from our finding that retigabine differentially affects the IsAHP decay rate in wild-type and *Hippocalcin* knockout mice, suggesting that multiple channel and calcium sensor combinations could give rise to a IsAHP. Molecular sAHP diversity is also reinforced by the previous findings that the pharmacology of the sAHP depends on the calcium source that activates it. For instance, UCL2077, a sAHP inhibitor, robustly blocks the IsAHP activated by calcium influx through L-type calcium channels in thalamic neurons, but has little effect when calcium influx is through T-type calcium channels in the same thalamic neurons (38). Together, our data suggest that multiple channels and sensors can give rise to a molecularly diverse (I)sAHP.

In vivo implications

Over the past decade, the KCNQ family of potassium channels has emerged as essential regulators of neuronal excitability. Mutations in either *Kcnq2* or *Kcnq3* are associated with benign familial neonatal convulsions, a form of

pediatric epilepsy. Currently, it is thought that KCNQ channels mediate the M-current (39,40), a potassium conductance that first activates at subthreshold potentials and increases as neurons approach their action-potential threshold. Consistent with this function, mice engineered with decreased KCNQ channel activity have smaller M-currents (41,42). However, we have also shown that CA3 pyramidal neurons in mice with reduced KCNQ levels have an additional reduction of the sAHP current, the major conductance underlying spike frequency adaptation in the hippocampus. This led us to propose that KCNQ channels may have a second significant identity in addition to their role in the M-current: as candidates for the elusive potassium channels that mediate the sAHP (17,18). In line with this proposal, we recently found that the specific sAHP inhibitor UCL2077 is also a blocker of KCNQ channels (32). Our finding that retigabine, a specific KCNQ channel activator, modifies the IsAHP kinetics in wild-type and Hippocalcin null neurons further supports our proposal that KCNQ channels contribute to the sAHP in hippocampus.

Additionally, it has now become apparent that not all KCNQ mutations lead to similar neurological deficits. For instance, a different set of *Kcnq2* channel mutations leads to benign familial neonatal convulsions, whereas another set leads to epileptic encephalopathy and Ohtahara syndrome (43,44). It is therefore possible that various *Kcnq* channel gating mutations differentially affect the M-current and sAHP, thus leading to a spectrum of neurological disorder severity. Lastly, a reliance of the sAHP activation on KCNQ channel kinetics would make the sAHP time-course less susceptible to calcium level fluctuations. As a result, changes in calcium levels as seen during aging or neurodegenerative disorders like prion disease (45) would primarily affect the sAHP amplitude but not necessarily its time-course. This is particularly important, as the sAHP time-course is critical for the development of spike frequency adaptation, a quiescence period that neurons enter after a surge of activity.

CONCLUSION

In this study, we combined KCNQ-specific pharmacology to investigate the relative contribution of KCNQ channel kinetics to the sAHP time-course in CA3 pyramidal neurons. We found that KCNQ channel kinetics limit the (I)sAHP activation and that the identity of the sAHP calcium sensor dictates the extent that KCNQ channel kinetics contribute to the IsAHP time-course. Overall, we propose a model in which the sAHP time-course is controlled by two events—slow calcium binding to a diffusible sensor, followed by the slow activation of KCNQ channels. Our work provides the first, to our knowledge, evidence for a role of KCNQ channel kinetics in the sAHP time-course.

SUPPORTING MATERIAL

One table and two figures are available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(12)01193-9.

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