Brief Communications

Visinin-Like Neuronal Calcium Sensor Proteins Regulate the Slow Calcium-Activated Afterhyperpolarizing Current in the Rat Cerebral Cortex

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Many neurons in the nervous systems express afterhyperpolarizations that are mediated by a slow calcium-activated potassium current. This current shapes neuronal firing and is inhibited by neuromodulators, suggesting an important role in the regulation of neuronal function. Surprisingly, very little is currently known about the molecular basis for this current or how it is gated by calcium. Recently, the neuronal calcium sensor protein hippocalcin was identified as a calcium sensor for the slow afterhyperpolarizing current in the hippocampus. However, while hippocalcin is very strongly expressed in the hippocampus, this protein shows a relatively restricted distribution in the brain. Furthermore, the genetic deletion of this protein only partly reduces the slow hyperpolarizing current in hippocampus. These considerations question whether hippocalcin can be the sole calcium sensor for the slow afterhyperpolarizing current. Here we use loss of function and overexpression strategies to show that hippocalcin functions as a calcium sensor for the slow afterhyperpolarizing current in the cerebral cortex, an area where hippocalcin is expressed at much lower levels than in hippocampus. In addition we show that neurocalcin δ, but not VILIP-2, can also act as a calcium sensor for the slow afterhyperpolarizing current. Finally we show that hippocalcin and neurocalcin δ both increase the calcium sensitivity of the afterhyperpolarizing current but do not alter its sensitivity to inhibition by carbachol acting through the $G\alpha_{q\text{-}11}$-PLCβ signaling cascade. These results point to a general role for a subgroup of visinin-like neuronal calcium sensor proteins in the activation of the slow calcium-activated afterhyperpolarizing current.

Introduction

Repetitive spiking by pyramidal cells of the hippocampus and cortex results in the development of a slow afterhyperpolarization (sAHP) that controls spike frequency accommodation (Madison and Nicoll, 1984; Schwindt et al., 1988b) and regulates neuronal gain (Higgs et al., 2006). Similar slow hyperpolarizing afterpotentials have also been reported in many other cell types, suggesting that sAHPs are ubiquitous regulators of neuronal excitability (Faber and Sah, 2003; Vogalis et al., 2003). A general property of these sAHPs is that they are regulated by neuromodulators signaling via $G\alpha_{q\text{-}11}$ or through the $G\alpha_s$/adenylate cyclase/cAMP/protein kinase A signaling cascade (Madison and Nicoll, 1982; Schwindt et al., 1988a; Pedarzani and Storm, 1993; Krause et al., 2002). Thus the sAHP is an important determinant of cellular excitability in a variety of cell types in the CNS and an important target for many neuromodulators.

There is now widespread consensus that the sAHP is mediated by a calcium-activated potassium current generally referred to as I_{sAHP} (Faber and Sah, 2003; Vogalis et al., 2003). Surprisingly, however, neither the ion channel(s) carrying I_{sAHP} nor the mechanism through which this current is gated by calcium are well understood. A recent breakthrough occurred with the discovery that the genetic deletion of the neuronal calcium sensor (NCS) hippocalcin results in the inhibition of I_{sAHP} in hippocampal pyramidal cells. This observation has led to the idea that hippocalcin is the calcium sensor for I_{sAHP} (Tzingounis et al., 2007). However, while I_{sAHP} is a broadly expressed cellular property in many regions of the brain, the expression of hippocalcin is much more restricted (Saitoh et al., 1994; Paterlini et al., 2000). Furthermore, hippocalcin deletion inhibits but does not abolish I_{sAHP} in hippocampal pyramidal cells. These considerations suggest that hippocalcin may not be the only NCS protein capable of functioning as a calcium sensor for I_{sAHP}. The cerebral cortex offers the opportunity to address this issue since hippocalcin is expressed in this area at levels considerably lower than in hippocampus (Saitoh et al., 1994), but cortical pyramidal cells display a robust and well characterized I_{sAHP}. In the present work, we have taken advantage of these properties to readdress the role of hippocalcin in the regulation of I_{sAHP}.

Materials and Methods

Experiments were conducted on cortical brain slices either acutely prepared or maintained in organotypic culture (Villalobos et al., 2004; Yan et al., 2009). Briefly, rats or mice during the second (organotypic) or third to fifth (acute) postnatal weeks were anesthetized with halothane or isoflurane and killed by decapitation. The brain was removed and cooled in ice-cold Ringer's solution of standard composition (in mM: 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 $CaCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose), supplemented with 10 mM HEPES and bubbled to saturation with 95%O_2, 5% CO_2. The brain was blocked and coronal brain slices of a

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nominal thickness of 300 μm were cut using a vibratome. For organotypic culture, slices were collected in sterile Ringer's solution and placed in culture essentially as described by Stoppini et al. (1991).

Electrophysiological recordings were obtained from pyramidal cells of layer II–III (shRNA experiment) or layer V (NCS expression experiments) of the medial prefrontal/anterior cingulate cortices as previously described (Villalobos et al., 2004, 2005; Yan et al., 2009). Slices were transferred one at a time to a recording chamber on the stage of a Nikon E600 microscope, where they were held in place by a platinum and nylon grid. Slices in the recording chamber were perfused continuously with Ringer's solution at 30 ± 1°C bubbled to saturation with 95% O_2 and 5% CO_2. Whole-cell recordings were obtained using a potassium-based intracellular solution (composition in mM: $KMeSO_4$ 120, KCl 5, NaCl 5, EGTA 0.02, HEPES 10, $MgCl_2$ 1, Na phosphocreatine 10, ATP magnesium salt 4, GTP Na salt 0.3, myo-inositol 30). Electrodes filled with this solution exhibited resistances ranging from 2 to 4 MΩ. Because holding currents and I_{sAHP} amplitudes were relatively small, series resistance was left uncompensated. All recording were conducted using a Multiclamp amplifier under the control of pClamp 9 (Molecular Devices).

Pyramidal neurons were transfected using either *in utero* electroporation (shRNA experiments) or particle mediate gene transfer (NCS expression experiments). *In utero* electroporation was conducted on embryonic mice at E14.5 as described by Saito (2006) and used a pool of five shRNAs targeting hippocalcin to maximize the suppression of hippocalcin expression (Parsons et al., 2009). These shRNAs were developed by the RNAi Consortium, and their expression was driven by the U6 promoter in the pLKO.1-puro vector (Mission RNAi, Sigma) (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). YFP in pCAG (Addgene) at a 4:1 ratio was electroporated with the shRNA to serve as a transfection marker. Particle-mediated gene transfer was conducted as previously described (Villalobos et al., 2004; Yan et al., 2009). Gold particles were coated with plasmids encoding either hippocalcin (in pCMV-Sport6, ATCC Cat. No. 10470251/MGC Image ID 5360894), neurocalcin δ (in pCMV-Sport6, ATCC Cat. No. MGC-36496/MGC Image ID 5365560), or VILIP-2 (in pcDNA3.1, a kind gift from Dr. W. A. Catterall, University of Washington, Seattle, WA) (Lautermilch et al., 2005) and EGFP (pEGFP-N1, Clontech) at a 4:1 ratio. In all these vectors, expression was driven by the CMV promoter, which effectively drives gene expression in this preparation (Béïque and Andrade, 2003). The PLCβ1 knock-out mouse (Kim et al., 1997) was a kind gift from Drs. R. K. Wong (SUNY Downstate Medical Center, Brooklyn, NY) and H. S. Shin (Korea Institute of Science and Technology, Seoul, Korea).

For experiments examining the effect of the shRNA targeting hippocalcin, we used cells in the untransfected side of the brain as controls, rather than non-YFP-expressing cells in the transfected side, because preliminary experiments indicated that detectable YFP expression in a cell *in vivo* did not unambiguously identify untransfected neurons in the transfected side (supplemental Fig. S2, available at www.jneurosci.org as supplemental material). Experiments aimed at examining the effects of expressing NCS proteins used paired recordings from neighboring layer V transfected and untransfected pyramidal neurons located within ~150 μm of each other. This approach was preferred to using mock transfections in separate slices for the controls because it allows for a more stringent cell match with respect to cell location and slice history. We have previously shown that transfection with EGFP has no detectable effect on I_{sAHP} (Yan et al., 2009) and the very low transfection efficiency of the gene gun assured that the overwhelming fraction of non-GFP-expressing cells correspond to untransfected neurons. All organotypic slice recordings were obtained 2–3 d after transfection.

Pyramidal cells were held at −60 mV and I_{sAHP} was triggered using a 100-ms-long depolarizing step in voltage clamp. In most experiments, we administered apamin (300 nM) to block I_{mAHP}. This allowed us to estimate the amplitude of I_{mAHP} for most cells and also to record I_{sAHP} in isolation. I_{sAHP} amplitude was determined ~300 ms after the end of the triggering step. The voltages reported are not corrected for the liquid junction potential. Most drugs were obtained from Sigma or Fisher. Tetrodotoxin was obtained from EMD Biosciences, while apamin was obtained from Tocris Bioscience. All drugs were administered to the slice dissolved in the bath. Statistical comparisons used *t* tests.

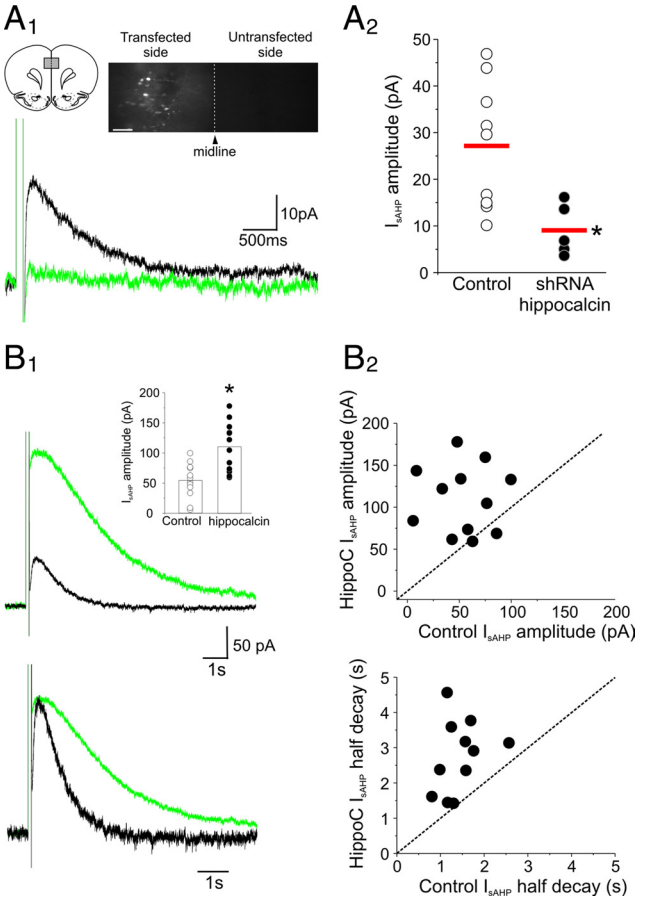

Figure 1. Hippocalcin expression regulates I_{sAHP}. ***A*$_1$,** Superimposed traces illustrating I_{sAHP} recorded from shRNA/YFP-transfected (green) and untransfected (black) pyramidal cells. Inset, Fluorescence image depicting transfected and untransfected cingulate cortices in a living brain slice at the approximate position illustrated in the diagram. Calibration bar = 100 μm. ***A*$_2$,** Graph summarizing the amplitude of I_{sAHP} in 5 shRNA/YFP-transfected and 9 control untransfected cells. *$p < 0.05$. ***B*$_1$,** Superimposed traces depicting I_{sAHP} recorded in a pair of hippocalcin-transfected (green) and neighboring untransfected (black) pyramidal cells in organotypic slices. Lower panel illustrates the same slices scaled to emphasize the change in the time course of I_{sAHP}. Inset, Effect of hippocalcin on the amplitude of I_{sAHP}. *$p < 0.001$. ***B*$_2$,** Graphs illustrating the effects of hippocalcin expression on the amplitude (upper plot) and decay (lower plot) of I_{sAHP} for each cell pair tested in this experiment. The half decay for only 11 cell pairs is depicted in the lower graph because it was not possible to estimate the half decay for I_{sAHP} in one of the control cells.

Results

In a first experiment, we used *in utero* electroporation to express shRNAs targeting hippocalcin in layer II–III pyramidal cells of cingulate cortex to ask whether hippocalcin played a role regulating I_{sAHP} in this area. Previous studies have shown the effectiveness of this shRNA approach to knockdown the expression of specific proteins (e.g., Bai et al., 2003; Parsons et al., 2009), and we confirmed the effectiveness of this strategy in the current experiments using immunohistochemistry to confirm the knockdown of hippocalcin expression in transfected cortical neurons of the upper layers of cortex (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). As illustrated in Figure 1*A*, transfection of shRNAs targeting hippocalcin resulted in a significant reduction in the amplitude of I_{sAHP} in YFP-expressing pyramidal cells when compared to the amplitude of I_{sAHP} in pyramidal cells matched for location on the (nontransfected) contralateral cortex.

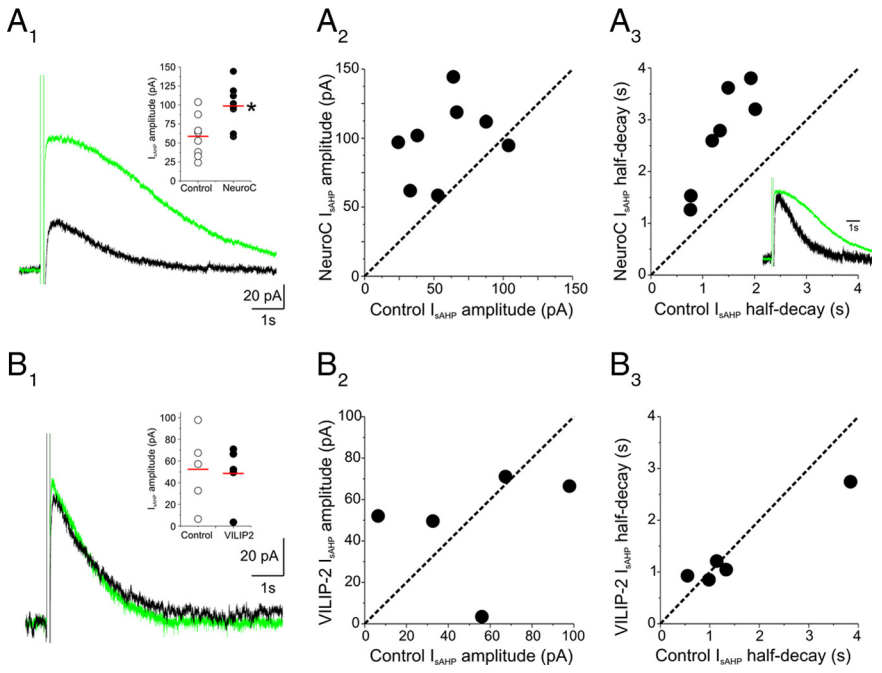

Figure 2. Neurocalcin δ, but not VILIP-2, increases the amplitude and prolongs the duration of I_{sAHP}. ***A*****1**, I_{sAHP} recorded in a pyramidal cell transfected with neurocalcin δ (green) and in a neighboring untransfected control pyramidal cell (black) in the same slice. Inset, Graph summarizing the effect of neurocalcin δ on the amplitude of I_{sAHP} in 8 cell pairs. *$p = 0.012$. ***A*****2**, ***A*****3**, Graphs illustrating the effects of neurocalcin δ expression on the amplitude (***A*****2**) and decay (***A*****3**) of I_{sAHP} for each cell pair. The half decay for only 7 cell pairs is depicted in ***A*****3** because it was not possible to estimate the half decay for I_{sAHP} in one of the control cells. ***A*****3**, Inset, Traces in ***A*****1** scaled to illustrate the slower time course of I_{sAHP} induced by neurocalcin δ. ***B*****1**, I_{sAHP} recorded in a cortical pyramidal cell transfected with VILIP-2 (green) and a neighboring control untransfected cell (black). Inset, Graph summarizing the amplitude of I_{sAHP} in control cells and in cells transfected with VILIP-2. ***B*****2**, ***B*****3**, Graphs illustrating the effects of VILIP-2 on the amplitude (***B*****2**) and decay (***B*****3**) of I_{sAHP} for each cell pair tested in this experiment. $N = 5$ cell pairs tested. $p = 0.49$.

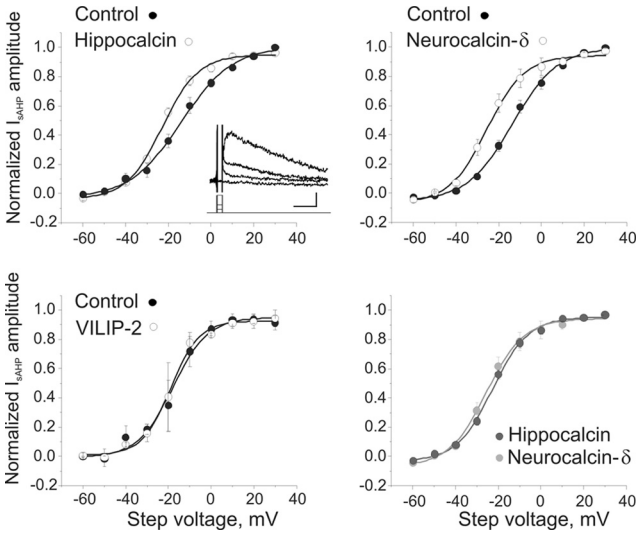

Figure 3. Effect of hippocalcin, neurocalcin δ and VILIP-2 on the apparent calcium sensitivity of I_{sAHP}. Inset, Voltage steps to −60 to +20 mV were used to produce graded increases in calcium influx and hence graded activations of I_{sAHP} (Gerlach et al., 2004). Calibration: 40 pA, 500 ms. Expression of hippocalcin and neurocalcin δ, but not of VILIP-2, resulted in an increase in the apparent calcium sensitivity of I_{sAHP}. However, the apparent calcium sensitivity curves for cells transfected with hippocalcin or neurocalcin δ were superimposable (lower right). Hippocalcin, $n = 15$ cell pairs; neurocalcin δ, $n = 6$ cell pairs, VILIP-2, $n = 4$ cell pairs.

To test further the possible role of hippocalcin in mediating I_{sAHP} in cortex, we transfected this NCS into pyramidal cells in cortical slices maintained in organotypic culture and assessed its effect on I_{sAHP} using paired recordings from neighboring transfected and untransfected pyramidal cells (Béïque et al., 2007; Yan et al., 2009). As illustrated in Figure 1*B*, expression of hippocalcin resulted in a large increase in the amplitude of I_{sAHP} (from 54 ± 8 to 110 ± 12 pA, $n = 12$ cell pairs, $p < 0.01$) that was accompanied by a significant slowing of its decay kinetics ($p < 0.01$). Since expression of hippocalcin had no significant effect on cell resting membrane potential, input resistance, or the amplitude of the apamin-sensitive calcium-activated potassium current I_{mAHP} (supplemental Table S1, available at www.jneurosci.org as supplemental material), we interpret these loss of function and overexpression experiments to indicate that hippocalcin regulates the amplitude of I_{sAHP} in cortical pyramidal cells.

We next considered the possibility that other members of the NCS protein family could act as calcium sensors for I_{sAHP}. Hippocalcin shares considerable sequence homology with the NCS proteins neurocalcin δ and VILIP1–3, and previous studies have suggested that these proteins may form a functionally distinct subfamily of visinin-like proteins (Burgoyne, 2007; Braunewell and Klein-Szanto, 2009). Therefore, we next transfected neurocalcin δ, a close homolog of hippocalcin (91% amino acid identity), and examined its effect on I_{sAHP}. As illustrated in Figure 2*A*, expression of neurocalcin δ, like hippocalcin, again resulted in a large increase in I_{sAHP} amplitude (from 58 ± 10 to 98 ± 10 pA, $n = 8$ cell pairs, $p < 0.01$) and slowing of I_{sAHP} decay kinetics ($p < 0.01$). In contrast, transfection of VILIP-2, a more distant member of the hippocalcin subfamily of NCS proteins (<70% amino acid identity), had no effect on either the amplitude or decay kinetics of I_{sAHP} (Fig. 2*B*) (control: 63 ± 13 pA, VILIP-2 59 ± 5 pA, $n = 5$ cell pairs, $p = 0.79$). Combined, these results indicate that I_{sAHP} can be regulated by at least two members of the visinin-like subfamily of NCS proteins.

Hippocalcin and neurocalcin δ are expressed in the cerebral cortex in a roughly overlapping pattern, suggesting coexpression at the cellular level (supplemental Fig. S4, available at www.jneurosci.org as supplemental material). Previous work has suggested that two or more NCS proteins exhibiting different calcium affinities may work in tandem to extend the dynamic range of calcium sensing (reviewed by Burgoyne, 2007). Therefore we considered the possibility that a hippocalcin and neurocalcin δ could sense different calcium concentrations to activate I_{sAHP}. If that was the case, it could be expected that the hippocalcin- and neurocalcin δ-enhanced I_{sAHP}s should differ in their calcium sensitivity. To test this idea, we examined the apparent calcium sensitivity of I_{sAHP} under control conditions and after transfection with hippocalcin or neurocalcin δ. As illustrated in Figure 3, both hippocalcin and neurocalcin δ, but not VILIP-2, shifted the apparent calcium sensitivity of I_{sAHP}. However, the calcium-sensitivity curves for hippocalcin-enhanced and neurocalcin δ-enhanced I_{sAHPs} are superimposable (Fig. 3*B*, inset). These results do not support the idea that hippocalcin and neurocalcin δ sense different concentrations of calcium.

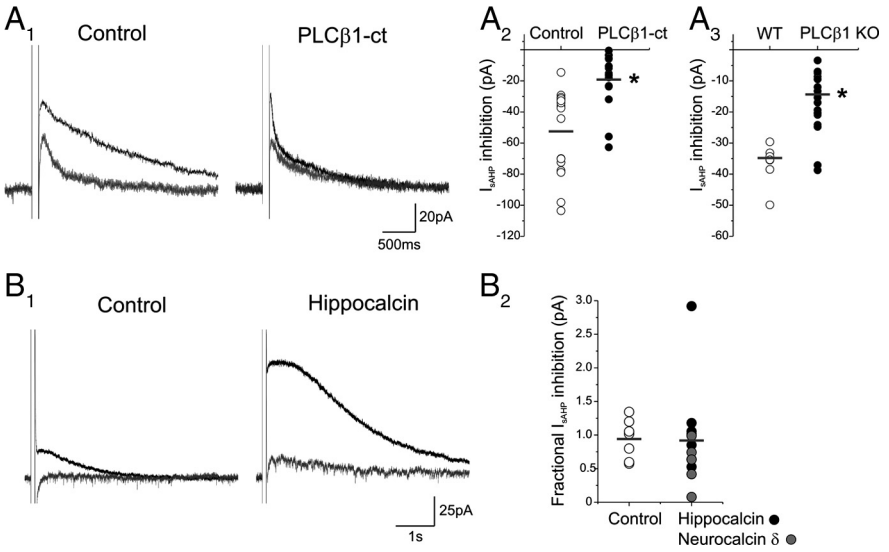

Figure 4. Effect of carbachol on I_{sAHP}. ***A1***, Superimposed traces depicting the effect of carbachol (30 μM) on I_{sAHP} in a pyramidal cell transfected with the $G\alpha_{q\text{-}11}$ dominant-negative PLCβ1-ct and a neighboring control cell. Control, Black; Carb, gray. ***A2***, Graph summarizing the effect of carbachol on I_{sAHP} in control and PLCβ1-ct-transfected pyramidal cells. *$p < 0.001$. ***A3***, Summary plot illustrating the effect of carbachol on I_{sAHP} in slices derived from wild-type and PLCβ1 knock-out mice. *$p < 0.001$. ***B1***, Effect of carbachol (30 μM) on a pyramidal cell transfected with hippocalcin and on a neighboring untransfected pyramidal cell. ***B2***, Graph plotting the carbachol (30 μM)-induced inhibition of I_{sAHP} in 7 control cells, 7 cells transfected with hippocalcin, and 5 cells transfected with neurocalcin δ. Please note that even in these slices derived from young animals, carbachol induced a slow afterdepolarization in a few cells (Yan et al., 2009), which resulted in a fractional inhibition of I_{sAHP} bigger than 1.

Little is known at the present time about the molecular identity of the channels carrying I_{sAHP}. This makes it difficult to unambiguously determine whether hippocalcin and neurocalcin δ facilitate the ion channels carrying the native current. As noted in the Introduction, one of the defining characteristics of I_{sAHP} is its inhibition by neuromodulators acting through G-protein-coupled receptors signaling through $G\alpha_s$ or $G\alpha_{q\text{-}11}$. Previous studies have shown that carbachol inhibits its I_{sAHP} in pyramidal cells of the cerebral cortex by acting on muscarinic $M1/M_3$ receptors (Gulledge et al., 2009). At a biochemical level, these receptors activate $G\alpha_{q\text{-}11}$ and PLCβ, leading to the breakdown of PtdIns(4,5)P_2. Therefore we first tested whether carbachol indeed inhibited I_{sAHP} by activating $G\alpha_{q\text{-}11}$ and PLCβ in these cells. As illustrated in Figure 4*A*, the ability of carbachol to inhibit I_{sAHP} was blocked by expression of the carboxy tail of PLCβ1 fused to GFP (PLCβ-ct), a dominant-negative construct that blocks $G\alpha_{q\text{-}11}$ signaling in central and peripheral neurons (Kammermeier and Ikeda, 1999; Yan et al., 2009). In addition, the ability of carbachol to inhibit I_{sAHP} was greatly reduced in animals in which PLCβ1, the predominant PLCβ isoform expressed in the cerebral cortex, had been deleted (Fig. 4*A3*) (Kim et al., 1997; Watanabe et al., 1998). These results indicated that carbachol inhibits I_{sAHP} in cortex through $G\alpha_{q\text{-}11}$ and, at least in part, PLCβ1. To examine the sensitivity of the NCS-enhanced I_{sAHP} to inhibition by $G\alpha_{q\text{-}11}$ signaling, we transfected pyramidal cells with hippocalcin or neurocalcin δ and again examined the effects of carbachol. As illustrated in Figure 4*B*, administration of carbachol (30 μM) inhibited I_{sAHP} in these cells in a manner that was not statistically different from control ($p = 0.94$), thus suggesting that the NCS-enhanced I_{sAHP} retains its ability to be inhibited by neuromodulators acting via $G\alpha_{q\text{-}11}$ and PLCβ.

Discussion

The identification of hippocalcin as a calcium sensor for I_{sAHP} in hippocampus, while making much-needed fresh inroads into our understanding of this current, raised a number of important mechanistic questions (Brown et al., 2007; Tzingounis et al., 2007). Most notably, the restricted expression of hippocalcin in the brain, and the only partial suppression of I_{sAHP} in the hippocalcin knock-out mouse, questioned whether hippocalcin alone could account for the calcium sensitivity of I_{sAHP} (Brown et al., 2007). In the current work, we used loss of function and overexpression approaches to show that hippocalcin regulates I_{sAHP} in the cerebral cortex, and show that neurocalcin δ, a closely related visinin-like NCS protein similarly expressed in this region, can also regulate I_{sAHP}. These results solve inconsistencies surrounding the identification of hippocalcin as the calcium sensor for I_{sAHP} and point to a broader role for NCS proteins in the regulation of this current.

The original suspicion that hippocalcin may not be the sole calcium sensor for I_{sAHP} was based not only on the presence of a residual I_{sAHP} in the hippocalcin knock-out, but also on the observation that this residual I_{sAHP} is relatively resistant to inhibition by norepinephrine acting through β-adrenergic receptors (Tzingounis et al., 2007). This led Brown et al. (2007) to suggest that I_{sAHP} may be gated through two parallel branches exhibiting differential sensitivity to inhibition by norepinephrine. In the current work, we show that carbachol inhibits I_{sAHP} in cortical pyramidal neurons by acting through $G\alpha_{q\text{-}11}$ and (at least in part) PLCβ1. Furthermore, we show that the native, the hippocalcin-enhanced, and the neurocalcin δ-enhanced I_{sAHP}s are all inhibited to a comparable extent by this signaling cascade. These results suggest that the $G\alpha_{q\text{-}11}$/PLCβ signaling cascade does not distinguish between hippocalcin- and neurocalcin δ-gated I_{sAHP}s. However, they do support the idea that hippocalcin and neurocalcin δ act on the native I_{sAHP}.

The main finding of the current report is that at least two distinct NCS proteins can act as calcium sensors for I_{sAHP}. But why should that be the case, especially since it seems likely that both these proteins are coexpressed in at least some cortical neurons? One possibility is suggested by the observation that NCS proteins with different calcium affinities can regulate a single molecular target to allow for an increase in the dynamic range of calcium sensing (reviewed by Burgoyne, 2007). In the current work, we find that while hippocalcin and neurocalcin δ both increase the apparent calcium sensitivity of I_{sAHP}, within the limits of this assay, the calcium sensitivities of these NCS-enhanced I_{sAHP}s are indistinguishable. As such, these results are inconsistent with the idea that hippocalcin and neurocalcin δ sense different calcium concentrations when they regulate I_{sAHP}. Further studies are needed to clarify and understand this apparent redundancy.

The observation that the genetic deletion of hippocalcin results in a reduction in the amplitude of I_{sAHP} has been interpreted to suggest that hippocalcin functions as calcium sensor for this current (Tzingounis et al., 2007). The results of the present experiments support this idea, while also extending such a role to neurocalcin δ. Specifically, we now show that hippocalcin and

neurocalcin δ increase not only the amplitude of I_{sAHP} but also its calcium sensitivity. Since such an increase in calcium sensitivity would be predicted for the overexpression of a calcium sensor, these results support the idea that these NCS proteins sense calcium as part of the gating of I_{sAHP}. Such an interpretation is consistent with previous studies showing that I_{sAHP} follows the time course of cytoplasmic calcium (Abel et al., 2004) and that hippocalcin shuttles from the cytoplasm to the membrane in response to rises in intracellular calcium, and does so with a time course compatible with a role in signaling I_{sAHP} (Markova et al., 2008). However, exactly how these visinin-like NCS proteins function in the context of gating I_{sAHP} is unclear. Clearly a more detailed understanding of the molecular mechanisms underlying I_{sAHP} will be required to understand how these visinin-like NCS proteins regulate this enigmatic current.

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