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Protein kinase C bidirectionally modulates I_h and hyperpolarization-activated cyclic nucleotide-gated (HCN) channel surface expression in hippocampal pyramidal neurons

Aaron D. Williams[1], Sangwook Jung[1] and Nicholas P. Poolos[1,2]

[1] *Department of Physiology and Biophysics, University of Washington*
[2] *Department of Neurology and Regional Epilepsy Center, University of Washington, Seattle, WA, USA*

Key points

- Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, particularly that of the HCN1 isoform, are enriched in the distal dendrites of hippocampal CA1 pyramidal neurons; these channels have physiological functions with respect to decreasing neuronal excitability.
- In the present study, we aimed to investigate phosphorylation as a mechanism controlling I_h amplitude and HCN1 surface expression in hippocampal principal neurons under normal physiological conditions.
- Tyrosine phosphorylation decreased I_h amplitude at maximal activation (maximal I_h), without altering HCN1 surface expression, in two classes of hippocampal principal neurons. Inhibition of serine/threonine protein phosphatases 1 and 2A decreased maximal I_h and HCN1 surface expression in hippocampal principal neurons.
- Protein kinase C (PKC) activation irreversibly diminished I_h and HCN1 surface expression, whereas PKC inhibition augmented I_h and HCN1 surface expression. PKC activation increased HCN1 channel phosphorylation.
- These results demonstrate the novel finding of a phosphorylation mechanism, dependent on PKC activity, which bidirectionally modulates I_h amplitude and HCN1channel surface expression in hippocampal principal neurons under normal physiological conditions.

Abstract Hyperpolarization-activated, cyclic nucleotide-gated (HCN) ion channels attenuate excitability in hippocampal pyramidal neurons. Loss of HCN channel-mediated current (I_h), particularly that mediated by the HCN1 isoform, occurs with the development of epilepsy. Previously, we showed that, following pilocarpine-induced status epilepticus, there are two independent changes in HCN function in dendrites: decreased I_h amplitude associated with a loss of HCN1 surface expression and a hyperpolarizing shift in voltage-dependence of activation (gating). The hyperpolarizing shift in gating was attributed to decreased phosphorylation as a result of a loss of p38 mitogen-activated protein kinase activity and increased calcineurin activity; however, the mechanisms controlling I_h amplitude and HCN1 surface expression under epileptic or normal physiological conditions are poorly understood. We aimed to investigate phosphorylation as a mechanism regulating I_h amplitude and HCN1 surface expression (i.e. as is the case for HCN gating) in hippocampal principal neurons under normal physiological conditions. We discovered that inhibition of either tyrosine phosphatases or the serine/threonine protein phosphatases 1 and 2A decreased I_h at maximal activation in hippocampal CA1

Aaron D. Williams and Sangwook Jung contributed equally to this work.

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pyramidal dendrites and pyramidal-like principal neuron somata from naïve rats. Furthermore, we found that inhibition of PP1/PP2A decreased HCN1 surface expression, whereas tyrosine phosphatase inhibition did not. Protein kinase C (PKC) activation reduced I_h amplitude and HCN1 surface expression, whereas PKC inhibition produced the opposite effect. Inhibition of protein phosphatases 1 and 2A and activation of PKC increased the serine phosphorylation state of the HCN1 protein. The effect of PKC activation on I_h was irreversible. These results indicate that PKC bidirectionally modulates I_h amplitude and HCN1 surface expression in hippocampal principal neurons.

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Corresponding author N. P. Poolos: University of Washington, Department of Neurology and Regional Epilepsy Center, Box 359745, 325 Ninth Avenue, Seattle, WA 98104, USA. Email: npoolos@u.washington.edu

Abbreviations CaN, calcineurin; GFX, GF 109203X; HCN, hyperpolarization-activated cyclic nucleotide-gated; OA, okadaic acid; MAPK, mitogen-activated protein kinase; PAO, phenylarsine oxide; PDA, phorbol 12,13-diacetate; PKC, protein kinase C; PLP, pyramidal-like principal; PP1/2A, protein phosphatase 1/2A; pSer, Ser phosphorylation; SE, status epilepticus; Ser, serine; Thr, threonine; TRIP8b, tetratricopeptide repeat-containing Rab8b interacting protein; Tyr, tyrosine.

Introduction

Hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels are voltage-gated ion channels that open in response to membrane hyperpolarization, and conduct an inward, mixed cationic (Na^+ and K^+) current (Craven and Zagotta, 2006). In pyramidal neurons of the cortex and hippocampus, where the HCN1 isoform is predominant, HCN1 channels are principally localized to the apical dendrites (Biel *et al.* 2009; Poolos 2012). HCN1 channels are non-inactivating and open at resting potential, reducing input resistance and decreasing the time window over which temporal summation can occur. The decreased temporal summation reduces action potential firing from excitatory input to the dendrites, giving HCN1 channels inhibitory control over neuronal excitability (Magee 1998, 1999).

Diminished HCN channel-mediated current (I_h) is associated with the latent period following a brain insult and preceding the onset of spontaneous seizures in several animal models of epilepsy, particularly the post-status epilepticus (SE) models (Shah *et al.* 2004; Zhang *et al.* 2006; Jung *et al.* 2007; Marcelin *et al.* 2009). However, the precise mechanisms controlling HCN1 downregulation post-SE are only partially understood. Our laboratory has found that, following the induction of SE in the pilocarpine model of epilepsy, dendritic HCN channels undergo two independent changes: a hyperpolarizing shift in the voltage-dependence of activation (gating) and a decrease in I_h amplitude at maximal activation. HCN1 gating is phosphorylation-dependent: increased calcineurin (CaN; protein phosphatase 2B) and decreased p38 mitogen-activated protein kinase (MAPK) activity are associated with a hyperpolarizing shift in HCN1 channel gating (Poolos *et al.* 2006; Jung *et al.* 2010). However, neither p38 MAPK activity nor CaN activity affects I_h amplitude.

The decrease in I_h amplitude immediately follows SE, is associated with the loss of HCN1 channel surface expression, and precedes any HCN1 transcriptional downregulation, suggesting that the decline is a result of post-translational modification (Jung *et al.* 2011; McClelland *et al.* 2011). The mechanisms that control I_h amplitude and HCN1 channel surface expression in neurons under either normal conditions or post-SE are poorly understood. However, phosphorylation is an almost universal modulator of the surface membrane expression of other ion channels, such as $GABA_A$ receptors (Terunuma *et al.* 2008; Goodkin *et al.* 2008), AMPA receptors (Rakhade *et al.* 2008) and Kv4.2 channels (Kim *et al.* 2007; Lugo *et al.* 2008), and therefore represents a potential mechanism. Notably, membrane surface expression of the closely-related channel HCN2 is inhibited by the receptor-like protein tyrosine (Tyr) phosphatase α in HEK293 cells (Huang *et al.* 2008). The mechanisms controlling HCN1 channel surface expression are probably also dependent upon phosphorylation, although little is known about phosphorylation regulation of HCN1 in pyramidal neurons under normal physiological conditions.

In the present study, we aimed to investigate phosphorylation as a mechanism regulating I_h amplitude at maximal activation (maximal I_h) and HCN1 channel surface expression in two types of hippocampal principal neurons. Using pharmacological tools, we modulated the Tyr and serine/threonine (Ser/Thr) phosphorylation states of HCN1 in CA1 principal neurons from rat hippocampal brain slices and determined that Tyr phosphorylation modulated maximal I_h alone, whereas Ser/Thr phosphorylation modulated both maximal I_h and

HCN1 surface expression. We then determined that the Ser/Thr kinase protein kinase C (PKC) bidirectionally modulated both maximal I_h and HCN1 surface expression. The present study represents a novel demonstration of phosphorylation-dependent modulation of maximal I_h and HCN1 surface expression in hippocampal CA1 principal neurons under normal physiological conditions.

Methods

Slice preparation

Acute hippocampal slices were prepared from 6- to 7-week-old male Sprague–Dawley rats, in accordance with the rules and regulations of the University of Washington Institutional Animal Care and Use Committee. Rats were anaesthetized by I.P. injection of a mixture of ketamine and xylazine, producing deep anaesthesia. Transcardial perfusion was carried out with ice-cold cutting saline comprising (in mM): 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 7 dextrose, 0.5 $CaCl_2$, 7 $MgCl_2$, 110 choline chloride, 1.3 ascorbic acid and 3 sodium pyruvate; then bubbled with 95% O_2–5% CO_2. Rats were then decapitated and their brains removed. After removal, the brains were hemisected along the longitudinal fissure. Parasagittal slices (400 μm thick) from the hippocampus were prepared using a Vibratome 1500 (The Vibratome Company, St Louis, MO, USA). Hippocampal slices were held for 10 min at 34°C in a recording chamber containing external recording solution comprising (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 10 dextrose, 2 $CaCl_2$, 2 $MgCl_2$, 1.3 ascorbic acid and 3 sodium pyruvate; then bubbled with 95% O_2–5% CO_2. Slices were then allowed to rest at room temperature for at least 60 min prior to recording.

Electrophysiology

Neurons were visualized using infrared differential-interference contrast imaging using a Axioskop microscope (Zeiss, Oberkochen, Germany) fitted with a 60X objective (Olympus, Tokyo, Japan). Pyramidal-like principal (PLP) neurons were identified by their characteristic pyramidal morphology, large soma (approximately twice or more the diameter of a CA1 pyramidal neuron) and somatic location in the stratum radiatum. A fuller description of the morphology of PLP neurons is provided by Bullis *et al.* (2007). Pipette resistances for cell-attached recordings were between 4–7 MΩ for somatic recordings and 12–15 MΩ for dendritic recordings. Cell-attached patch recordings were made with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA), sampled at 2 KHz and filtered at 500 Hz. All data were collected and analysed with custom software written for the Igor Pro analysis environment (Wavemetrics, Lake Oswego, OR, USA). For recording, slices were kept at 29–31°C and continuously bathed in external solution. Pipettes were filled with a solution comprising (in mM): 120 KCl, 20 TEA-Cl, 5 4-aminopyridine, 10 Hepes, 2 $CaCl_2$, 1 $MgCl_2$ and 1 $BaCl_2$.We measured I_h using cell-attached voltage-clamp recordings. I_h was elicited by 1500 ms voltage commands to a maximum of ∼150 mV. Maximal I_h amplitude was measured at the end of the current trace elicited by this –150 mV command. I_h activation was quantified by measuring the amplitude of the tail current that followed each voltage command, and normalizing it to the tail current amplitude at maximal activation (I/I_{max}). Leak subtraction was performed by acquiring current traces with voltage commands that provoked no I_h activation; four times as many leak traces were collected as I_h traces, and these were averaged and subtracted from each I_h trace. Pipette capacitance was compensated at the amplifier to diminish artefacts in the current traces. I_h amplitudes are presented without correction for estimated patch area; however, pipette tip diameter (∼1 μm) was held constant, and each investigator performed his own control series to minimize the effects of differences in pipette fabrication. All supplies were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise noted. A recent study has described potential artefacts in cell-attached voltage-clamp measurements under the conditions of very high input resistance and very high current density (Williams and Wozny, 2011). We estimated the maximum error in command potential at –150 mV to be ∼1.7 mV, which would not impact upon the findings reported here.

Surface biotinylation assay

Surface membrane protein expression was measured using a biotinylation protocol (Jung *et al.* 2011).We prepared hippocampal slices and incubated them for 45 min at 4°C in extracellular recording solution containing 1 mg ml^{-1} sulfo-NHS-SS-biotin (Pierce, Rockford, IL, USA) and then in extracellular solution with 1 μM lysine to block all reactive sulfo-NHS-SS-biotin in excess. The CA1 regions were microdissected on dry ice and homogenized in buffer comprising (in mM): 50 Tris-HCl, 5 EDTA, 1 PMSF, 1 sodium orthovandate, 50 NaCl, 10 EGTA, 2 sodium pyrophosphate and 4 paranitrophenylphosphate; as well as 4 μg ml^{-1} aprotonin, 20 μg ml^{-1} leupeptin and 1% Triton X-100 (Roberson *et al.* 1999). The homogenates were centrifuged at 16,900 × *g* for 15 min at 4°C, and the supernatant was harvested. After measurement of protein concentration with a BCA assay protocol and normalization of the inputs for total protein content, the supernatant was incubated with NeutrAvidin Plus beads

(Pierce) overnight at 4°C and then washed three times with homogenization buffer. After centrifugation at 16,900 × *g* for 5 min, the supernatant was discarded and the beads resuspended in Laëmmli buffer (final concentration of 4% SDS, 20 mM dithiothreitol) and boiled. Total protein and biotinylated surface protein levels were analysed using western blotting. To quantify the changes in HCN1 surface expression, we first quantified the ratio of HCN1 channel protein surface expression between drug-treated and control brain slices. We then quantified the total HCN1 channel (surface + intracellular) protein expression as the ratio between drug-treated and control brain slices, normalized the surface HCN1 expression ratio by the total HCN1 expression ratio, and reported this surface/total ratio as the final result. Normal blot-to-blot variability in band intensities was controlled for by analyzing band intensities for control and treated samples within the same gel. Total HCN1 expression was not significantly changed by drug treatment in any sample. As an additional control for protein loading, we quantified total β-actin expression in GF109203X (bisindolylmaleimide I; GFX)-treated slices, which was not significantly different (105 ± 7% control, $n = 10$), demonstrating that protein loading was not significantly different between control and drug-treated samples.

Immunoprecipitation

Tissue homogenates from CA1 hippocampal regions were prepared as described for the surface biotinylation protocol. Immunoprecipitation of HCN1 subunits from the supernatant was then carried out: anti-HCN1 antibody (10 μg) (Millipore, Billerica, MA, USA) was incubated with the supernatant at 4°C overnight. Protein A/G-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were then added to the incubation mixture, incubated for an additional 3 h, and washed three times with homogenization buffer. After centrifugation at 16,900 × *g* for 5 min, the supernatant was discarded and the beads resuspended in Laëmmli buffer (with final concentration of 4% SDS, 20 mM dithiothreitol) and boiled. HCN1 and phosphoserine protein levels were analysed using western blotting.

Western blotting

Samples from experimental and control groups were loaded and run on a 10% acrylamide gel (Bio-Rad Laboratories, Redmond, WA, USA), transferred to a nitrocellulose membrane and incubated with HCN1 antibody (dilution 1:1000; Millipore) or phosphoserine antibody (dilution 1:1000; Millipore), followed by incubation in anti-rabbit secondary antibody (1:1000; Life Technologies, Carlsbad, CA, USA), and then visualized by enhanced chemiluminescence and film exposure. Three different protein loading amounts were used in each condition to confirm that signal detection was in the linear range, as reported previously (Jung *et al.* 2010).

Statistical analysis

Statistical significance between data sets containing two independent groups was evaluated using an unpaired Student's *t* test. All hypothesis testing was performed with $\alpha = 0.05$. Statistical analysis was conducted using either Igor Pro, version 4.09A (WaveMetrics, Inc., Portland, OR, USA) or InStat, version 3.b (GraphPad, La Jolla, CA, USA). All data points are displayed as the mean ± SEM and statistically significant differences are indicated.

Results

Tyr phosphorylation diminishes I_h

We began by studying the effects of broad-spectrum manipulation of phosphorylation on hippocampal principal neurons from naïve animals, as a starting point for understanding modulation of maximal I_h. To investigate Tyr phosphorylation as a potential mechanism, we began by performing cell-attached patch-clamp recordings at the somata of hippocampal PLP neurons, a subtype of pyramidal neuron with somata localized to the stratum radiatum, and with a high somatic density of I_h (unlike other types of hippocampal pyramidal neurons). I_h in PLP neurons demonstrates high density and voltage-dependence of activation, as well as activation kinetics and regulation by p38 MAPK that are similar to those for I_h in the dendrites of CA1 pyramidal neurons (Poolos *et al.* 2006; Bullis *et al.* 2007). The high somatic density of I_h in PLP neurons, and large size of PLP somata compared to pyramidal dendrites, make I_h measurements technically easier in the soma of PLP neurons than in pyramidal dendrites; the similar I_h characteristics between the two cell types mean that the I_h measurements in PLP neurons provide a useful adjunct to measurements of I_h in pyramidal dendrites. We began the present study in PLP neurons, and confirmed key findings in CA1 pyramidal neurons. However, the functional role of PLP neurons has not yet been determined, and we did not examine this further in the present study.

In PLP neurons, maximal I_h was significantly reduced to 45% of control after 30 min of treatment with 10 μM phenylarsine oxide (PAO), a general Tyr phosphatase inhibitor that increases phosphorylation at Tyr residues (control: 31 ± 2.4 pA, $n = 23$; PAO: 14 ± 3.4 pA, $n = 10$; $P < 0.01$) (Fig. 1*A*). As measured at the half-maximal point ($V_{1/2}$), I_h voltage-dependent activation underwent a slight, but significant, hyperpolarizing shift in PLP neurons from

PAO-treated brain slices (control: –98 ± 1.7 mV, $n = 23$; PAO: –106 ± 1.8 mV, $n = 10$; $P < 0.05$) (Fig. 1*B*).

We next investigated whether inhibition of Tyr phosphorylation would increase I_h in PLP neurons. We applied the broad-spectrum kinase inhibitor genistein, an inhibitor of Tyr kinases, at one of two doses (either 1 μM or 20 μM) (for specificity and potency of genistein for Tyr kinase inhibition, see O'Dell *et al.* 1991) to brain slices from naïve animals for 30 min, and measured the effect on I_h in PLP somata. Genistein did not have a significant effect at either dose on maximal I_h (1 μM genistein: 27 ± 4.6 pA, $n = 7$; 20 μM genistein: 25 ± 6.9 pA, $n = 5$) (Fig. 1*C*) or on voltage-dependent activation (1 μM genistein: –104 ± 2.9 mV, $n = 7$; 20 μM genistein: –106 ± 4.5 mV, $n = 5$) (Fig. 1*D*). These results suggest that HCN channels are at a very low basal phosphorylation state under normal conditions, and cannot undergo significant further dephosphorylation.

We next investigated whether I_h in hippocampal CA1 pyramidal apical dendrites was similarly affected by

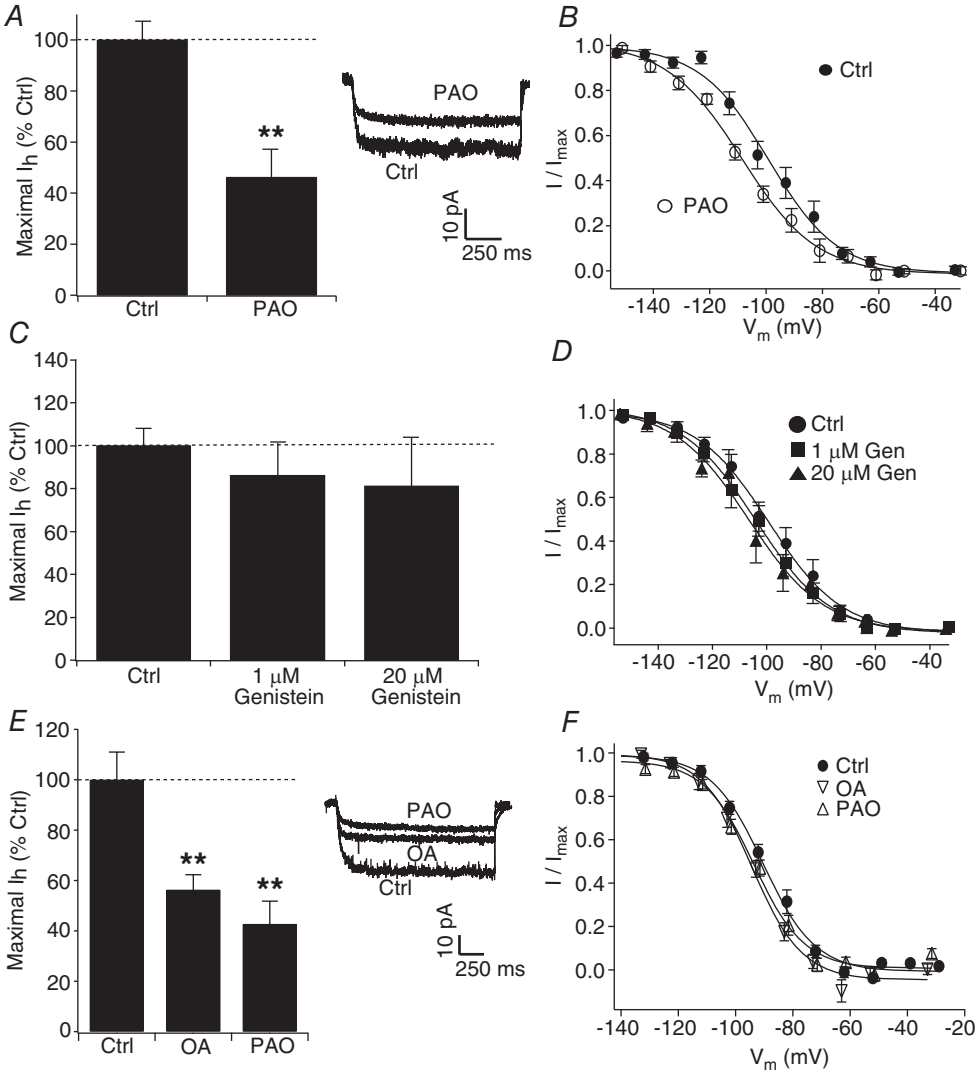

Figure 1. I_h decreases after either PP1/PP2A inhibition or Tyr phosphatase inhibition
A, I_h amplitudes at maximal activation obtained at the somata of hippocampal PLP neurons from PAO-treated hippocampal slices were significantly reduced compared to control (Ctrl). Representative current traces are shown at voltage commands of approximately −150 mV. I_h values shown as a percentage of control to facilitate comparison among experiments. ***B***, voltage-dependent activation in PLP neurons was significantly hyperpolarized after 30 min of treatment with 10 μM phenylarsine oxide. ***C***, I_h at maximal activation in hippocampal PLP neurons was not significantly altered after 30 min of treatment with either 1 μM or 20 μM genistein. ***D***, voltage-dependent activation in PLP neurons was not significantly altered after 30 min of treatment with either 1 μM or 20 μM genistein. ***E***, I_h amplitudes obtained in CA1 pyramidal neuronal dendrites from OA- and PAO-treated hippocampal slices were significantly reduced compared to control. ***F***, voltage-dependent activation of dendritic I_h in OA- and PAO-treated hippocampal slices was similar to control.

modulation of Tyr phosphorylation using cell-attached patch clamp recordings. We found that dendritic I_h (at an average of 174 ± 5 μm from the soma) was significantly reduced to 43% of control after 30 min of treatment with 10 μM PAO (control: 42 ± 4.6 pA, $n = 13$; PAO: 18 ± 3.8 pA, $n = 10$; $P < 0.01$) (Fig. 1*E*). Unlike in PLP neurons, I_h voltage-dependent activation was not significantly altered in pyramidal dendrites from PAO-treated brain slices (control: –90 ± 1.5 mV, $n = 13$; PAO: –93 ± 1.2 mV, $n = 10$) (Fig. 1*F*). These results show that increased Tyr phosphorylation diminishes I_h amplitude in both hippocampal CA1 pyramidal dendrites and PLP neurons to a similar extent.

PP1/2A inhibition diminishes I_h and HCN1 surface expression

Previously, we showed that activation of the Ser/Thr protein phosphatase 2B (PP2B; CaN) downregulated HCN channel gating without affecting maximal I_h (Jung *et al.* 2010). We next considered the other two Ser/Thr phosphatase families, PP1 and PP2A, as potential mechanisms modulating I_h amplitude. To investigate this possibility, we bathed hippocampal slices for 30 min in 50 nM okadaic acid (OA), an inhibitor of both PP1 and PP2A, and performed cell-attached patch-clamp recordings in the dendrites of hippocampal CA1 pyramidal neurons (at 162 ± 3 μm from the soma). A concentration of 50 nM OA significantly reduced maximal I_h to 57% of control (OA: 24 ± 2.5 pA, $n = 10$; $P < 0.01$) (Fig. 1*E*), without a significant change in I_h voltage-dependent activation (control: –90 ± 1.4 mV, $n = 13$; OA: –94 ± 1.5 mV, $n = 10$) (Fig. 1*F*), thus demonstrating that I_h is reduced by both Tyr and Ser/Thr phosphorylation-dependent mechanisms.

In a previous study, we demonstrated that the loss of I_h amplitude in CA1 pyramidal neuron dendrites at 1 h post-SE was accompanied by a similar decrease in HCN1 channel surface expression; however, the mechanisms leading to this diminished surface expression are unclear (Jung *et al.* 2011). In light of the discovery that increased Tyr phosphorylation leads to decreased maximal I_h, we next investigated whether Tyr phosphatase inhibition diminishes I_h by reducing HCN1 channel neuronal surface expression, as determined by a surface biotinylation assay (see Methods). To validate our surface biotinylation assay, we first aimed to replicate the previous finding that PKC activation increases $GABA_A$ α4 subunit surface expression in hippocampal tissue (Abramian *et al.* 2010). Using our assay, PKC activation by 60 min of bath application of 10 μM phorbol 12,13-diacetate (PDA) produced a significant increase in $GABA_A$ α4 subunit surface expression (total expression: 66 ± 10% of control; surface expression: 156 ± 27% of control; ratio surface/total: 237 ± 15% total; $n = 3$; $P < 0.05$) (Fig. 2*A*), consistent with the previously reported increase of 294 ± 91%. We probed both surface and total fractions with a β-actin antibody to confirm that the surface fraction was not contaminated with cytoplasmic proteins. No β-actin staining was observed in the surface fraction (Fig. 2*A*).

We then used our surface biotinylation assay to measure HCN1 surface expression. HCN1 surface expression in tissue from CA1 hippocampus treated with 10 μM PAO for 60 min was unchanged (total expression: 96 ± 5% of control; surface expression: 97 ± 6% of control; surface/total: 102 ± 5% total; $n = 5$) (Fig. 2*B*). These results demonstrate that increased Tyr phosphorylation does not diminish I_h by reducing HCN1 surface expression; instead, Tyr phosphorylation may diminish I_h by reducing HCN1 single-channel conductance, either as a direct effect or via other signalling intermediaries.

To confirm whether the effect of PP1/2A inhibition on I_h was a result of changes in HCN1 surface expression, we similarly performed a surface biotinylation assay. By contrast to the lack of effect of increased Tyr phosphorylation on HCN1 channel surface expression, OA treatment (50 nM) for 60 min significantly diminished HCN1 surface expression in hippocampal CA1 tissue (total expression: 108 ± 10% of control; surface expression: 77 ± 16% of control; ratio surface/total: 70 ± 9% total; $n = 5$; $P < 0.05$) (Fig. 2*C*). These results show that increased Ser/Thr phosphorylation contributes to diminished I_h by decreasing HCN1 surface expression.

PKC modulates I_h and HCN1 surface expression in hippocampal neurons

We next turned to the identification of a specific Ser/Thr kinase responsible for modulating I_h and HCN1 surface expression in hippocampal neurons. We considered PKC as a prime candidate: previous studies have identified PKC as a mechanism directly regulating the function of Kv4.2 channels and $GABA_A$ receptors in hippocampal neurons (Hoffman and Johnston, 1998; Poisbeau *et al.* 1999). A few studies have also demonstrated that PKC acts as a mediator of other modulators of I_h, such as neurotensin, diacylglycerol kinase, phospholipase C and orexin A (Cathala and Paupardin-Tritsch, 1997; Fogle *et al.* 2007; Carr *et al.* 2007; Li *et al.* 2010).

To test the hypothesis that PKC modulates I_h in hippocampal neurons, we bathed hippocampal slices for 30 min in 10 μM PDA, an activator of PKC, and performed cell-attached patch-clamp recordings in PLP somata and the dendrites of hippocampal CA1 pyramidal neurons (at 171 ± 6 μm from the soma). PDA significantly reduced maximal I_h in PLP somata to 57% of control (control: 36 ± 2.3 pA, $n = 43$; PDA: 20 ± 1.7 pA, $n = 7$; $P < 0.01$)

(Fig. 3*A*); I_h voltage-dependent gating underwent a hyperpolarizing shift (control: –98 ± 1.7 mV, $n = 23$; PDA: –108 ± 4.4 mV, $n = 7$; $P < 0.05$) (Fig. 3*B*). We also investigated whether application of 1 μM GFX, a PKC inhibitor (Hama *et al.* 2004), would increase I_h in PLP somata. There was no significant change in maximal I_h after 30 min of application of 1 μM GFX; however, maximal I_h was significantly increased after 60 min of incubation to 158% of control (30 min GFX: 35 ± 6.1 pA, $n = 8$; 60 min GFX: 57 ± 9.5 pA, $n = 10$, $P < 0.01$) (Fig. 3*A*). No

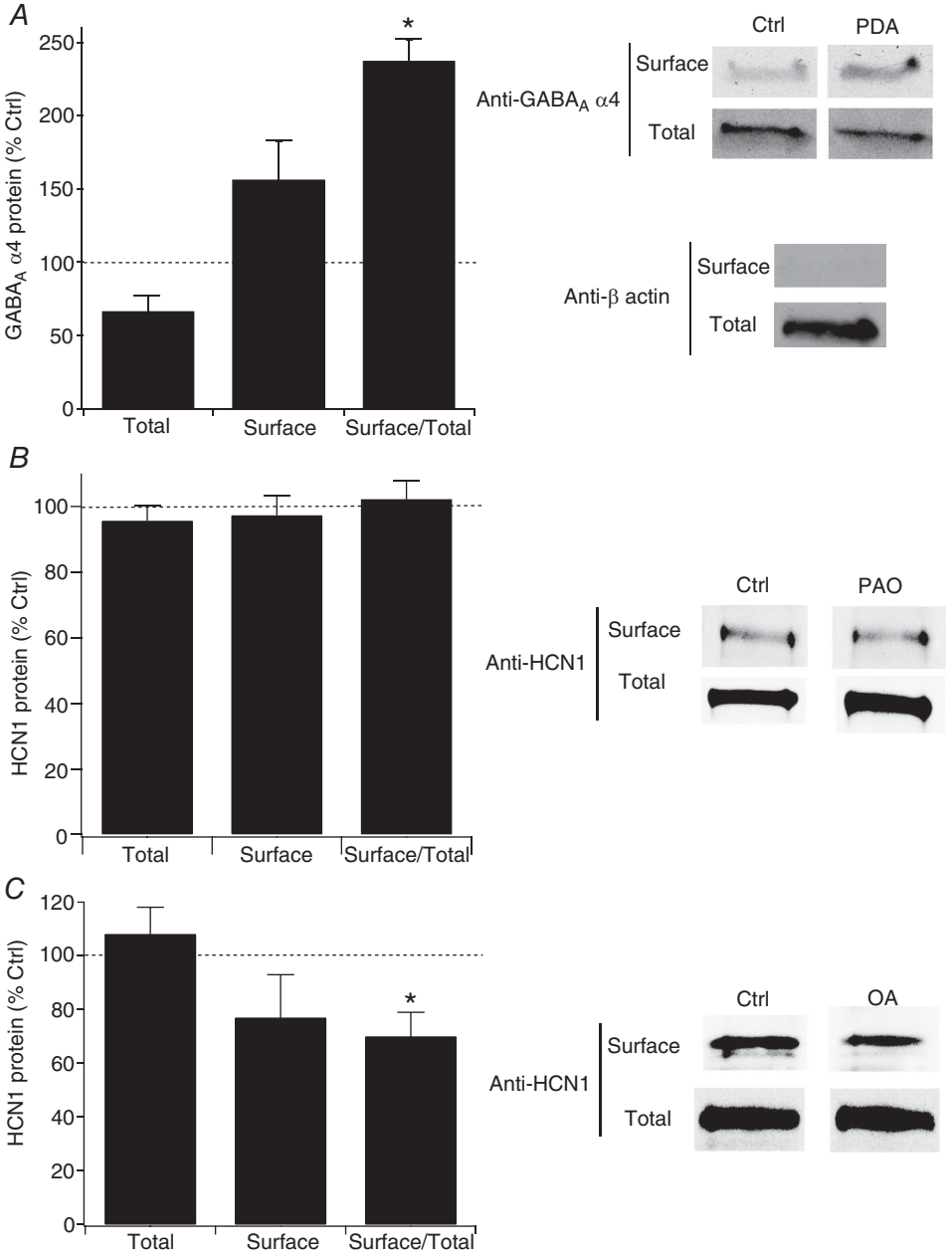

Figure 2. PP1/PP2A inhibition reduces HCN1 channel surface expression but Tyr phosphatase inhibition does not

A, GABA$_A$ α4 subunit surface expression in the hippocampal CA1 area was significantly increased after 60 min of treatment with 10 μM PDA. Representative western blots of surface and total GABA$_A$ α4 subunit protein levels are shown in each condition. β-actin protein expression is found only in the total homogenate and not in the surface fraction, as determined by surface biotinylation assay. The absence of actin staining confirms that the surface fraction is not contaminated with cytoplasmic proteins. ***B***, surface expression of HCN1 channel protein from CA1 hippocampal tissue homogenates was unchanged in PAO-treated tissue compared to control. Representative western blots of surface and total HCN1 protein are shown. ***C***, surface HCN1 channel protein expression was decreased in OA-treated hippocampal slices.

significant change in gating was observed after incubation in 1 μM GFX for either 30 min (not shown) or 60 min (Fig. 3*B*) (control: –95 ± 1.6 mV, n = 43; 30 min GFX: –103 ± 3.3 mV, n = 8; 60 min GFX: –98 ± 3.6 mV, n = 10). These results show that PKC activity can bidirectionally modulate I_h amplitude in hippocampal principal neurons; however, the effect of PKC inhibition on I_h develops more slowly than that of PKC activation.

We then confirmed that PDA significantly reduced maximal I_h in hippocampal CA1 pyramidal dendrites (as it did in PLP neurons) to 43% of control (control: 42 ± 4.6 pA, n = 13; PDA: 18 ± 2.9 pA, n = 7; $P < 0.01$) (Fig. 3*C*); however, the hyperpolarizing shift in $V_{1/2}$ was not observed in pyramidal dendrites (control: –90 ± 1.5 mV, n = 13; PDA: –92 ± 3.6 mV, n = 7) (Fig. 3*D*). Notably, the magnitude of change in I_h from PKC activation is similar to that with inhibition of the Ser/Thr phosphatases PP1/2A with OA. Thus, PKC activation significantly reduces I_h amplitude in both PLP somata and hippocampal CA1 pyramidal dendrites.

PKC modulation of I_h amplitude is irreversible

We then aimed to confirm that the action of PDA on I_h amplitude was dependent on PKC activation. We bathed hippocampal slices in 1 μM GFX for 30 min, and then in both 1 μM GFX and 10 μM PDA for 30 min, and measured I_h at the soma of PLP neurons using cell-attached patch clamp electrophysiology (Fig. 4*A*). Subsequent to this treatment, maximal I_h was significantly increased to 141% of control, similar to that seen with 60 min of GFX application alone (control: 36 ± 2.3 pA, n = 43; GFX pre-treated: 51 ± 9.0 mV, n = 6; $P < 0.05$)

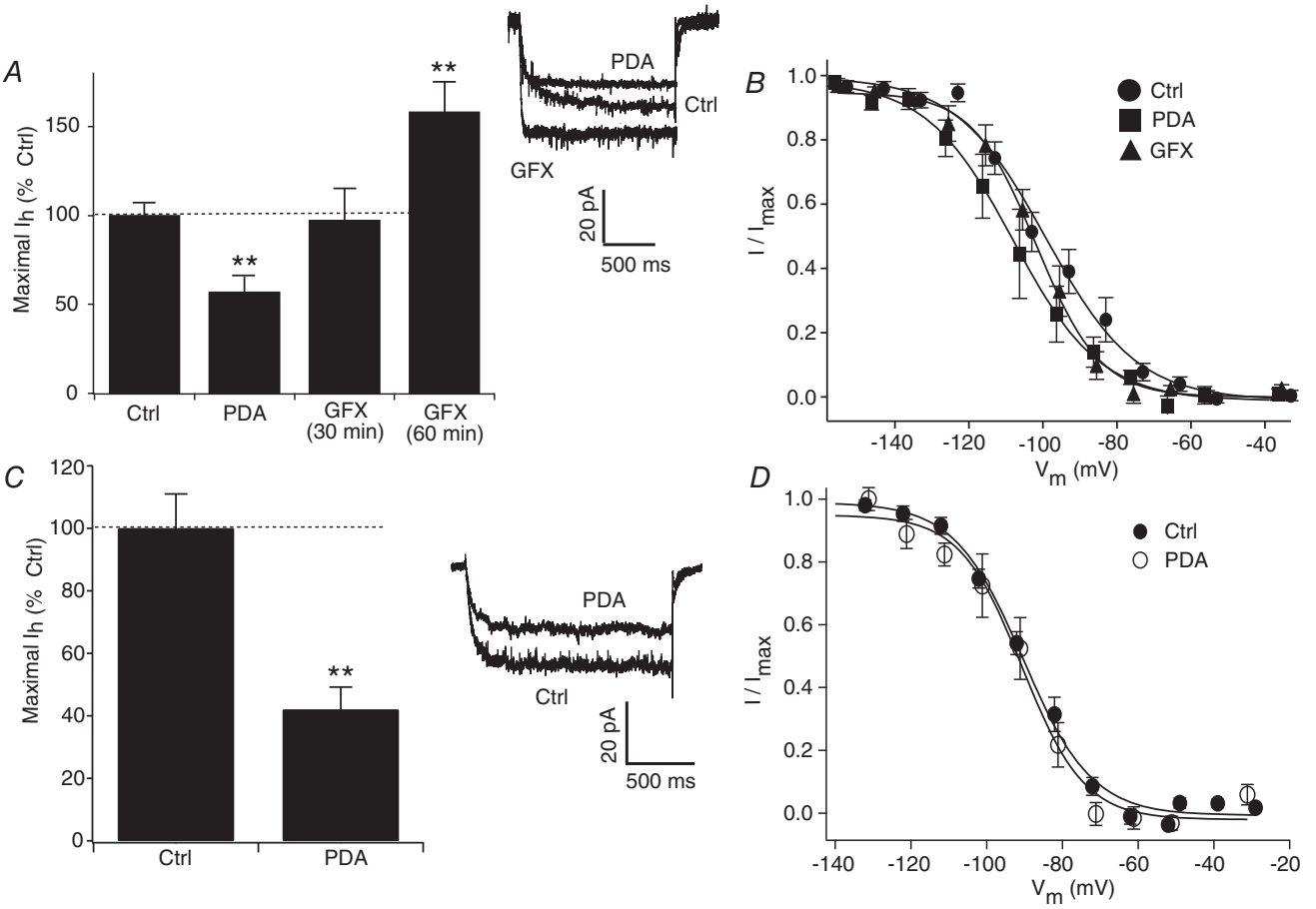

Figure 3. PKC activity bidirectionally modulates I_h
A, maximal I_h in PLP neurons was significantly reduced after 30 min of treatment with 10 μM PDA. I_h was significantly increased after 60 min of treatment with 1 μM of the PKC inhibitor GF109203X. Representative current traces are shown at voltage commands of approximately −150 mV. ***B***, voltage-dependent activation in PLP neurons was significantly hyperpolarized after treatment with PDA but not after treatment with GF109203X. ***C***, maximal I_h obtained at CA1 pyramidal neuronal dendrites from PDA-treated hippocampal slices was significantly reduced compared to control. ***D***, voltage-dependent activation of dendritic I_h in PDA-treated hippocampal slices was similar to control.

(Fig. 4*B*). This demonstrates that GFX blocks the action of PDA, suggesting that the action of PDA depends on PKC activation. No significant change in gating was observed (control: –95 ± 1.6 mV, $n = 43$; GFX pre-treated: –93 ± 6.3 mV, $n = 6$) (Fig. 4*C*).

Finally, we aimed to determine whether the PDA-induced decrease in maximal I_h is reversible. We bathed hippocampal slices in 10 μM PDA for 30 min (to induce the previously-observed decrease in I_h), then in 1 μM GFX for 60 min, and measured I_h at the soma of PLP neurons (Fig. 4*D*). Unexpectedly, we found that that the PKC inhibitor did not reverse the PKC activator-induced decrease in maximal I_h (control: 36 ± 2.3 pA, $n = 43$; PDA pre-treated: 20 ± 2.6 mV, $n = 6$; $P < 0.05$) (Fig. 4*E*). By contrast to treatment with PDA alone (Fig. 3*B*), no significant change in gating was observed (control: –95 ± 1.6 mV, $n = 43$; PDA pre-treated: –89 ± 4.5 mV, $n = 6$) (Fig. 4*F*), suggesting that the GFX treatment at least partially reversed the PDA-induced hyperpolarizing shift in $V_{1/2}$ in PLP neurons.

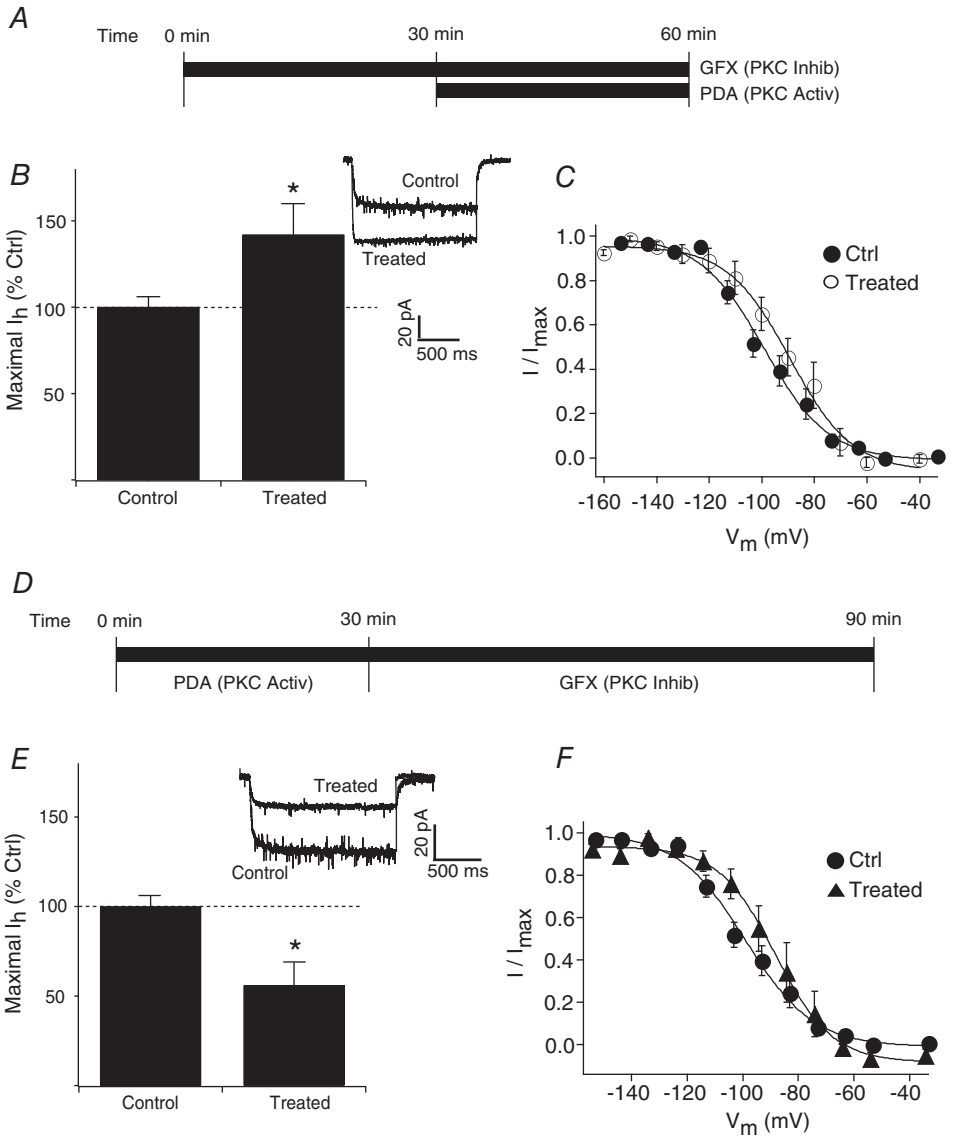

Figure 4. PKC modulation of I_h is irreversible
A, Paradigm for the GF109203X pre-treatment experiment. Hippocampal slices were treated for 30 min with 1 μM GF109203X, followed by 30 minute treatment with both 1 μM GF109203X and 10 μM PDA. ***B***, Maximal I_h in PLP neurons was significantly increased in the GF109203X pre-treatment experiment. ***C***, No significant change in gating was observed in the GF109203X pre-treatment condition. ***D***, Paradigm for the PDA pre-treatment experiment. Hippocampal slices were treated for 30 min with 10 μM PDA, followed by 60 minute treatment with 1 μM GF109203X. ***E***, Maximal I_h in PLP neurons remained significantly decreased in the PDA pre-treatment experiment. ***F***, No significant change in gating was observed in the PDA pre-treatment condition.

PKC activity modulates HCN1 channel phosphorylation and HCN1 surface expression

To confirm that PKC activation affected the phosphorylation state of HCN1, we probed immunoprecipitated HCN1 protein with a pan-phosphoserine antibody. These experiments confirmed that 60 min of treatment with 10 μM PDA significantly increased the Ser phosphorylation (pSer) state of HCN1 (total HCN1: 112 ± 7% of control; pSer: 153 ± 8% of control, $P < 0.05$; pSer/HCN1: 138 ± 9% of control, $P < 0.05$; $n = 5$) (Fig. 5*A*). As a positive control for the sensitivity of the phosphoserine antibody, we confirmed that 60 min of treatment with 50 nM OA also significantly increased the pSer state of HCN1 (total HCN1: 111 ± 13% of control; pSer: 175 ± 15% of control, $P < 0.05$; pSer/HCN1: 165 ± 19% of control, $P < 0.05$; $n = 5$) (Fig. 5*B*). These results demonstrate that inhibition of PP1/2A or activation of PKC decreased I_h in hippocampal principal neurons, which was associated with increased pSer of HCN1.

Finally, we aimed to determine whether the PKC activation-induced decrease in I_h amplitude was correlated with decreased HCN1 channel surface expression. We found that HCN1 channel surface expression in hippocampal CA1 tissue was diminished by PKC activation with 10 μM PDA. As measured by the previously-described surface biotinylation assay, although the HCN1 total protein expression was unchanged (108 ± 8% of control); PDA decreased HCN1 surface expression (57 ± 8% of control, $P < 0.01$) and HCN1 surface expression was significantly reduced when normalized to total HCN1 protein expression (55 ± 11% total, $P < 0.05$) (Fig. 6*A*). This decrease in surface/total expression was comparable in magnitude to the decrease in I_h amplitude following PKC activation.

Conversely, we found that PKC inhibition increases HCN1 surface expression. HCN1 surface expression was significantly increased after 60 min of bath application of 1 μM GFX to hippocampal CA1 tissue (total expression: 97 ± 8.2% control; surface expression: 114 ± 7.0% control; surface/total: 122 ± 9.8% total; $n = 10$; $P < 0.05$) (Fig. 6*B*).

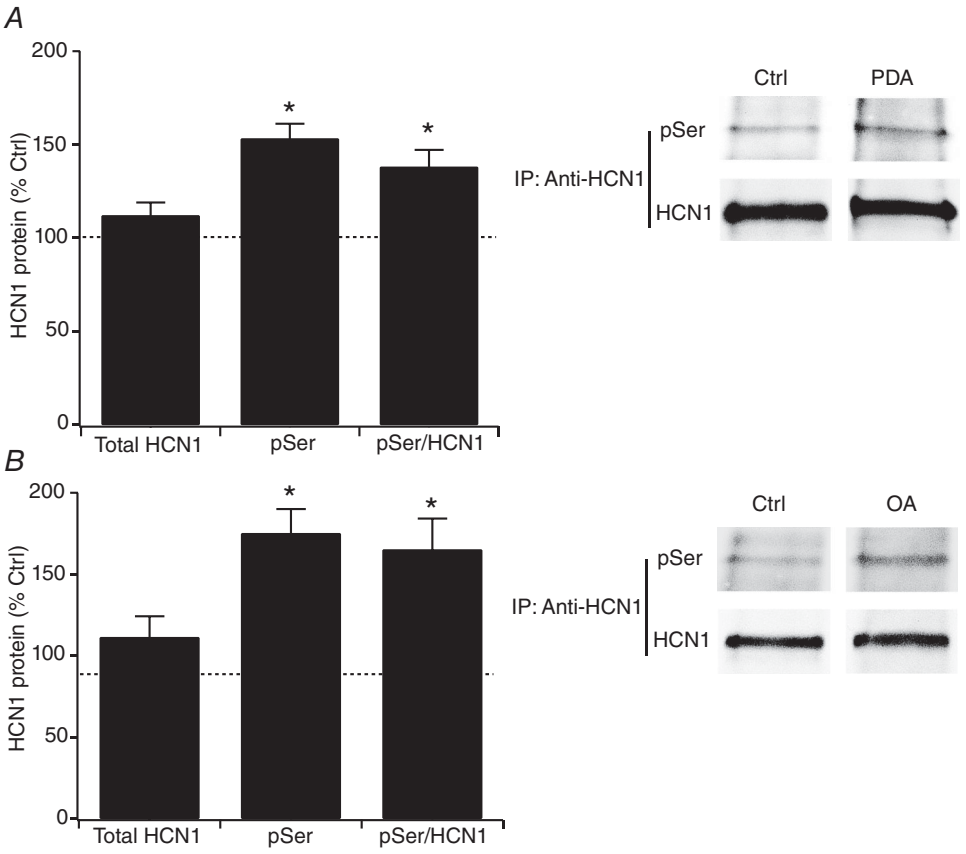

Figure 5. HCN1 Ser phosphorylation increases following both PKC activation and PP1/2A inhibition
A, Ser phosphorylation of HCN1 channel subunits was increased in PDA-treated hippocampal slices. Representative western blots of pSer and HCN1 protein levels are shown in each condition after immunoprecipitation with anti-HCN1 antibody. ***B***, Ser phosphorylation of HCN1 channel subunits was increased in OA-treated hippocampal slices.

These results demonstrate that PKC activity bidirectionally modulates HCN1 surface expression in hippocampal CA1 neurons, as is the case with I_h amplitude.

Discussion

Despite the importance of HCN1 channels in regulating neuronal excitability in the cortex and hippocampus, there is little understanding of how maximal I_h amplitude and HCN1 surface expression are dynamically modulated in neurons. Our previous studies have shown that I_h gating is phosphorylation-dependent. In the present study, we aimed to uncover evidence for phosphorylation modulation of maximal I_h and HCN1 channel surface expression in hippocampal principal neurons. Here, we showed (i) that Tyr phosphorylation modulated I_h amplitude in hippocampal CA1 pyramidal dendrites and PLP neurons, without altering HCN1 surface expression; (ii) that PKC modulated both maximal I_h and HCN1 surface expression in hippocampal CA1 pyramidal dendrites and PLP neurons; (iii) that the PKC-induced change in I_h was irreversible; and (iv) that both PP1/2A and PKC directly modulated the pSer state of HCN1. The present study is the first to demonstrate a phosphorylation-dependent mechanism regulating I_h amplitude and HCN1 surface expression in pyramidal neurons.

Phosphorylation regulation of HCN channels

In previous studies, the effects of Tyr phosphorylation upon I_h current and gating are mixed and depend upon the HCN channel isoform (HCN1–4) and cell type (i.e. neurons, or a heterologous system such as HEK293) Our results are consistent with the previous finding that Tyr kinase B activation through binding of brain-derived neurotrophic factor reduced I_h amplitude in respiratory neurons within the pre-Bötzinger complex, where HCN1 is the predominant isoform (Thoby-Brisson *et al.* 2003). Our finding that neuronal I_h is unchanged following Tyr kinase inhibition by genistein is also consistent with a similar finding using HCN1 expressed in *Xenopus* oocytes (Yu *et al.* 2004). The reason for the decrease in maximal I_h following Tyr phosphatase inhibition remains unclear; however, our finding that Tyr phosphatase inhibition did not alter HCN1 surface expression may suggest a decrease in single-channel conductance. Additionally, Tyr phosphatase inhibition may have a myriad of downstream

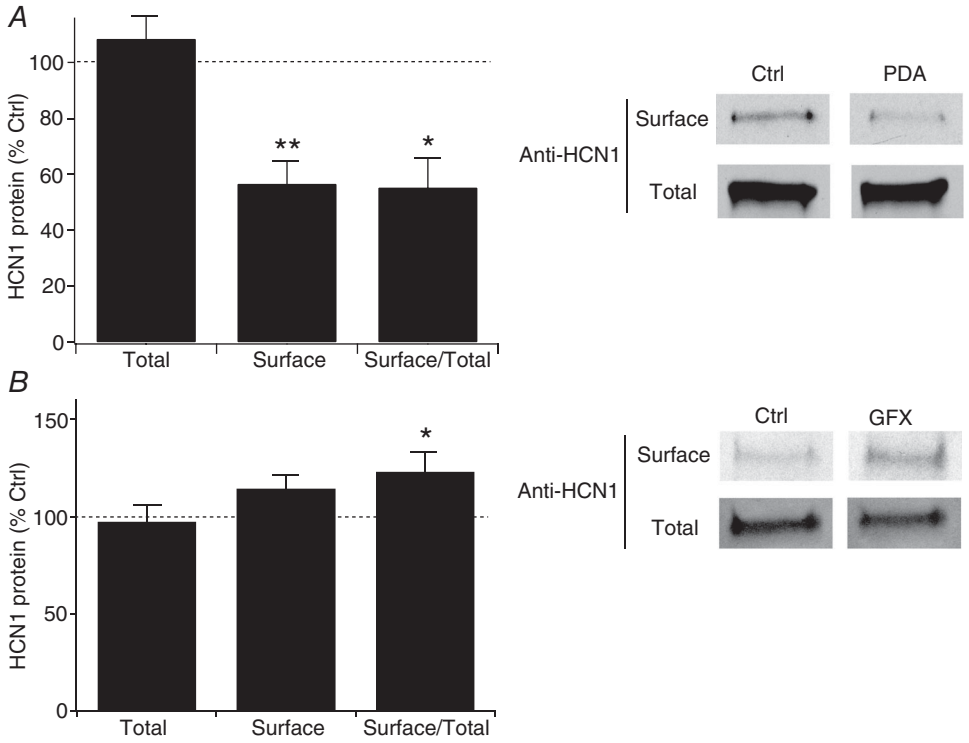

Figure 6. PKC activity bidirectionally modulates HCN1 surface expression
A, HCN1 channel surface expression in the hippocampal CA1 area was significantly reduced after 60 min of treatment with 10 μM PDA. Representative western blots of surface and total HCN1 protein levels are shown in each condition. ***B***, HCN1 channel surface expression in hippocampal CA1 area was significantly increased after 60 min of treatment with 1 μM GF109203X. Representative western blots of surface and total HCN1 protein levels are shown in each condition.

effects on signalling and accessory proteins that, in turn, mediate the effects on I_h conductance; one (or more) of these accessory proteins or pathways could be an intermediary for the decrease in I_h.

These results build upon previous studies concerning the PKC regulation of I_h in heterologous expression systems. In one study, application of 4β-phorbol 12-myristate,13-acetate, an activator of PKC (Goel *et al.* 2007), to excised inside–out patches from HCN1-transfected *Xenopus* oocytes reduced maximal I_h by ∼80%, and caused a +20 mV shift in voltage-dependence of activation (Fogle *et al.* 2007). Recently, another study found that bath application of 4β-phorbol 12-myristate,13-acetate decreased maximal I_h by ∼30–40% in both HEK293 and N1E-115 transfected with either rat or human HCN1, as measured by inside–out cell-excised patch clamp electrophysiology (Reetz and Strauss, 2013). Both studies speculated that the decrease in maximal I_h was primarily the result of a reduction in the number of surface-expressed HCN1 channels. Our results successfully replicate the reduction in I_h following PKC activation, and demonstrate for the first time that this loss of I_h results from diminished HCN1 surface expression. The present study also demonstrates the novel finding that this effect is bidirectional in hippocampal CA1 principal neurons; although PKC activation decreases I_h and HCN1 surface expression, PKC inhibition increases I_h and HCN1 surface expression. Furthermore, our results show that activation of PKC increases the pSer state of HCN1.

Although the changes in I_h and HCN1 surface expression induced by PKC activation are quantitatively similar, our surface biotinylation data include HCN1 channel protein from the entire CA1 area, and not just from hippocampal pyramidal dendrites. The CA1 area could provide additional sources of HCN1 protein, such as from presynaptic terminals or interneurons, which were not assessed in our electrophysiology experiments. However, elegant electron microscopy experiments have demonstrated that HCN1 channel protein expression is significantly enriched in the distal dendrites (compared to proximal dendrites) and in dendritic shafts (compared to dendritic spines) of pyramidal neurons and far outweighs that from presynaptic terminals (Lörincz *et al.* 2002). This is because HCN1 channels are expressed throughout the dendritic shafts of pyramidal neurons and not just at synaptic terminals. This is also supported by immunohistochemistry for HCN1 in the hippocampus, which shows a HCN1 channel density gradient throughout the stratum radiatum and lacunosum moleculare that reflects the subcellular gradient in pyramidal neurons (Lörincz *et al.* 2002). Thus, it appears that the changes in HCN1 surface expression described by our surface biotinylation assay are primarily dendritic in origin.

Unexpectedly, our results demonstrate that the effects of PKC phosphorylation of HCN1 are acutely irreversible, in that 1 h of treatment with GFX does not reverse the PDA-induced decrease in maximal I_h. There are several potential mechanisms that could cause this irreversibility; for example, phosphorylation could cause a conformational change in HCN1 rendering the phosphosite inaccessible. Alternatively, phosphorylation could trigger internalization and compartmentalization of HCN1. In either case, phosphorylation could affect I_h and HCN1 surface expression by altering HCN1 co-assembly with one or more trafficking proteins, such as tetratricopeptide repeat-containing Rab8b interacting protein (TRIP8b) or filamin A (Santoro *et al.* 2004; Gravante *et al.* 2004). TRIP8b, for example, has at least nine N-terminal splice variants; some of these promote HCN1 surface expression, whereas others diminish HCN1 surface expression (Lewis *et al.* 2009; Santoro *et al.* 2009). PKC could diminish I_h and HCN1 surface expression in hippocampal pyramidal neurons by altering the co-assembly of HCN1 and one of these TRIP8b splice variants. The identification of the PKC phosphosites within the HCN1 protein would help determine the specific mechanism by which PKC activity modulates HCN1 surface expression. However, phosphosite identification is beyond the scope of the present study, although we do plan to investigate this question in the near future.

Potential relevance to epileptogenesis

Our discovery of a phosphorylation mechanism controlling maximal I_h and HCN1 surface expression in pyramidal neurons has potential implications as a mechanism in epileptogenesis. In a previous study, we demonstrated that maximal I_h and HCN1 surface expression are decreased in the hippocampal CA1 area at 1 h post-SE in the rat pilocarpine model of temporal lobe epilepsy; however, the mechanisms controlling these changes were unclear (Jung *et al.* 2011). Our results provide a potential mechanism for this downregulation, and are consistent with the discovery of increased hippocampal PKC activity in rodent models of epilepsy (Grabauskas *et al.* 2006). It remains to be clarified whether increased PKC activity in the hippocampal CA1 area post-SE is responsible for the contribution of diminished HCN1 expression and function to epileptogenesis. Our results are the first demonstration of a phosphorylation-induced change in I_h amplitude and HCN1 surface expression in pyramidal neurons, and present a potential mechanism regulating diminished HCN1 function and expression both under normal conditions and post-SE in the rat pilocarpine model of temporal lobe epilepsy.

References

Abramian AM, Comenencia-Ortiz E, Vithlani M, Tretter EV, Sieghart W, Davies PA & Moss SJ (2010). Protein kinase C phosphorylation regulates membrane insertion of $GABA_A$ receptor subtypes that mediate tonic inhibition. *J Biol Chem* **285**, 41795–41805.

Biel M, Wahl-Scott C, Michalakis S & Zong X (2009). Hyperpolarization-activated cation channels: from genes to function. *Physiol Rev* **89**, 847–885.

Bullis JB, Jones TD & Poolos NP (2007). Reversed somatodendritic I_h gradient in a class of rat hippocampal neurons with pyramidal morphology. *J Physiol* **579**, 431–443.

Carr DB, Andrews GD, Glen WB & Lavin A (2007). Alpha2-noradrenergic receptor activation enhances excitability and synaptic integration in rat prefrontal cortex pyramidal neurons via inhibition of HCN currents. *J Physiol* **584**, 437–450.

Cathala L & Paupardin-Tritsch D (1997). Neurotensin inhibition of the hyperpolarization-activated cation current (I_h) in the rat substantia nigra pars compacta implicates the protein kinase C pathway. *J Physiol* **503**, 87–97.

Craven KB & Zagotta WN (2006). CNG and HCN channels: two peas, one pod. *Annu Rev Physiol* **68**, 375–401.

Fogle KJ, Lyashchenko AK, Turbendian HK & Tibbs GR (2007). HCN pacemaker channel activation is controlled by acidic lipids downstream of diacylglycerol kinase and phospholipase A2. *J Neurosci* **27**, 2802–2814.

Goel G, Makkar HPS, Francis G & Becker K (2007). Phorbol esters: structure, biological activity, and toxicity in animals. *Int J Toxicol* **26**, 279–288.

Goodkin HP, Joshi S, Mtchedlishvili Z, Brar J & Kapur J (2008). Subunit-specific trafficking of $GABA_A$ receptors during status epilepticus. *J Neurosci* **28**, 2527–2538.

Grabauskas G, Chapman H & Wheal HV (2006). Role of protein kinase C in modulation of excitability of CA1 pyramidal neurons in the rat. *J Neurosci* **139**, 1301–1313.

Gravante B, Barbuti A, Milanesi R, Zappi I, Viscomi C & DiFrancesco D (2004). Interaction of the pacemaker channel HCN1 with filamin A. *J Biol Chem* **279**, 43847–43853.

Hama H, Hara C, Yamaguchi K & Miyawaki A (2004). PKC signaling mediates global enhancement of excitatory synaptogenesis in neurons triggered by local contact with astrocytes. *Neuron* **41**, 405–415.

Hoffman DA & Johnston D (1998). Downregulation of transient K^+ channels in dendrites of hippocampal CA1 pyramidal neurons by activation of PKA and PKC. *J Neurosci* **18**, 3521–3528.

Huang J, Huang A, Zhang Q, Lin YC & Yu HG (2008). Novel mechanism for suppression of hyperpolarization-activated cyclic nucleotide-gated pacemaker channels by receptor-like tyrosine phosphatase-α;. *J Biol Chem* **283**, 29912–29919.

Jung S, Jones TD, Lugo JN, Sheerin AH, Miller JW, D'Ambrosio R, Anderson AE & Poolos NP (2007). Progressive dendritic HCN channelopathy during epileptogenesis in the rat pilocarpine model of epilepsy. *J Neurosci* **27**, 13012–13021.

Jung S, Bullis JB, Lau IH, Jones TD, Warner LN & Poolos NP (2010). Downregulation of dendritic HCN channel gating in epilepsy is mediated by altered phosphorylation signaling. *J Neurosci* **30**, 6678–6688.

Jung S, Warner LN, Pitsch J, Becker AJ & Poolos NP (2011). Rapid loss of dendritic HCN channel expression in hippocampal pyramidal neurons following status epilepticus. *J Neurosci* **31**, 14291–14295.

Kim J, Jung SC, Clemens AM, Petralia RS & Hoffman DA (2007). Regulation of dendritic excitability by activity-dependent trafficking of the A-type K^+ channel subunit $K_v4.2$ in hippocampal neurons. *Neuron* **54**, 933–947.

Lewis AS, Schwartz E, Chan CS, Noam Y, Shin M, Wadman WJ, Surmeier DJ, Baram TZ, Macdonald RL & Chetkovich DM (2009). Alternatively spliced isoforms of TRIP8b differentially control h channel trafficking and function. *J Neurosci* **29**, 6250–6265.

Li B, Chen F, Ye J, Chen X, Yan J, Li Y, Xiong Y, Zhou Z, Xia J & Hu Z (2010). The modulation of orexin A on HCN currents of pyramidal neurons in mouse prelimbic cortex. *Cereb Cortex* **20**, 1756–1767.

Lörincz A, Notomi T, Tamás G, Shigemoto R & Nusser Z (2002). Polarized and compartment-dependent distribution of HCN1 in pyramidal cell dendrites. *Nat Neurosci* **5**, 1185–1193.

Lugo JN, Barnwell LF, Ren Y, Lee WL, Johnston LD, Kim R, Hrachovy RA, Sweatt JD & Anderson AE (2008). Altered phosphorylation and localization of the A-type channel, Kv4.2 in status epilepticus. *J Neurochem* **106**, 1929–1940.

Magee JC (1998). Dendritic hyperpolarization-activated currents modify the integrative properties of hippocampal CA1 pyramidal neurons. *J Neurosci* **18**, 7613–7624.

Magee JC (1999). Dendritic I_h normalizes temporal summation in hippocampal CA1 neurons. *Nat Neurosci* **2**, 508–514.

Marcelin B, Chauvière L, Becker A, Migliore M, Esclapez M & Bernard C (2009). h channel-dependent deficit of theta oscillation resonance and phase shift in temporal lobe epilepsy. *Neurobiol Dis* **33**, 436–447.

McClelland S, Flynn C, Dubé C, Richichi C, Zha Q, Ghestem A, Esclapez M, Bernard C & Baram TZ (2011). Neuron-restrictive silencer factor-mediated hyperpolarization-activated cyclic nucleotide gated channelopathy in experimental temporal lobe epilepsy. *Ann Neurol* **70**, 454–464.

O'Dell TJ, Kandel ER & Grant SGN (1991). Long-term potentiation in the hippocampus is blocked by tyrosine kinase inihibitors. *Nature* **353**, 558–560.

Poisbeau P, Cheney MC, Browning MD & Mody I (1999). Modulation of synaptic $GABA_A$ receptor function by PKA and PKC in adult hippocampal neurons. *J Neurosci* **19**, 674–683.

Poolos NP, Bullis JB & Roth MK (2006). Modulation of h-channels in hippocampal pyramidal neurons by p38 mitogen-activated protein kinase. *J Neurosci* **26**, 7995–8003.

Poolos NP (2012). Hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channelopathy in epilepsy. In *Jasper's Basic Mechanisms of the Epilepsies*. Noebels JL, Avoli M, Rogawski MA, Olsen RW, Delgado-Escueta AV, eds, pp. 85–96. Oxford University Press, New York , NY.

Rakhade SN, Zhou C, Aujla PK, Fishman R, Sucher NJ & Jensen FE (2008). Early alterations of AMPA receptors mediate synaptic potentiation induced by neonatal seizures. *J Neurosci* **28**, 7979–7990.

Reetz O & Strauss U (2013). Protein kinase C activation inhibits rat and human hyperpolarization activated cyclic nucleotide gated channel (HCN)1-mediated current in mammalian cells. *Cell Physiol Biochem* **31**, 532–541.

Roberson ED, English JD, Adams JP, Selcher JC, Kondratick C & Sweatt JD (1999). The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. *J Neurosci* **19**, 4337–4348.

Santoro B, Wainger BJ & Siegelbaum SA (2004). Regulation of HCN channel surface expression by a novel c-terminal protein–protein interaction. *J Neurosci* **24**, 10750–10762.

Santoro B, Piskorowski RA, Pian P, Hu L, Liu H & Siegelbaum SA (2009). TRIP8b splice variants form a family of auxiliary subunits that regulate gating and trafficking of HCN channels in the brain. *Neuron* **62**, 802–813.

Shah MM, Anderson AE, Leung V, Lin X & Johnston D (2004). Seizure-induced plasticity of h channels in entorhinal cortical layer III pyramidal neurons. *Neuron* **44**, 495–508.

Terunuma M, Xu J, Vithlani M, Sieghart W, Kittler J, Pangalos M, Haydon PG, Coulter DA & Moss SJ (2008). Deficits in phosphorylation of $GABA_A$ receptors by intimately associated protein kinase C activity underlie compromised synaptic inhibition during status epilepticus. *J Neurosci* **28**, 376–384.

Thoby-Brisson M, Cauli B, Champagnat J, Fortin G & Katz DM (2003). Expression of functional tyrosine kinase B receptors by rhythmically active respiratory neurons in the pre-Bötzinger complex of neonatal mice. *J Neurosci* **23**, 7685–7689.

Williams SR & Wozny C (2011). Errors in the measurement of voltage-activated ion channels in cell-attached patch-clamp recordings. *Nature Comms* **2**, 242.

Yu HG, Lu Z, Pan Z & Cohen IS (2004). Tyrosine kinase inhibition differentially regulates heterologously expressed HCN channels. *Pflüg Arch Eur J Physiol* **447**, 392–400.

Zhang K, Peng B & Sanchez RM (2006). Decreased I_h in hippocampal area CA1 pyramidal neurons after perinatal seizure-inducing hypoxia. *Epilepsia* **47**, 1023–1028.

Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

AW, SJ and NP conceived and designed the experiments. AW, SJ and NP collected and analysed the data. AW, SJ and NP interpreted the results. AW and NP prepared the manuscript. All authors approved the final version of the manuscript submitted for publication.

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