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Original Paper

Protein Kinase C Activation Inhibits Rat and Human Hyperpolarization Activated Cyclic Nucleotide Gated Channel (HCN)1 - Mediated Current in Mammalian Cells

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Key Words
Protein kinase C • Hyperpolarization-activated cyclic nucleotide-gated channel • HEK293 cells • N1E-115 cells

Abstract
Background/Aims: Hyperpolarization activated cyclic nucleotide gated 1 (HCN1) channels determine neuronal excitability in several brain regions. In contrast to HCN2 and HCN4, HCN1 is less sensitive to cAMP and the number of other known modulators is limited. One of those, the protein kinase C (PKC), showed opposing effects on mouse HCN1 channels expressed in *Xenopus* oocytes. ***Methods:*** In order to study PKC effects on HCN1 mediated currents in a mammalian environment we expressed rat HCN1 or human HCN1 in human embryonic kidney (HEK293) cells and rat HCN1 in murine neuroblastoma (N1E-115) cells. We recorded the resulting I_h before and during the application of the membrane permeable non-metabolizable PKC-activator 4βPMA in cell-attached mode of the patch-clamp technique, leaving the intracellular environment intact. ***Results:*** 4βPMA reduced maximal HCN1 mediated currents to about 60-70 % and slowed its activation, but left its voltage sensitivity unchanged. The effect was neither due to species-related differences nor restricted to HEK293 cells, because it was comparable for human and rat HCN1 in HEK293 and for rat HCN1 in N1E-115 cells. However, pre-treatment with the PKC blocker GF109203X abolished 4βPMA induced I_h changes. Disrupting the intracellular environment by recording in whole-cell mode drastically reduced the 4βPMA effect. ***Conclusion:*** PKC activation reduces and slows I_h in non-neuronal and neuronal mammalian cells transfected with rat or human HCN1 if the intracellular content remains intact.

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Introduction

Hyperpolarization-activated, cyclic nucleotide-gated (HCN) channels underlying I_h regulate the neuronal excitability in many regions of the mammalian brain including the neocortex and hippocampus [1]. In these regions, HCN1, as one of the four tetramer-forming subunits found in mammals, is predominant in pyramidal neurons [2]. In general, known determinants of HCN1 function - and therewith neocortical excitability - include total protein expression, surface expression, channel properties and tetramer formation [1]. Fast alterations in HCN1 mediated currents, in particular, likely arise from changes in channel properties such as gating. Although the gating of HCN1 channels is virtually insensitive to the canonical HCN channel modulator cAMP, a depolarizing shift in HCN1 voltage dependence can occur apart from the action of cyclic nucleotides in *Xenopus* oocytes by e. g. allosteric gating by phospatidylinositol-4,5-biphosphate [3] or by protein kinase C (PKC) activation with 4β-phorbol 12-myristate 13-acetate (4βPMA) [4] and in hippocampal pyramidal neurons by p38MAP kinase [5]. However, changes in the maximum current without shifts in voltage dependence can likewise underlie fast alterations of HCN1 mediated currents [6, 7]. Interestingly, the activation of conventional and novel isoforms of PKC by 4βPMA affected both, gating and maximum current, of HCN1 channels expressed in *Xenopus* oocytes, although in an opposing manner [4]. In the *Xenopus* expression system PKC activation depolarized voltage sensitivity of HCN1 mediated currents, but suppressed its maximum. Therewith, the net effect - apart from the newly opened voltage window - remains unclear. In order to expand the knowledge on this HCN1 modulation and, in particular, to test the PKC action in a mammalian system, we chose the well characterized rat (r)HCN1 [6] and human (h)HCN1 [8] expressed in HEK293 cells. A synopsis of our data suggests that PKC overall inhibits HCN1-mediated currents in mammalian cells due to a reduction in maximal current. In contrast to data on murine HCN1 expressed in *Xenopus* oocytes PKC does not influence gating.

Materials and Methods

Cell culture and transfection

HEK293 cells (DSMZ, Braunschweig, Germany) or N1E-115 cells (ATCC, Wesel, Germany) were cultured at 37°C (95 % O_2 and 5 % CO_2) in DMEM (Dulbecco´s modified eagle medium) including 4.5 g/L D-Glucose, L-glutamine and 3.7 g/L $NaHCO_3$ supplemented with 10 % fetal calf serum, 1 % penicillin/streptomycin (all from Pan biotech GmbH, Aidenbach, Germany) and 1 % L-glutamine (for N1E-115 cells DMEM with pyruvate instead of $NaHCO_3$ and without supplemental L-glutamine). Cells were detached by use of 2.5 % trypsin (life technologies GmbH, Darmstadt, Germany) and passaged regularly. One day after HEK293 cells or N1E-115 cells were plated at low density on poly-L-lysine coated cover slips they were transfected with pIRES2_dsRed-rHCN1 (NM_053375.1) or with (human) hHCN1 (in pcDNA 3.1; kind gift of Juliane Stieber) in a co-transfection with pIRES2_eGFP (3:1 ratio) by 2 M $CaPO_4$ for 15 min as described previously [6]. In the used rHCN1 construct the base pairs 75 – 2807 exactly resembled the respective published sequence of rHCN1 as proofed by sequencing. Culture passages from 1 to 20 were used.

Patch-Clamp Recordings

2 - 4 days after transfection HEK293 cells (for N1E-115 cells 2 - 3 days after transfection) on their cover slips were placed into a recording chamber. The actual I_h magnitude (the channel presence in the membrane) appeared tightly related to the time after transfection and the fluorescent dye (see below) seemed to negatively influence cell viability, leaving only a small time frame for measurements. Cells were constantly superfused with extracellular solution comprising (in mM): 120 NaCl, 10 KCl, 0.5 $MgCl_2$, 10 HEPES, 10 glucose (all from Carl Roth GmbH and Co. KG, Karlsruhe, Germany), 1.8 $CaCl_2$ (Merck, Darmstadt, Germany), 10 TEA and 2.5 4-aminopyridine (both from Sigma-Aldrich, Munich, Germany) (pH adjusted with NaOH to 7.4). HEK293 cells expressing rHCN1 were identified by red fluorescence with an inverted microscope (Axiovert S100; Zeiss, Oberkochen, Germany). Pipettes were pulled (P-97 micropipette puller; Sutter Instruments, Novato, CA, USA) to a resistance of 2.0 - 3.5 MΩ. The intracellular solution comprised (in

mM) 120 K-gluconate, 10 Na-phosphocreatine, 11 EGTA, 2 Mg^{2+}ATP, 0.3 Tris-GTP (Sigma-Aldrich), 10 KCl, 1 $MgCl_2$, 1$CaCl_2$, 10 HEPES, adjusted with KOH (Carl Roth) to pH 7.2 and was kept on ice for one day. For some whole cell experiments on HEK293 cells a modified cAMP containing intracellular solution was used (in mM): 120 K-methylsulphate ($KMeSO_4$) (ICN Biomedical Inc, California, USA), 20 KCl, 14 Na-phosphocreatine, 4 NaCl, 0.5 EGTA, 10 HEPES, 4 Mg^{2+}-ATP, 0.3 Tris-GTP and 0.1 cAMP (Sigma-Aldrich) (pH adjusted with KOH to 7.2). Experiments were conducted at room temperature and recorded with an EPC-10 amplifier (HEKA, Lambrecht, Germany) controlled by PatchMaster v2.32 software (HEKA). 4β-phorbol 12-myristate 13-acetate (4βPMA) (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) was dissolved in 881.68 µl of 99.8 % DMSO (Sigma-Aldrich) and GF109203X (R&D Systems) was dissolved in 2.35 ml 99.8 % DMSO to a stock concentration of 10 mM. Stocks were diluted with extracellular solution to a final concentration of 0.2 µM or 1 µM 4βPMA or GF109203X / 0.02 % or 0.1% DMSO respectively.

Data and Statistical Analysis

Data were analyzed with FitMaster (HEKA) and Origin 8.5 (Origin Labs, Northampton, MA, USA). For the analysis of the experiments in whole cell mode, recordings with a holding current larger than 200 pA were excluded. An offline linear leak subtraction was performed only for estimating the I_h amplitude in cells measured in whole cell mode. In cell attached mode the leak current was negligible, so no leak subtraction was applied. For analysis of the voltage dependence of the current, tail current amplitudes of all voltage steps were normalized separately for each cell and plotted against the preceding voltage step. Each data set was fitted with a Boltzmann function: $I(V) = ([A_1 - A_2]/1+ e ([V-V_{1/2}]/k)+ A_2)$ resulting in the floating parameters $V_{1/2}$, the voltage at which half of the maximal current was activated and k, the slope of the fitted curve. Calculated liquid junction potentials of 9.3 mV were not corrected for. The activation kinetics were fitted in cell attached recordings by a single exponential equation $y(x) = y_0 + A_1 (1 – e^{-x/\tau 1})$ and for the whole cell recordings by a double exponential equation $y(x) = y_0 + A_1 (1 – e^{-x/\tau 1}) + A_2 (1 – e^{-x/\tau 2})$ (as integrated in FitMaster), resulting in one or two (fast and slow) time constants τ. Statistical tests were performed with the nonparametric test for paired data (Wilcoxon signed rank test) in case of non-normal distribution and with the paired t-test in case of normal distribution. Results were regarded as statistical significant if $P < 0.05$. Error bars represent SEM.

Results

4βPMA reduced and slowed rHCN1 or hHCN1 mediated I_h in HEK293 cells

To study PKC – HCN1 interactions in a mammalian system we utilized human embryonic kidney (HEK) 293 cells transfected with rHCN1 or hHCN1. By recording I_h in the cell-attached mode we aimed to conserve the intracellular content and the respective signaling pathways in particular. Under those conditions I_h conductance for rHCN1 ranged between 60 and 880 pS. Given a single rHCN1 channel conductance of 680 fS [9] we simultaneously recorded from 88 to 1294 rHCN1 channels. Bath applying the membrane permeable 4βPMA enabled us to activate the intracellular PKC without affecting the extracellular HCN1 domains under the attached pipette. Such treatment reduced the maximal rHCN1 mediated current to about 73 %: the application of 1 µM reduced the maximal I_h amplitude from I_{hctrl} = 51.7 ± 11.0 pA to $I_{h4\beta PMA}$ = 37.9 ± 8.0 pA (n = 6, Wilcoxon signed-rank test: $P < 0.05$; Fig. 1A/B) and 0.2 µM from I_{hctrl} = 45.8 ± 6.6 pA to $I_{h4\beta PMA}$ = 33.6 ± 7.5 pA (n = 8, paired t- test, $P = 0.003$). Although the currents were relatively small, their individual amplitude was not correlated to the magnitude of the 4βPMA effect (r = –0.22).

The I_h reduction was accompanied by a slowing of I_h activation (Fig. 1B). The rHCN1 current activation was best fitted with a single exponential equation at –130 mV. The resulting time constant increased from τ_{ctrl} = 66 ± 16.0 ms under control conditions to $\tau_{4\beta PMA}$ = 104.7 ± 25.9 ms during 1 µM 4βPMA application ($P < 0.05$; Fig. 1C/D) and from τ_{ctrl} = 85 ± 9.9 ms under control conditions to $\tau_{4\beta PMA}$ = 95.4 ± 12.2 ms during 0.2 µM 4βPMA application ($P < 0.05$). However, in contrast to the two-electrode voltage clamp recordings in *Xenopus* oocytes [4], 4βPMA did not change the voltage of half maximal current activation ($V_{1/2}$) by ($V_{1/2ctrl}$ = –96.0 ± 3.3 mV vs. $V_{1/24\beta PMA}$ = –99.0 ± 3.0 mV, n = 6, Wilcoxon signed-rank test:

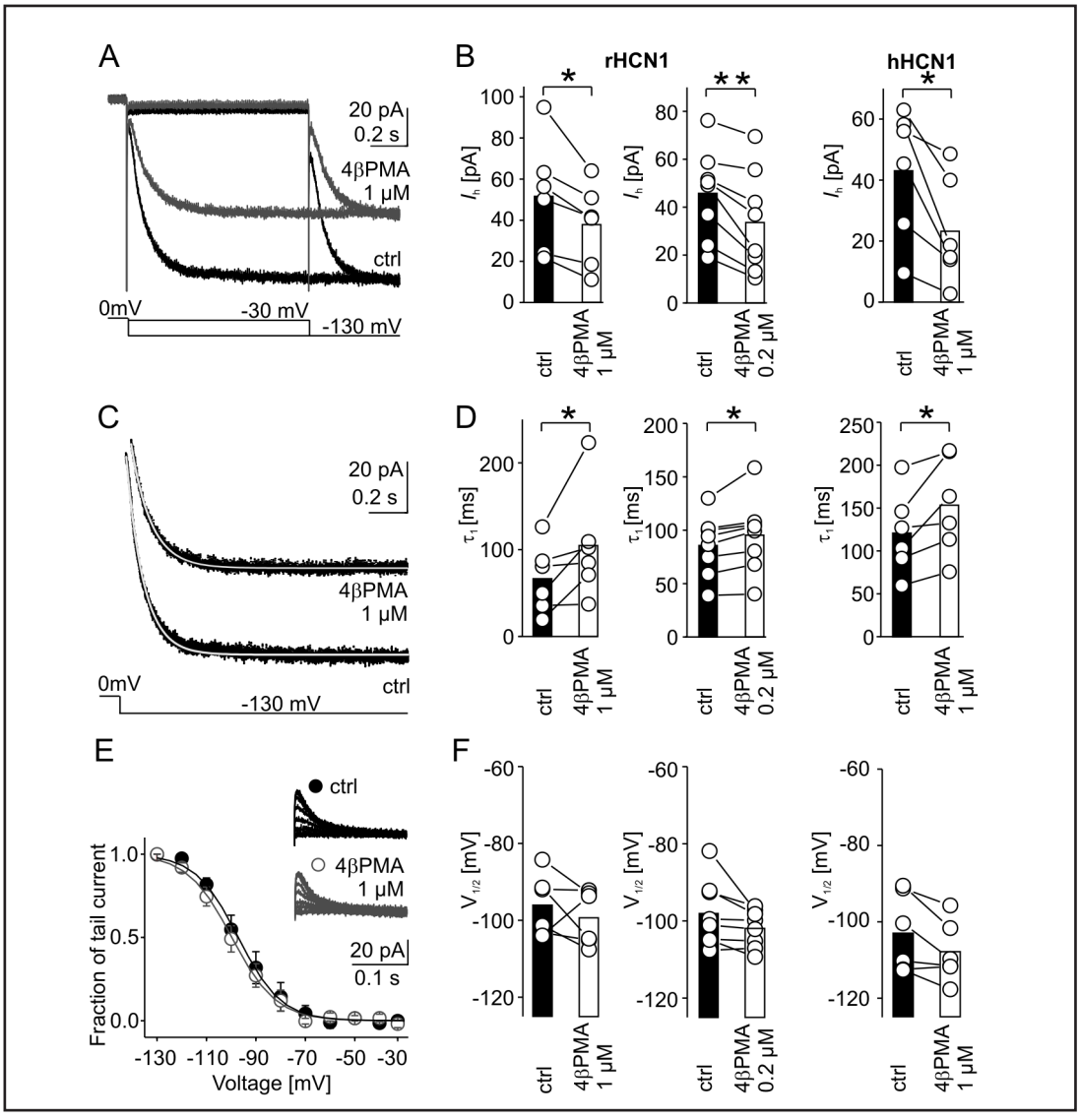

Fig. 1. 4βPMA induced PKC activation reduced maximum rHCN1 or hHCN1 mediated I_h and slowed their activation in mammalian cells with intact intracellular content. A. Traces of rHCN1 mediated I_h at voltage steps of –30 mV and –130 mV as indicated in the pulse protocol at the bottom recorded in HEK293 cells in cell attached mode before (black trace) and during the application of 1 μM 4βPMA (grey trace). B. Population data of maximum I_h measured at the end of a 1-sec pulse to –130 mV demonstrate the decrease of I_h after 4βPMA addition. C. Distinct data points represent the time course of I_h activation at –130 mV under control conditions (lower trace) and after 4βPMA application (upper trace). Both are mathematically described by a single exponential function (light grey line). D. Population data of time constants of I_h activation reveal a considerable slowing. E. Voltage dependence of I_h activation. The tail currents after different preceding command potentials (insets) were normalized to the maximal tail current for each cell. For the graph values were averaged, plotted against the preceding voltage and fitted with the Boltzmann equation. F. Population data of half maximal current activation voltages ($V_{1/2}$) estimated for individual cells before and during 4βPMA application. Control values are represented as filled black, values under 4βPMA as open white columns throughout all figures. *denotes significance level P < 0.05 throughout all figures.

P = 0.3; Fig. 1E/F under 1 μM 4βPMA or $V_{1/2ctrl}$ = –98.0 ± 3.0 mV vs. $V_{1/24\beta PMA}$ = –101.9 ± 1.7 mV n = 8, paired t-test: P = 0.07 under 0.2 μM 4βPMA). These data suggest that activation of PKC results in a suppression of maximal rHCN1 mediated current accompanied by a slowing of its activation.

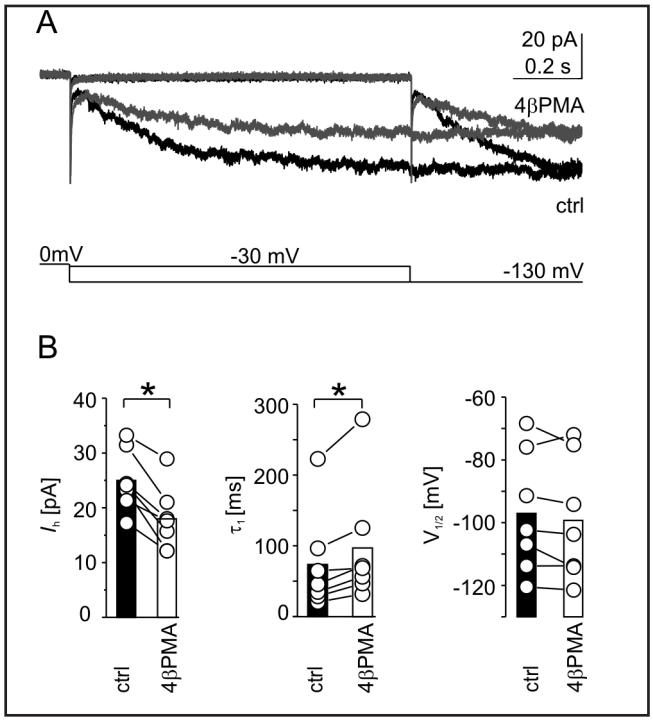

Fig. 2. 4βPMA affects rHCN1 in N1E-115 cells as in HEK293 cells. A. Traces of rHCN1 mediated I_h in N1E-115 cells at voltage steps of –30 mV and –130 mV recorded under the same conditions as in HEK293 cells before (black trace) and after the application of 1 µM 4βPMA (grey trace). B. Population data of maximum I_h amplitude measured at –130 mV demonstrate the decrease of I_h by 1µM 4βPMA (left); of single exponential function time constants of I_h activation reveal a considerable slowing by 1µM 4βPMA (middle); and of unperturbed $V_{1/2}$s estimated for individual cells under control conditions and under 1µM 4βPMA (right).

In order to investigate whether species related differences or incompatibilities influenced our results, we subsequently transfected HEK293 cells with hHCN1. Analysis of the resulting currents yielded a comparable effect of 4βPMA on rHCN1 and hHCN1 mediated I_h: 1µM 4βPMA reduced the maximal hHCN1 mediated I_h amplitude to about 57 % from I_{hctrl} = 43 ± 8.7 pA to $I_{h4\beta PMA}$ = 24.9 ± 8.0 pA (at –130 mV, n = 7, Wilcoxon signed-rank test P = 0.03, Fig. 1B). Likewise, the time constant of I_h activation increased from τ_{ctrl} = 120.8 ± 19.6 ms under control conditions to $\tau_{4\beta PMA}$ = 153.1 ± 23.2 ms during 1 µM 4βPMA application (n = 7, Wilcoxon signed-rank test P = 0.03, Fig. 1D). Finally, as for rHCN1, 4βPMA did not change the $V_{1/2}$ of the hHCN1 mediated I_h ($V_{1/2ctrl}$ = –102.9 ± 4.3 mV vs. $V_{1/24\beta PMA}$ = –107.9 ± 3.2 mV, n = 7, Wilcoxon signed rank test: P = 0.06, Fig. 1F). This data suggest that reducing the amplitude of HCN1 mediated I_h without shifting the $V_{1/2}$ by 4βPMA in mammalian cells is independent of species-related differences and that PKC / PKC metabolites might not act via C-terminal loci that are non-homologous between both species (AA721 – AA748).

4βPMA similarly affects rHCN1mediated I_h in neuroblastoma cells of the mouse (N1E-115)

The 4βPMA effects on HCN1 presented so far might be HEK293-specific and not transferable to other mammalian cells including pyramidal neurons, in which HCN1 channels predominate. Therefore we studied the PKC effect on HCN1 in a more neuronal and assumingly less embryonic environment - the murine neuroblastoma cell line N1E-115.

By reproducing the cell attached measurements we showed that 4βPMA similarly affected rHCN1 in N1E-115 cells and HEK293: 4βPMA (1 µM) reduced the maximal amplitude of rHCN1 mediated I_h to about 72 % from I_{hctrl} = 25.0 ± 2.1 pA to $I_{h4\beta PMA}$ = 17.9 ± 2.2 pA (n = 7, Wilcoxon-ranked test P = 0.02, Fig. 2A/B) without shifting the $V_{1/2}$ ($V_{1/2ctrl}$ = –97.4 ± 7.3 mV vs. $V_{1/24\beta PMA}$ = –99.2 ± 7.4 mV, n = 7, Wilcoxon signed rank test: P = 0.2, Fig. 2B). The I_h activation slowed as indicated by the increase in activation time constant from τ_{ctrl} = 73.4 ± 26.7 ms under control conditions to $\tau_{4\beta PMA}$ = 97.1 ± 32.3 ms during 1 µM 4βPMA application (P = 0.02, Fig. 2B). The comparability of 4βPMA effects on HCN1 in N1E-115 and HEK293 cells suggest that PKC act on HCN1 channels in mammalian cells independent of the cellular environment or developmental stage.

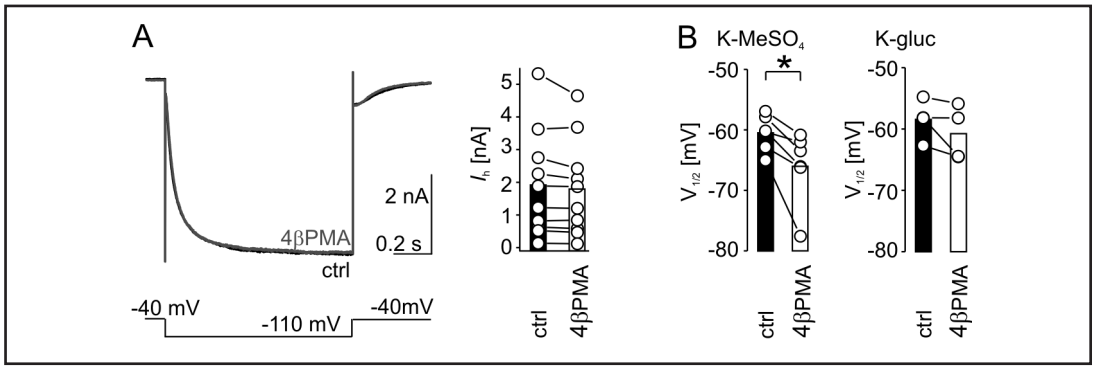

Fig. 3. Disruption of intracellular content impaired the 4βPMA effects. A. Left: Traces of I_h in HEK293 cell expressing rHCN1 recorded in the whole cell mode at voltage steps to –110 mV with (grey traces) and without (black traces) 1 µM 4βPMA. Right: Population data of maximal I_h amplitude at –110 mV. B. Voltage dependence of I_h activation estimated in whole cell configuration using K-$MeSO_4$- (left) or K-gluconate- (right) based intracellular solution. Individual Boltzmann fits of tail current fractions were used for estimating the plotted $V_{1/2}$s.

An intact intracellular environment is a prerequisite for 4βPMA to exert its modulatory effect on rHCN1 mediated I_h

To test whether 4βPMA modulation of rHCN1 channels requires an intact intracellular environment, we next applied 1 µM 4βPMA during whole cell patch clamp recordings of rHCN1 transfected HEK293 cells. Such disruption of the proper intracellular environment prevented the main 4βPMA effect: 4βPMA did not change the maximum amplitude of I_h (I_{hctrl} = 1.9 ± 0.5 nA to $I_{h4\beta PMA}$ = 1.79 ± 0.46 nA, n = 10, P = 0.11; Fig. 3A). This prevention persisted when looking separately to experiments with differing intracellular solutions (see Materials and Methods, for K-gluconate based solution: I_{hctrl} = 1.9 ± 0.8 nA to $I_{h4\beta PMA}$ = 1.85 ± 0.8 nA, n = 4, P = 0.6; K-$MeSO_4$ based solution: I_{hctrl} = 1.9 ± 0.7 nA to $I_{h4\beta PMA}$ = 1.75 ± 0.6 nA, n = 6, P = 0.09). Further, 4βPMA did not affect the I_h activation kinetics. As in our previous study [6], whole-cell recorded rHCN1 current activation better matched to a double exponential fit. Both time constants remained comparable under 4βPMA, when using K-gluconate based intracellular solution (τ_{1ctrl} = 21.4 ± 2.1 ms vs. $\tau_{14\beta PMA}$ = 25.9 ± 4.1 ms, n = 4, Wilcoxon signed-rank test: P = 0.4; τ_{2ctrl} = 96.9 ± 14.1 ms vs. $\tau_{24\beta PMA}$ = 100.4 ± 20.4 ms, n = 4, P = 1) at full current activation (–110 mV). Also the voltage dependence of activation was stable under 4βPMA, when using K-gluconate based intracellular solution ($V_{1/2\ ctrl}$ = –58.4 ± 1.6 mV and $V_{1/24\beta PMA}$ = –60.8 ± 2.2 mV, n = 4, P = 0.125; Fig. 3B). However, during the 10 min of 4βPMA application the current activation slowed when recorded with K-$MeSO_4$ based intracellular solution: τ_{1ctrl} = 19.21 ± 4.7 ms vs. $\tau_{14\beta PMA}$ = 27.4 ± 2.5 ms, n = 6, Wilcoxon signed-rank test, P = 0.06; τ_{2ctrl} = 78.76 ± 13.3 ms vs. $\tau_{24\beta PMA}$ = 103.4 ± 14.3 ms, n = 6, P = 0.09). This might be explained by the slight but consistent shift to more hyperpolarized potentials. Here, the $V_{1/2}$ decreased from $V_{1/2\ ctrl}$ = –60.5 ± 1.23 mV to $V_{1/24\beta PMA}$ = –66.0 ± 2.5 mV, n = 6, P < 0.05; Fig. 3B). Because the latter changes in voltage sensitivity were restricted to recordings with K-$MeSO_4$ based intracellular solution and resemble the known "run down" of HCN-channels we attribute them to the recording conditions rather than to a 4βPMA effect. Such K-$MeSO_4$ induced I_h reduction could explain the gradual enhancement of the input resistance found in rat CA1 pyramidal neurons [10, 11]. Taken together, the disruption of the intracellular environment prevented the 4βPMA effects observed in intact cells.

4βPMA induced I_h changes are mediated via PKC

We here used the high affinity stereoselective agonist of the cysteine-rich C-1 DAG/4β-phorbol-binding pocket 4βPMA to exclude receptor-mediated cleavage of phosphatidylinositol 4,5-bisphosphate (4,5-PIP2) and changes in calcium signaling [4].

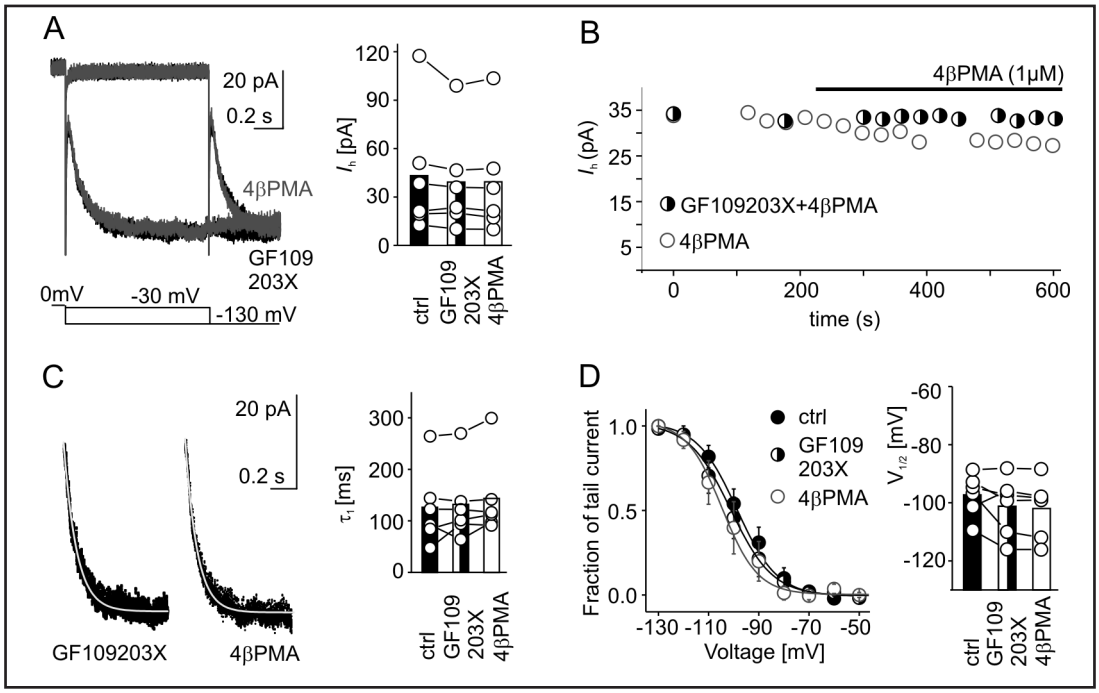

Fig. 4. The PKC inhibitor GF109203X prevented the 4βPMA effects. A. Inhibiting PKC prevented the 4βPMA-induced reduction of rHCN1 current amplitude when recorded in cell-attached mode. Left: rHCN1 mediated I_h at voltage steps to –30 mV and –130 mV in a HEK293 cell was comparable when 1 μM 4βPMA (grey trace) was applied under PKC blockade by GF109203X (black trace). Right: Population data of maximal I_h amplitudes estimated as in Fig. 1A. B. Time course of I_h amplitudes at –100 mV following the application of 1μM 4βPMA (horizontal grey line) to untreated (open circles) or PKC blocked (GF109203X, halved black and white circles) in both cases exemplarily in one cell. C. Prevention of 4βPMA-induced rHCN1 current activation alteration by PKC inhibition. Left: Distinct data points represent the time course of I_h activation at –130 mV during the application of 1 μM GF109203X (left trace) and after additional 1 μM 4βPMA application (right trace) with the respective single exponential function (light grey line). Right: Population data of time constants of I_h activation for each condition. D. Left: Voltage dependence of rHCN1 mediated I_h activation estimated and averaged over all patches as described in Fig. 1B. Right: Population data of individual $V_{1/2}$ estimated separately for each patch. Note, that also PKC inhibition itself (halved black and white circles and column) left all I_h parameters unchanged.

However, 4βPMA may have a number of effects beyond PKC activation: it might also activate ERK, all three MAPK subfamilies, in particular p38 [12], Akt/PKB [13] or Rac2 [14], the latter two in a PI3K-independent manner. Therefore we tested whether 4βPMA modulated rHCN1-mediated I_h specifically via its PKC activation properties by sequentially applying the bisindolylmaleimide derivative GF109203X (1 μM), a potent and selective inhibitor of PKC for >10 minutes followed by 4βPMA. Such treatment prevented the I_h amplitude reduction ($I_{hGF109203X}$ = 39.2 ± 13.0 pA vs. $I_{h4\beta PMA}$ = 39.2 ± 14.0 pA, n = 6, Wilcoxon signed-rank test, P = 0.84; Fig. 4A/B) in cell-attached recordings of HEK293 cells. It also impeded the 4βPMA effect on the kinetics of I_h activation. Fitting I_h activation at the voltage step of –130 mV using a single exponential equation revealed no 4βPMA induced changes in the kinetics of the channel ($\tau_{GF109203X}$ = 131.56 ± 29.5 ms vs. $\tau_{4\beta PMA}$ = 143.12 ± 32.1 ms, P = 0.22, Fig. 4C). As expected from the initial cell attached recordings (Fig. 1C), 4βPMA did not alter the $V_{1/2}$ ($V_{1/2GF109203X}$ = –101.1 ± 4.15 mV vs. $V_{1/24\beta PMA}$ = –101.9 ± 4.2 mV P = 0.09) when applied subsequent to GF109203X (Fig. 4D). However, the inhibition of basal PKC activity by GF109203X did not affect the maximal I_h (I_{hctrl} = 43.4 ± 15.8 pA vs. $I_{hGF109203X}$ = 39.2 ± 13.0 pA, P = 0.16; Fig. 4A), voltage sensitivity ($V_{1/2ctrl}$ = –97.3 ± 2.98 mV vs. $V_{1/2GF109203X}$ = –101.1 ± 4.15 mV, P = 0.2, Fig.

4D) or time course of I_h activation (τ_{ctrl} = 125.86 ± 30.8 ms vs. $\tau_{GF109203X}$ = 131.56 ± 29.5 ms, P = 0.8; Fig. 4C), suggesting a rather low basal PKC activity in HEK293 under our conditions. Above results strengthen our argument that PKC activation modulates HCN1 mediated I_h and exclude a direct 4βPMA action of on HCN1 channels.

Discussion

Against the background of apparently opposing effects of PKC activation on HCN1 (and HCN2) mediated currents in *Xenopus* oocytes [4] we here investigated the effects of PKC activation in mammalian systems. We found that 4βPMA reduces the maximum I_h in rHCN1 and hHCN1 expressing HEK293 cells and in rHCN1 expressing N1E cells. Therewith PKC activation overall inhibits I_h in mammalian cells in contrast to the at least partially activating effect in *Xenopus* oocytes [4]. As discussed in detail below, these changes are most likely attributable to the diverging expression systems, in particular to developmental (oocyte vs. more differentiated embryonic/neuroblastoma) stages or to phylogenetical (amphibian vs. mammal) origin.

The 4βPMA effect in the mammalian cells was specifically attributable to PKC activation, since it was prevented by pre-incubation of the PKC inhibitor GF109203X and mediated by intracellular compounds. Basal PKC activity - despite putatively being influential in other systems - did not modulate the rHCN1 mediated I_h in HEK293 cells. The reduction of the maximal I_h qualitatively resembles the one found in *Xenopus* oocytes [4]. Although HCN1 channels may posses phosphorylation sites with a predicted high likelihood of interaction [5], a direct phosphorylation of the channel was rendered unlikely, because the 4βPMA effects persisted when most cytoplasmatically exposed serines, threonines and tyrosines were removed by site directed mutagenesis [4]. Further following the convincing hypothesis of Fogle et al. [4], suppression of maximal I_h likely arises from membrane enrichment with proendocytotic acidic lipids such as arachidonic acid and / or phosphatidic acid followed by an enhanced retrieval of HCN channels from the plasma membrane [4]. Such dynamic regulation of surface expression also underlies the activity dependent HCN1 regulation [15, 16]. Taken together, a reduction in the number of available channels seems plausible to explain the reduction of the maximal I_h, however, our data cannot exclude changes in single channel current or open probability.

In contrast to the findings in *Xenopus* oocytes [4], the gating of neither rHCN1 nor hHCN1 channels was affected by 4βPMA in HEK293 or N1E-115 cells. We can not exclude that this difference between the expression systems is in part due to the more depolarized basic value of the $V_{1/2}$ in the *Xenopus* oocytes and therewith a more "active state" of the mHCN1 channels expressed in *Xenopus* oocytes (–66 mV vs. –95 to –100 mV in this study). Reasons for variations in $V_{1/2}$ are manifold (partially discussed in Battefeld et al., [6]). However, following the above mentioned hypothesis reduction of maximal I_h [4] due to an increase in at least one of the acidic lipids would imply a shift of the channels voltage sensitivity. The lack of shift in the mammalian systems investigated by us might be due to HCN channel "context dependence" [17] suggested by different properties of identical HCN2/4 channels expressed in different systems [18]. Indeed, both expression systems (HEK293 and *Xenopus* oocytes) differ in many putatively relevant aspects: Whereas a large number of PKC isoforms are present in *Xenopus* oocytes (PKCα, β, δ, ζ, ε, η, θ, μ) [19, 20], only some (PKCα, β, δ, ε) were detected endogenously in HEK293 [21].

Distinctions in intracellular content might further result in different interaction with essential downstream players as diacylglycerol kinase or cytoplasmic/independent phospholipaseA2, finally leading to a lack of PKC induced increase in the levels of phosphatidic acid and arachidonic acid or an imbalance of both. Although tempting, it is impossible to pin down the effect to either of these major pathways, because both were necessary for the effects on gating and maximal current, as shown in the blocking experiments [4]. Therefore, it seems more likely that the "direct" interaction of acidic lipids with the channels differs

between the expression systems, maybe due to varying conditions in the channel vicinity, e. g. the availability of putatively required additional proteins [as for p38MAPK – 22]. Finally, even though we ensured the rHCN1 sequence, we cannot exclude different HCN1 protein modulations by posttranslational modifications and/or protein-protein interactions [23, 24].

Although our results in HEK293 and N1E-115 cells are not directly transferrable, it is interesting to note that PKC activation inhibits I_h in several regions of the central nervous system. As in HEK293 cells PKC reduced the maximal conductance of mainly HCN2/3 containing channels when activated by neurotensin receptors in the substantia nigra [25], and of mainly HCN1 containing channels in pyramidal neurons following noradrenergic receptor stimulation in the prefrontal cortex [26] or following metabotrobic glutamate receptor stimulation in the hippocampus [11], notably without evidence for a change in the voltage dependence of activation. Such PKC-induced modulation of I_h in somatosensory neurons might - if present - link the IFN signaling to neuronal excitability changes [7]. Notably, biophysical characteristics of HCN1 channels expressed in HEK293 cells (in particular their $V_{1/2}$) are closely related to neuronal HCN channels when recorded at dendrites of pyramidal cortical and hippocampal neurons in cell attached mode [9, 27, 28]. This apparent phenomenological resemblance might specify HEK293 or N1E-115 cells as model systems to further elucidate neuronal mechanisms of PKC actions.

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