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Low affinity block of native and cloned hyperpolarization-activated I_h channels by Ba^{2+} ions

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Abstract

Ba^{2+} is commonly used to discriminate two classes of ion currents. The classical inward-rectifying K^+ current, I_{Kir}, is blocked by low millimolar concentrations of Ba^{2+}, whereas the hyperpolarization-activated cation current, I_h, is assumed not to be sensitive to Ba^{2+}. Here we investigated the effects of Ba^{2+} on I_h currents recorded from rat hippocampal CA1 pyramidal neurons, and on cloned I_h channels composed of either HCN1 or HCN2 subunits transiently expressed in Human Embryonic Kidney (HEK) 293 cells. The results show that low millimolar concentrations of Ba^{2+} reduce the maximal I_h conductance (IC_{50}~3–5 mM) in both CA1 pyramidal neurons and in HEK 293 cells without specificity for HCN1 or HCN2 subunits. In addition, Ba^{2+} decreases the rate of activation and increases the rate of deactivation of I_h currents. Neither the half-maximal voltage of activation, V_h, nor the reversal potential of the I_h channels were affected by Ba^{2+}. The combined results suggest that Ba^{2+}, at concentrations commonly used to block I_{Kir} currents, also reduces the conductance of I_h channels without subunit specificity, and affects the kinetics of I_h channel gating.

Keywords: Ba^{2+}; Hyperpolarization-activated cation channels; In situ patch clamp; HEK 293 cells

1. Introduction

Hyperpolarization-activated I_h channels are a subset of voltage-gated ion channels which are expressed in both peripheral and central neurons (Pape, 1996; Robinson and Siegelbaum, 2003). I_h currents have been identified as a component of the anomalous inward rectification (an increase of conductance upon hyperpolarization) observed in many neurons (DiFrancesco, 1981a; Pape, 1996). I_h currents are distinguished from the classical inward-rectifying K^+ currents (I_{Kir}) by the fact that I_h channels are permeable to both Na^+ and K^+, resulting in a reversal potential well above the equilibrium potential of K^+, and I_h currents activate more slowly than I_{Kir} currents (DiFrancesco, 1981b; Pape, 1996; Robinson and Siegelbaum, 2003). Furthermore, it is widely assumed that I_{Kir} currents can be distinguished from I_h currents by their sensitivity to Ba^{2+} ions (Robinson and Siegelbaum, 2003). However, there are a number of studies which show that low millimolar concentrations of Ba^{2+} can reduce I_h currents recorded from a variety of preparations (Takahashi, 1990; Kamondi and Reiner, 1991; Bayliss et al., 1994; Wollmuth, 1995). In addition, we observed in a previous study that Ba^{2+} blocks I_h currents recorded from cell-attached patches in rat hippocampal CA1 pyramidal neurons (van Welie et al., 2002).

A family of four subunits, HCN1–4, has been cloned which underlies the molecular diversity of I_h channels (Ludwig et al., 1998; Santoro et al., 1998; Monteggia et al., 2000). When expressed in heterologous expression systems, each subunit gives rise to a hyperpolarization-activated inward current, albeit with distinct functional properties. It has been shown that the HCN subunits are differentially distributed among neurons, giving rise to a functional heterogeneity (Moosmang et al., 1999, 2001; Santoro et al., 2000). In hippocampus, HCN1 and HCN2 are predom-

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inantly expressed, whereas HCN3 and HCN4 are present at very low levels, if present at all (Moosmang et al., 1999; Monteggia et al., 2000; Santoro et al., 2000; Bender et al., 2001). In this study, we examined the effect of Ba^{2+} on I_h currents in rat hippocampal CA1 pyramidal neurons, and tested whether the block by Ba^{2+} is subunit-specific by expressing HCN subunits in Human Embryonic Kidney (HEK) 293 cells.

2. Materials and methods

2.1. Slice preparation

Hippocampal slices were prepared as described previously (van Welie et al., 2004). Briefly, male Wistar rats (14–28 days old) were decapitated and parasagittal slices (250 μm) of the hippocampus were cut on a vibroslicer (725M, Campden Instruments, Loughborough, UK). Slices were allowed to recover for 1 h at 31 °C in artificial cerebrospinal fluid containing (in mM): 120 NaCl, 3.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, and 25 glucose, continuously bubbled with 95% O_2 and 5% CO_2 (pH 7.4). Slices were kept at room temperature until use.

2.2. Cell culture

Human Embryonic Kidney 293 (HEK 293) cells were maintained in minimum essential medium (MEM) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were plated on 12 mm cover slips, and were transiently transfected 2 days later with expression vectors for mHCN1, mHCN2, or hHCN2 using the calcium phosphate precipitation method. Results obtained with mHCN2 and hHCN2 did not differ significantly (data not shown). To detect transfected cells, a vector encoding Enhanced Green Fluorescent Protein was cotransfected at a ratio of 1:5. Medium was refreshed every 2–3 days, and cells were used for experiments 1–3 days after transfection.

2.3. Electrophysiology

Whole-cell voltage clamp recordings were made from CA1 pyramidal neurons in hippocampal slices, which were visualized using infrared differential interference contrast microscopy on a Zeiss FS2 microscope with a

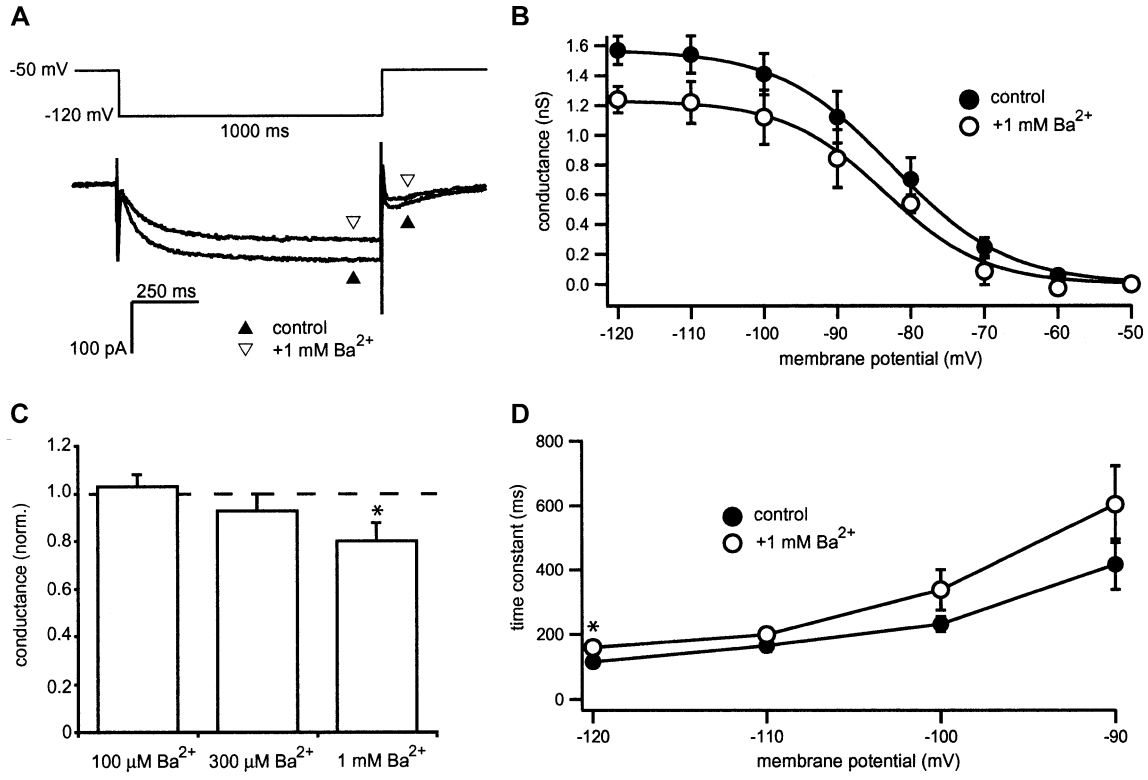

Fig. 1. Ba^{2+} ions reduce the maximal conductance and reduce the rate of activation of I_h currents in hippocampal CA1 pyramidal neurons. (A) I_h currents recorded from a whole-cell voltage clamped pyramidal neuron in the absence and presence of 1 mM Ba^{2+}. (B) Conductance of I_h versus membrane potential in the presence and absence of 1 mM Ba^{2+}. The maximal conductance in the presence of 1 mM Ba^{2+} is decreased, but the V_h and V_c are unchanged. Solid curves represent the fit to the Boltzmann equation (see Materials and methods). Data points represent the mean±S.E.M of 6–10 cells. (C) Concentration-dependence of the block of maximal conductance of I_h by Ba^{2+}. Bars represent the mean±S.E.M. of 6 cells. Asterisk indicates $P<0.05$. (D) Time constant of activation of the I_h currents is increased by Ba^{2+}. Asterisk indicates significant difference from control. Data points represent the mean±S.E.M. of 4–6 cells. Absence of error bars indicates that the S.E.M. is smaller than the symbol size.

VX44 CCD camera (PCO, Kelheim, Germany). Since a large part of I_h in hippocampal CA1 pyramidal neurons is located in the distal dendrites, a cut was placed in stratum radiatum 80–120 μm parallel to the CA1 pyramidal cell layer, under visual guidance using a dissecting microscope in order to isolate the somatic compartment and minimize space clamp errors (van Welie et al., 2004). Patch pipettes were pulled from borosilicate glass and had a resistance of 2–4 MΩ when filled with (in mM): 140 K-gluconate; 10 HEPES; 5 EGTA; 0.5 $CaCl_2$; 2 Mg-ATP; 10 sucrose (pH 7.4 with KOH). Currents were activated by hyperpolarizing voltage steps (1000 ms) from a holding potential of −50 mV.

Whole-cell voltage clamp recordings from HEK 293 cells expressing either HCN1 or HCN2 were made using an Olympus IX70 inverted microscope with Hoffmann modulation contrast. Transfected cells were detected by the expression of Enhanced Green Fluorescent Protein using standard epifluorescence. Cells were continuously superfused with external solution containing (in mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (pH 7.3 with NaOH). Borosilicate glass pipettes had a resistance of 2–4 MΩ when filled with (in mM): 105 K-gluconate, 30 KCl, 0.5 $CaCl_2$, 5 EGTA, 10 HEPES, and 2 Mg-ATP (pH 7.3 with KOH). Currents were activated by hyperpolarizing voltage steps (1000 ms for HCN1 and 2500 ms for HCN2) from a holding potential of −50 mV.

In all experiments, series resistance was compensated for at least 80%. Current signals in voltage clamp were filtered at 333 Hz and sampled at 1 kHz using an EPC9 amplifier and Pulse software (HEKA Electronik, Lambrecht, Germany). $BaCl_2$ was applied via bath perfusion. Experiments were performed at room temperature.

2.4. Data analysis

Off-line linear leak correction of the currents recorded from HEK 293 cells was performed using the estimated impedance from a 10 mV prepulse recorded with each current trace. Leak correction of the currents recorded from hippocampal slices was performed off-line using a custom-made procedure in Igor Pro (Wavemetrics, Lake Oswego, USA).

Conductance was calculated assuming

$$g(V) = I_h(V)/(V - V_{rev})$$

where V_{rev} is the reversal potential of the I_h current. Parameter estimates of the voltage-dependent activation were obtained by fitting the voltage-dependence of the conductance to the Boltzmann equation:

$$g(V) = g_{max}/(1 + \exp(V_h - V)/V_c)$$

where g_{max} is the maximal conductance, V_h is the voltage for half-maximal activation and V_c is proportional to the slope of the curve.

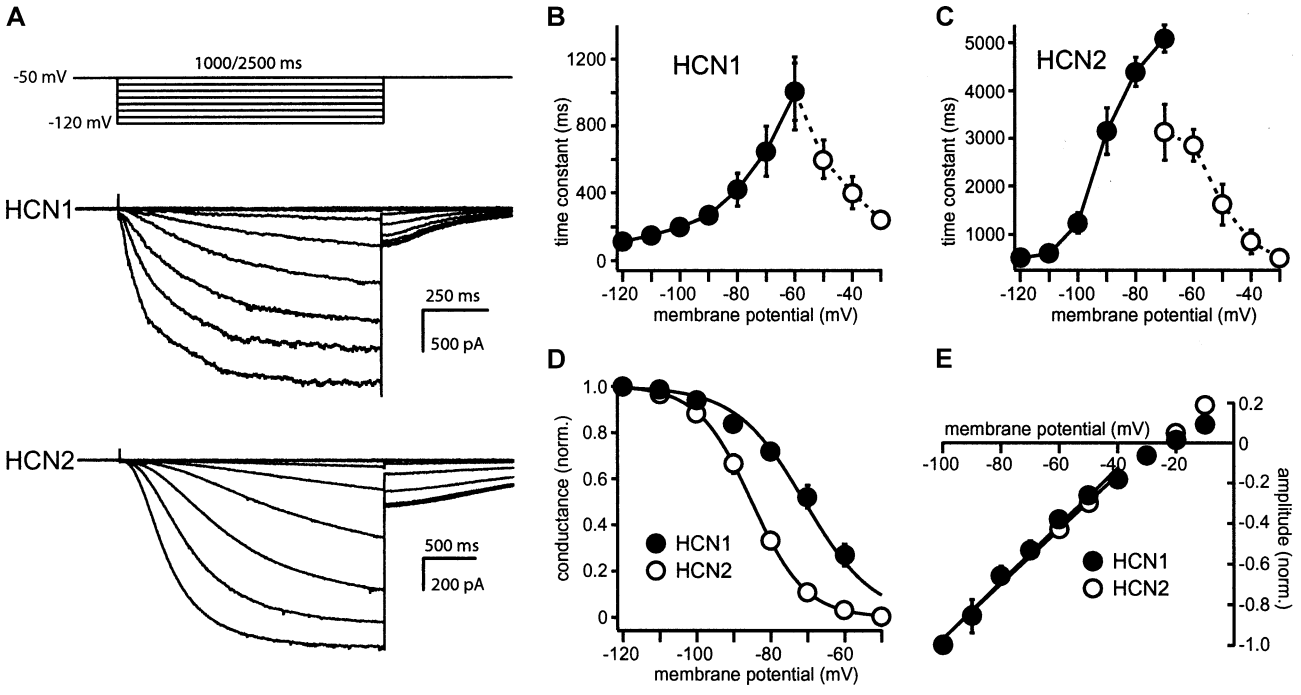

Fig. 2. Properties of HCN1 and HCN2 channels expressed in HEK 293 cells. (A) Family of current traces recorded in whole-cell voltage clamped HEK 293 cells transiently expressing HCN1 or HCN2. Note that the HCN2 currents are much slower than the HCN1 currents. This is more clearly demonstrated and quantified in panels (B) and (C), which show the voltage-dependence of the time constant for activation (●) and deactivation (○) for HCN1 and HCN2, respectively. Data points represent the mean±S.E.M. of 4–10 cells. (D) Normalized conductance of HCN1- and HCN2-mediated ion currents versus membrane potential. Solid curves represent the fit to the Boltzmann equation (see Materials and methods). Points represent the mean±S.E.M. of 8–9 cells. (E) *I–V* curves of the HCN1- and HCN2-mediated ion currents. Solid lines are linear fits from −100 to −40 mV, and the reversal potential was obtained by extrapolation. Points represent mean±S.E.M. of 8–9 cells. Absence of error bars indicates that the S.E.M. is smaller than the symbol size.

Parameter estimates of the concentration-dependence of the block of maximal conductance by Ba^{2+} were obtained by fitting the Hill equation:

$$g(Ba^{2+}) = g_{max} \Big/ \left(1 + \left(IC_{50} / [Ba^{2+}]\right)^{nH}\right)$$

where g_{max} is the maximal conductance, IC_{50} is the concentration of Ba^{2+} producing half-maximal block and n_H is the Hill coefficient.

The rate of activation was obtained by fitting a single exponential function to the rising phase of the ion current. The rate of deactivation was obtained by fitting a single exponential function to the deactivation of the ion current upon repolarization from a near-maximal activated current at −100 mV. Reversal potentials were obtained by linear extrapolation of the amplitudes recorded from a series of step depolarizations from a near-maximal activated current at −100 mV. Data analysis was performed using Igor Pro (Wavemetrics, Oregon, USA). All results are expressed as mean±S.E.M. of n independent experiments. Comparisons were made using Student's t-test unless indicated otherwise. $P<0.05$ was used to indicate a significant difference.

3. Results

3.1. Ba^{2+} reduces I_h conductance in hippocampal CA1 pyramidal neurons

Hyperpolarization-activated I_h currents were recorded from whole-cell voltage clamped hippocampal CA1 pyramidal neurons by step hyperpolarizations from a holding potential of −50 mV. The inward currents activated slowly, did not inactivate over a period of 1000 ms (Fig. 1A), and were blocked by 50 μM of the selective I_h antagonist ZD7288 (not shown; van Welie et al., 2004). The Boltzmann fit of the voltage-dependent I_h conductance yielded a potential of half-maximal activation (V_h) of −82.5±1.0 mV (n=10) and a slope factor (V_c) of 7.8±0.4 mV (n=10). The maximal conductance amounted to 1.57±0.09 nS (n=10). Bath application of Ba^{2+} reduced the maximal conductance in a concentration-dependent manner (Fig. 1B, C). At 1 mM the maximal conductance was reduced to 1.23±0.04 nS (n=10), which is 80±7% of control ($P<0.05$; Fig. 1C). The V_h and V_c in the presence of Ba^{2+} (−83.8±1.2 mV and 6.8±0.9 mV, respectively; n=10) were not significantly different from those in the absence of Ba^{2+}. Apart from the reduction of maximal conductance, the application of Ba^{2+} also resulted in a slower activation of the ion current (Fig. 1A, D). At −120 mV, the time constant of activation of the ion current was increased from 116±9 ms (n=10) to 160±19 ms (n=10) in the presence of 1 mM Ba^{2+} ($P<0.05$, Fig. 1D).

3.2. Ba^{2+} reduces HCN1 and HCN2 expressed in HEK 293 cells

Since hippocampal CA1 pyramidal neurons express both HCN1 and HCN2 subunits (Santoro et al., 2000), the effects of Ba^{2+} were examined on HCN1- and HCN2-mediated I_h currents in HEK 293 cells. Hyperpolarization of HEK 293 cells transiently expressing either HCN1 or HCN2 resulted in slowly activating, non-inactivating inward currents (Fig. 2A).

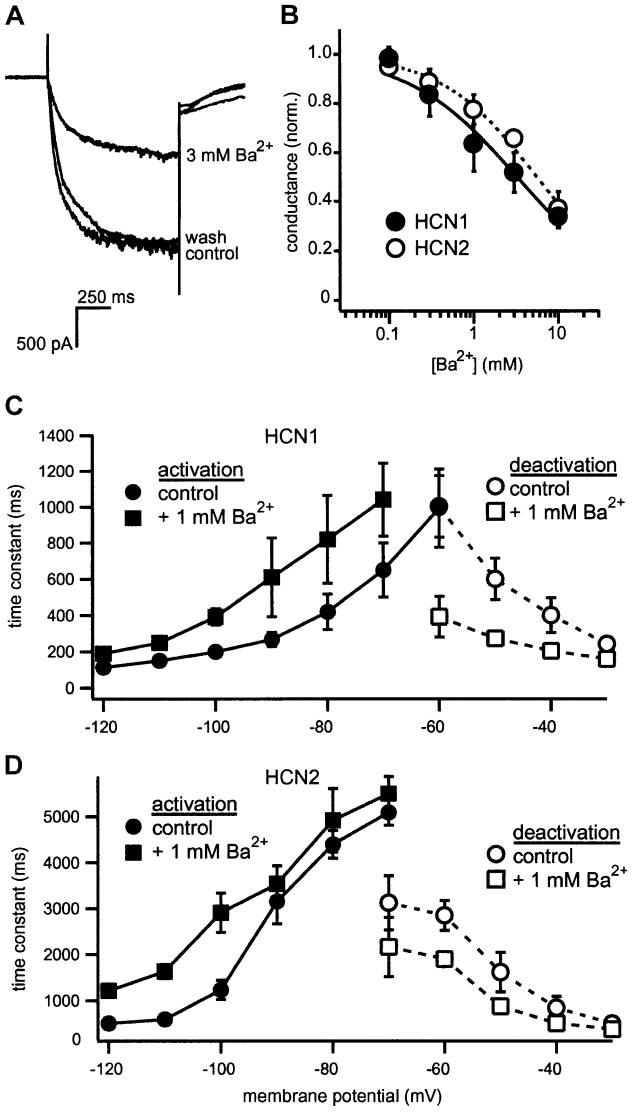

Fig. 3. Ba^{2+} reduces the maximal conductance of HCN1 and HCN2. (A) Currents recorded from an HEK 293 cell transiently expressing HCN1 in the presence and absence of 3 mM Ba^{2+}. (B) Concentration-dependence of the block of maximal conductance of HCN1- and HCN2-mediated ion currents by Ba^{2+}. Solid and dashed curves represent the fit to the Hill equation (see Material and methods). Data points represent mean±S.E.M. of 3–5 cells. (C) Ba^{2+} increases the time constant of activation, and decreases the time constant of deactivation of HCN1-mediated ion currents. Solid and open circles represent the time constant of activation and deactivation in the absence of Ba^{2+}, and solid and open squares represent those in the presence of 1 mM Ba^{2+}. Data points represent mean±S.E.M. of 4–10 cells. (D) Same as in panel (C), but for HCN2-mediated ion currents. Absence of error bars indicates that the S.E.M. is smaller than the symbol size.

The rate of both activation and deactivation of HCN1 was 2–10 fold faster, depending on the membrane potential, than those of HCN2 (Fig. 2B, C). The Boltzmann fit of the voltage-dependent conductance yielded a V_h and V_c of −73.4±2.4 mV and 9.5±0.8 mV (n=8) for HCN1, and −85.2±1.2 mV and 7.1±0.6 mV (n=9) for HCN2 (Fig. 2D). The reversal potentials of HCN1 (−27.9±1.0 mV, n=8) and HCN2 (−28.6±1.1 mV, n=9) were not different (Fig. 2E).

Application of Ba^{2+} resulted in a rapid, reversible, and concentration-dependent reduction of the HCN1- and HCN2-mediated ion current (Fig. 3A, B). The IC_{50} of Ba^{2+} for the block of the maximal HCN1 conductance amounted to 3.2±0.6 mM (n=4 for all data points) and the Hill coefficient amounted to 0.68±0.10 (n=4). For HCN2, the IC_{50} and Hill coefficient amounted to 5.5±0.6 mM and 0.77±0.07, respectively (n=4). In the presence of 1 mM Ba^{2+}, the maximal conductance of HCN1 was 63±11% of control, and that of HCN2 was 78±5% of control (Fig. 3B), without change in V_h and V_c. The reversal potential of HCN1- and HCN2-mediated ion currents were not affected by Ba^{2+} (−27.4±2.4 mV and −28.2±0.9 mV for HCN1 and HCN2, respectively, in the presence of 1 mM Ba^{2+}).

Parallel to the effects of Ba^{2+} on the I_h current in hippocampal CA1 pyramidal neurons, Ba^{2+} also affected the time constant of activation of HCN1- and HCN2-mediated currents (Fig. 3C, D). At −120 mV, the time constant of activation of the HCN1-mediated ion current increased from 113±21 ms to 190±19 ms (P<0.05; n=10) in the presence of 1 mM Ba^{2+} (Fig. 1D). The time constant of activation of the HCN2-mediated ion current at −120 mV increased from 509±52 ms to 1220±17 ms (P<0.05; n=4) in the presence of 1 mM Ba^{2+}. In addition, Ba^{2+} caused an increase in the rate of deactivation (Fig. 3C, D). The time constant of deactivation upon repolarization of the near-maximal activated HCN1-mediated current at −100 mV to −30 mV decreased from 241±19 ms to 159±21 ms (P<0.05; n=4, Fig. 3C) in the presence of 1 mM Ba^{2+}. Similarly, the HCN2-mediated current deactivated more rapidly in the presence of 1 mM Ba^{2+}, as indicated by the decrease of the time constant of deactivation from 508±27 ms to 387±25 ms (P<0.05; n=4, Fig. 3D).

4. Discussion

The combined results show that Ba^{2+} reduces the conductance of both I_h channels native to hippocampal CA1 pyramidal neurons and cloned I_h channels transiently expressed in HEK 293 cells, and in addition Ba^{2+} decreases the rate of activation and increases the rate of deactivation of the ion current. The estimated IC_{50} of Ba^{2+} for blocking I_h is in the low millimolar range, which is similar to the concentrations normally used to block I_{Kir}. It should therefore be taken into account that Ba^{2+} may mask a fraction of I_h channels when using Ba^{2+} in experiments to investigate the physiological role of I_h and/or I_{Kir}.

The IC_{50} of Ba^{2+} for block of HCN1 and HCN2 was estimated from the fit of the concentration–effect curves and was slightly different between HCN1 and HCN2 (3.2 mM and 5.5 mM, respectively; Fig. 3B). Although this suggests that the reduction of I_h by Ba^{2+} does depend on the specific subunit composition of I_h, the difference in IC_{50} is too small for using Ba^{2+} as a probe for subunit composition of I_h in native tissue. We were unable to obtain full concentration–effect curves of Ba^{2+} in hippocampal slices because of the poor solubility of Ba^{2+} in artificial cerebrospinal fluid. Nevertheless, the fraction of the amplitude remaining in the presence of 1 mM Ba^{2+} in hippocampal CA1 pyramidal neurons (80±7%) is in the same order of magnitude as in HEK 293 cells expressing either HCN1 or HCN2 (63±11% and 78±5%, respectively). This supports the notion that HCN1 and HCN2 subunits are the main molecular components of I_h channels in hippocampal CA1 pyramidal neurons.

Using a concatenated construct encoding HCN1 connected to HCN2, it has been shown that the currents through heteromeric HCN1/HCN2 channels activated with kinetics similar to homomeric HCN1 channels, but that the V_h of heteromeric channels is closer to that of homomeric HCN2 channels (Ulens and Tytgat, 2001). Based on a comparison of these kinetic parameters with those obtained from I_h in native cells, it was suggested that the I_h in hippocampal CA1 pyramidal cells consists of heteromeric assembly of HCN1 and HCN2 (Ulens and Tytgat, 2001). Our results are in close agreement with these suggestions: the time constant of activation of I_h in pyramidal cells (116 ms) is similar to that of homomeric HCN1 (113 ms), whereas the V_h of I_h in pyramidal cells (−82.5 mV) is similar to that of homomeric HCN2 (−85.2 mV). We did not examine the effects of Ba^{2+} on heteromeric assemblies of HCN subunits because the simultaneous expression of individual subunits without the help of tandem constructs will most likely lead to a heterogeneous population of channels. Nevertheless, the similar efficacies of Ba^{2+} on both native and cloned I_h channels strongly suggest that Ba^{2+} exerts its action independent of subunit composition and assembly. From a study of the biophysical properties of I_h from rod photoreceptors from tiger salamanders, it was concluded that Ba^{2+} acts inside the permeation pathway of the I_h channel, perhaps at one of the ion binding sites at a relative external location (Wollmuth, 1995). Given the homology of the ion permeation pathway between the HCN subunits (Santoro et al., 1998) and the similar effects of Ba^{2+} on native and cloned I_h channels, it is to be expected that the binding site of Ba^{2+} is a conserved feature among HCN subunits, and perhaps indeed a general binding site for ions.

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