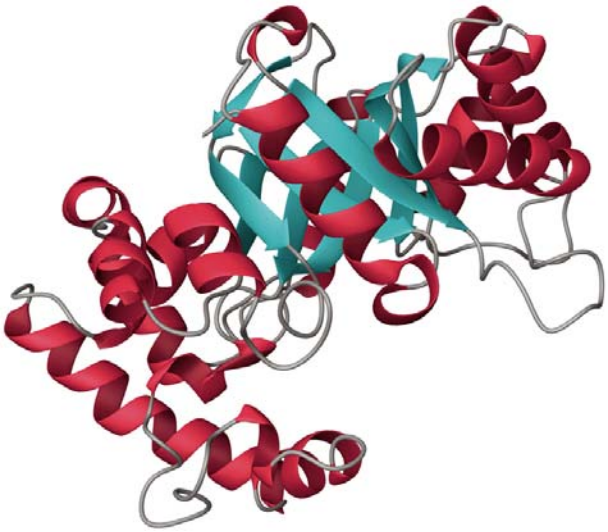
MILLIPORE

hHCN1-HEK293 Recombinant Cell Line

cat. #CYL3040

Revision 1



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Contents:

Licensing Statement

The CMV promoter is covered under U.S. Patents 5,168,062 and 5,385,839 and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center, Iowa City, IA 52242, USA.

Use of IRES is covered by U.S. Patent 4,937,190 and is limited to use solely for research purposes. Any other use of IRES requires a license from Wisconsin Alumni Research Fund (WARF)

The bovine growth hormone (bgh) polyadenylation signal is patented under U.S. Patent No. 5,122,458, European Patent No. 0 173 552, and Japanese Patent No. 6-83669. Use of the bgh polyadenylation signal found in screening systems sold by Upstate requires a license from Research Corporation Technologies, Inc. (RCT). After purchasing these materials from Upstate, you must contact RCT within 30 days to obtain a commercial license. The bgh polyadenylation signal cannot be used until a commercial license is obtained. Contact Jennifer Caldwell, Ph.D., at Research Corporation Technologies, Inc., 101 North Wilmot Road, Suite 600, Tucson, AZ 85711-3335, USA Tel: 1-520-748-4400, Fax: 1-520-748-0025.

Product description:
Recombinant HEK293 cell line expressing the human hyperpolarization-activated, cyclic nucleotide-gated potassium channel 1 (HCN1).

Format:
2 x 1 ml aliquots containing 1.20 x 10^6 cells/ml in 10% DMSO at passage 15.

Mycoplasma Testing:
The cell line has been screened using the MycoSensor™ PCR Assay Kit (Stratagene) to confirm the absence of Mycoplasma species.

Functional Validation:
HEK293 cells expressing hHCN1 were characterised in terms of their biophysical and pharmacological properties using whole-cell patch clamp techniques and IonWorks™ HT electrophysiology.

The biophysical properties are typical of hHCN1 both in terms of $V_{½}$ of activation (-86 ± 3 mV, n=11) and the time course of activation (e.g. 349 ± 130 ms at -110 mV and 104 ± 32 ms at -140 mV, n=6). Under whole-cell recording conditions the mean current amplitude was 2.7 ± 0.5 nA at -130 mV (n =10) and reversal potential measurements indicated permeability to both Na^+ and K^+ (P_{Na}/P_K around 0.2) typical of HCN channels.

Currents were dose-dependently inhibited by the selective HCN blocker ZD7288 (IC_{50} value of 44 µM) and the inorganic cation Caesium (Cs^+, IC_{50} value of 114 µM) in line with reported literature values. The currents were also insensitive (<10% inhibition) to the tyrosine kinase inhibitor genistein (100 µM) although the same concentration markedly inhibited hHCN2 currents (approximately 66% inhibition, Millipore PrecisION™ HCN2-HEK293 recombinant cell line, CYL3041). These differences in sensitivity have been noted by others and support the notion that this cell line selectively expresses hHCN1 channels.

Channel expression, monitored using IonWorks™ HT, is robust over at least 40 passages. For example 76% of cells expressed outward current >500 pA at passage 40 (n=123) with a mean current amplitude of 1.41 nA.

IonWorks™ HT is a trademark of Molecular Devices Corporation

Introduction.

Four different hyperpolarization-activated, cyclic nucleotide-modulated (HCN) channel isoforms have been cloned (HCN1-4) that can be distinguished by differing sensitivity to modulators such as cAMP, different $V_{½}$ values of activation and different activation kinetics. Even though they are structurally similar to Kv channels they are activated by strong hyperpolarizing voltages rather than depolarizing voltages. As such the channels are active at or around the resting potential and contribute to pacemaker activity both in the heart and CNS. There is considerable overlap in the expression of these isoforms in different tissues (Moosmang *et al.*, 2001, Stieber *et al.*, 2005) and it is highly likely that they can form heteromultimers (Altomare *et al.*, 2003, Chen, Wang and Siegelbaum 2001, Brewster *et al.*, 2005)

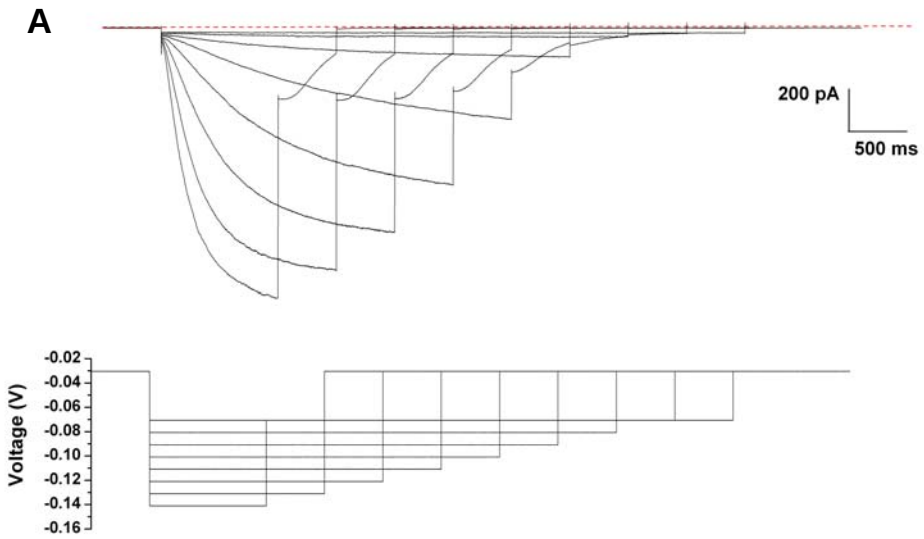
Altered HCN channel expression has been implicated in increased neuronal hyperexcitability following seizures (Brewster *et al.*, 2005) and reduced expression may in fact *trigger* seizures as demonstrated for example in a rat epilepsy absence model (Kole *et al.*, 2007). Hence developing drugs to target these channels may be useful in the treatment of some forms of epilepsy. HCN1 channels also appear to have a role in some models of neuropathic pain where the spontaneous firing of damaged nerves and therefore the sensation of pain can be alleviated by the selective HCN blocker ZD7288 (Chaplan *et al.*, 2003).

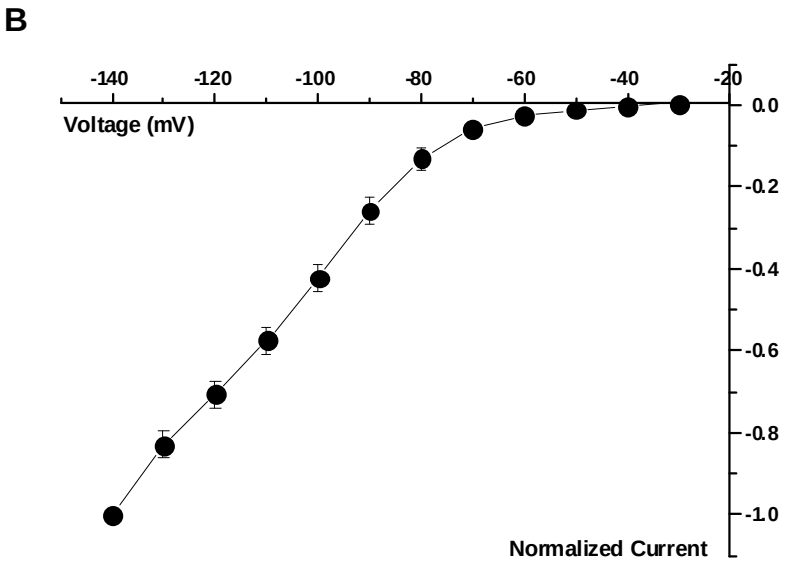
Electrophysiological Properties of the hHCN1 Current.

Conventional Whole-Cell Patch Clamp Electrophysiology.

Current/Voltage Relationship:
The basic electrophysiological properties of hHCN1 were examined using the voltage protocol shown in **Figure 1A** (lower panel). Hyperpolarizing steps to various voltages were applied from a holding potential of -30 mV (1 every 20 s) of varying durations, sufficient to approach the steady state current level at each voltage. The voltage was then stepped back to -70 mV for 500 ms prior to returning to the holding potential. Example current traces are shown in the upper panel. Using this type of protocol it was possible to measure the current/voltage relationship (**Figure 1B**), calculate the $V_{½}$ of activation (**Figure 2**) and measure time constants of activation (**Figure 3**). It can be seen that the channels only slowly begin to open when the voltage is stepped from the holding potential to around -60 mV. Increasing the magnitude of the hyperpolarizing steps from -60 mV to -140 mV in 10 mV increments leads to progressively larger and faster activating currents. The I/V relationship (**Figure 1B**) was linear with no evidence of inactivation during the hyperpolarizing pulse. These features are typical for HCN1 currents (see for example Chen, Wang and Siegelbaum, 2001). The mean current amplitude at -130 mV was 2.7 ± 0.5 nA (n =10) and thus the channel is functionally expressed at an appropriate level for manual and automated patch clamp applications.

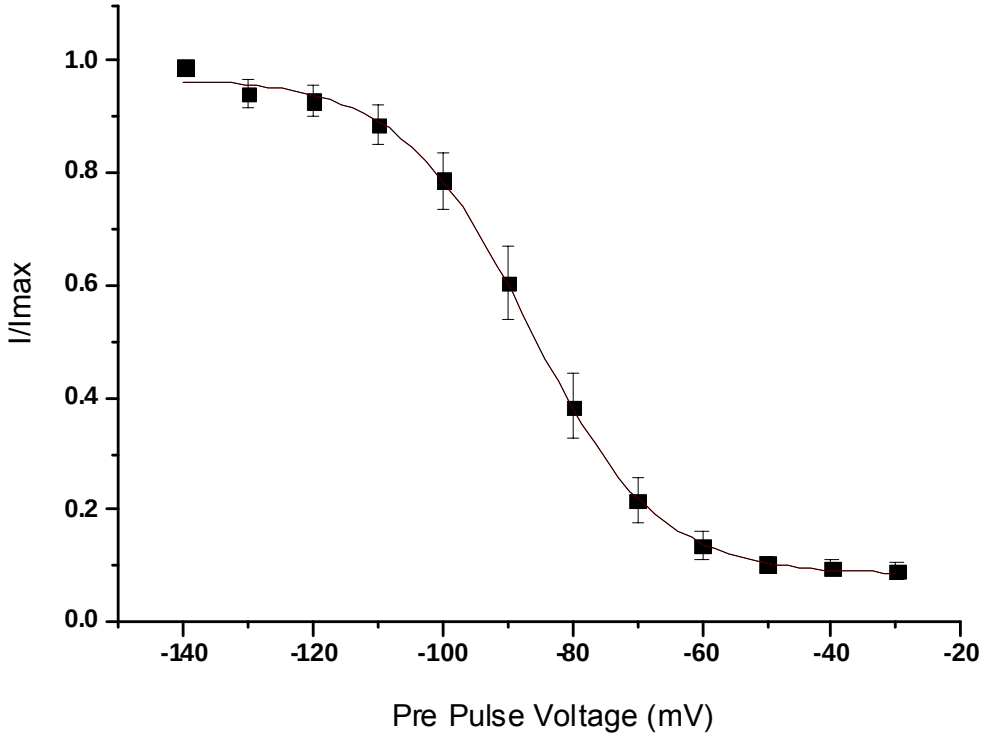
Figure 1. I/V relationship. The upper panel in **A** represents typical current traces obtained by applying the voltage protocol in the lower panel (see text for details). Red dotted line indicates zero current level. The maximum current amplitude was measured on stepping to the various hyperpolarizing potentials and normalized to the maximum current amplitude obtained at -140 mV. Mean data from 10 cells is plotted in **B**.





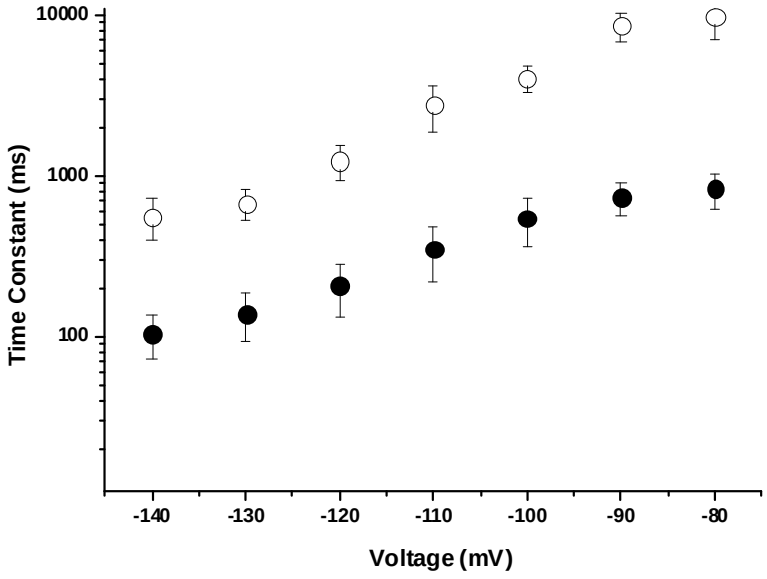
Voltage-Dependence of Channel Activation:
Using the protocol described in **Figure 1A** it was possible to obtain activation curves for 11 individual cells by measuring the instantaneous current on stepping back to -70 mV after the test potential. The mean data is shown in **Figure 2** and could be described by a Boltzmann equation giving an estimated $V_{½}$ of -86 ± 3mV and a slope of 7.8 ± 0.6 mV (n = 11). In the majority of studies comparing the voltage dependence of activation of the two channels, HCN1 typically begins to activate at voltages approximately 20 mV more depolarized than HCN2 and consequently has a more positive $V_{½}$ of activation (Wainger *et al.*, 2001, Chen *et al.*, 2005, Ulens and Tytgat 2001). Consistent with this, the value reported here is approx 17 mV more positive than the data obtained for Millipore's PrecisION™ hHCN2-HEK293 recombinant cell line (catalogue number CYL3041) recorded under identical conditions ($V_{½}$ of -102.6 ± 1.3, slope 8.4 ± 0.4, n = 7, see datasheet). In addition, the absolute value of $V_{½}$ falls within the range of reported values using similar methods and recording conditions; around -80 mV (Chen *et al.*, 2005, Yu *et al.*, 2004) to just over -90 mV (-94 mV, Moosmang *et al.*, 2001).

Figure 2. Activation Curve. The instantaneous current on stepping back to -70 mV from various test potentials was measured for each cell and normalized to the instantaneous current obtained with a pre pulse to -140 mV. The mean data from 11 cells is shown that could be described by a Boltzmann equation giving an estimated $V_{½}$ of -86.3 ± 3mV and a slope of 7.8 ± 0.6 mV.



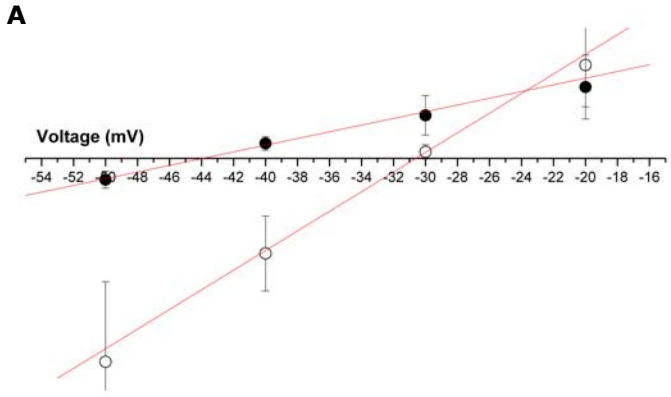
Time Constant of Activation:
The data could typically be described by a single exponential at voltages more depolarized than -110 mV and by 2 exponentials at more hyperpolarized voltages. However, the amplitude of the fast component was significantly greater than the slow component at all voltages in agreement with Chen, Wang and Siegelbaum, 2001 who reported that the fast component accounted for around 80% of the total amplitude at all voltages tested. This is also in accordance with Ishii *et al* 2001 who found that the slow component of activation accounts for <10% of the total amplitude. Hence, it is the fast time constant that is shown in **Figure 3** (solid circles) for comparison with the predominant time constant of activation of hHCN2 (open circles). It is clear that hHCN1 activates 5-10 fold faster than hHCN2, consistent with the difference reported by Wainger *et al.*, 2001 (4-6 fold faster) and Chen *et al.*, 2001 (10-fold faster). The absolute values are also similar to previous reports. For example Altomare *et al.*, 2003 reported a value of around 200 ms at -105 mV, Chen *et al.*, around 300 ms at -105 mV and Moroni *et al.*, 2001 around 322 ms at -95 mV. These values are comparable to the value at -110 mV of 349 ± 130 ms (n=6) obtained in the present study. In conclusion, currents activated with hyperpolarizing steps activate along time courses reported previously for HCN1.

Figure 3. Time Constants of Activation. On stepping to various hyperpolarizing voltages from a holding potential of -30 mV, hHCN currents activated following an exponential time course after an initial delay. hHCN1 time constants obtained by exponential fits decreased with increasing hyperpolarizing steps (solid circles) and at a given voltage were around 4-5x less than hHCN2 time constants (open circles).



Reversal Potential:
HCN channels conduct both K^+ and Na^+ and the reversal potential is around -25 mV under physiological conditions with a permeability ratio P_{Na}/P_K of 0.2-0.3 (see review of Biel *et al.*, 2002). In **Figure 4** the reversal potential was measured either using 137 mM Na^+/4 mM K^+ external and nominally 0 mM Na^+/140 mM K^+ internal (solid circles) or, as in most experiments involved in characterization, 110 mM Na^+/30 mM K^+ external and 10 mM Na^+/140 mM K^+ internal (open circles). Under these conditions the reversal potential was around -44 mV and –31 mV respectively (calculated by measuring the instantaneous current on stepping to various test potentials after a pre pulse to -140 mV to fully activate the channels). These values indicate that the channels are indeed permeable to both cations with a P_{Na}/P_K ratio of roughly between 0.1 and 0.2, calculated using the Goldman-Hodgkin-Katz voltage equation according to Stieber *et al.*, 2005 and with the standard ionic conditions routinely used in the characterization of this cell line.

Figure 4. Reversal Potential Measurements. The reversal potential was calculated either in low K^+ external solutions (solid circles in **A**) or high K^+ external solutions (open circles in **A**) by using the voltage protocols in **B** and **C**. The data is the mean of 3 and 4 cells respectively. Currents were activated by a hyperpolarizing voltage prior to stepping to various voltages negative and positive to the reversal potential i.e. where the instantaneous current crosses the zero current level (red dotted lines in **B** and **C**). In **B**, in low external K^+ conditions, the current clearly reverses near -40 mV whereas in high K^+ external conditions in **C**, the current reverses near -30 mV.



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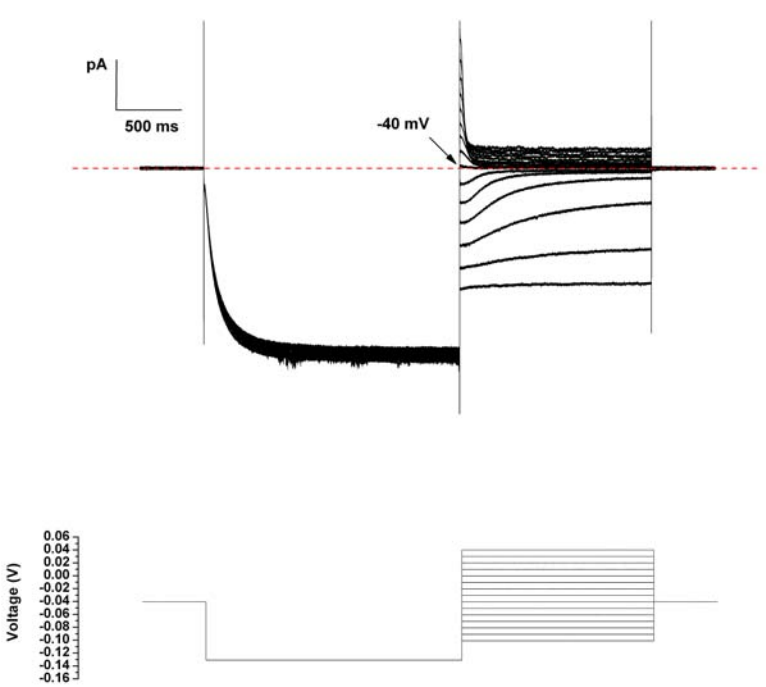

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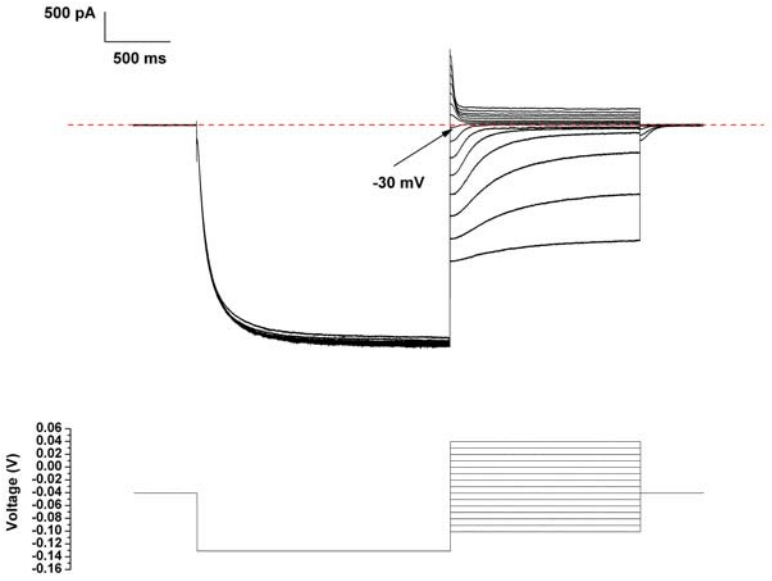
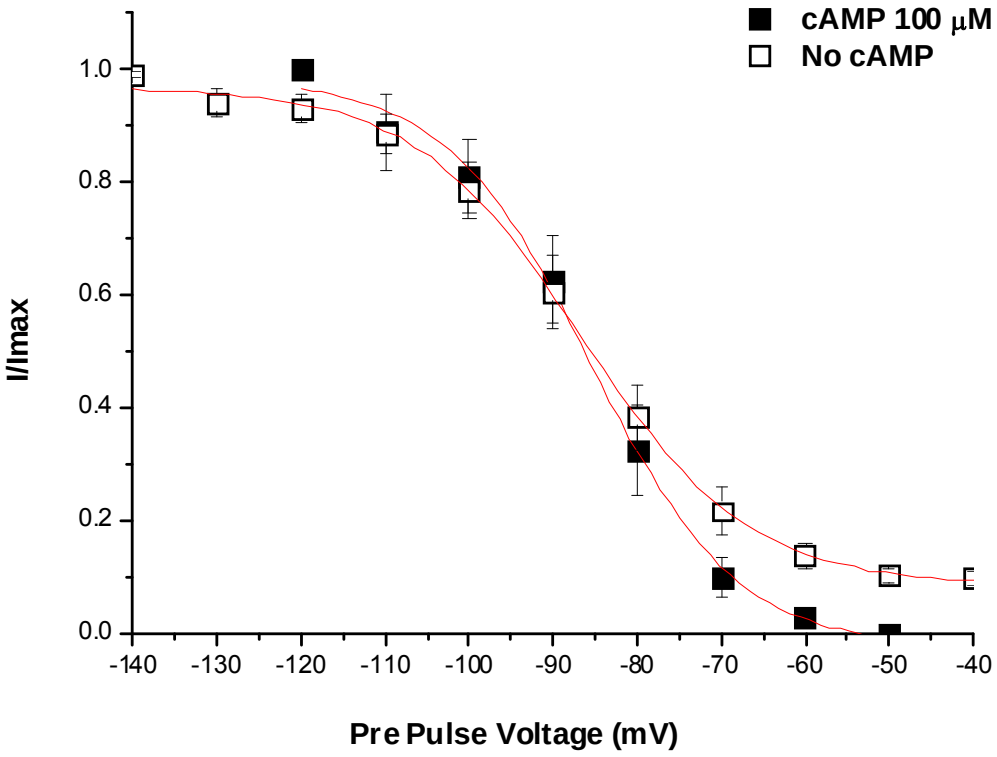


Pharmacology – Adenosine 3',5'-cyclic monophosphate (cAMP):
HCN1 channels are relatively insensitive to cAMP unlike HCN2 (see Millipore datasheet CYL3041-M1) and HCN4 channels (Wang *et al* 2002). For example, in inside out patches cAMP (10 µM) was reported to shift the $V_{½}$ of activation by only around +6 mV (Viscomi *et al.*, 2001), a value similar to the maximal positive shift reported by Chen, Wang & Siegelbaum, 2001 of +4 mV. Adding cAMP (100 µM) in the pipette and comparing with $V_{½}$ activation data under control conditions in separate cells (**Figure 2**), a shift in $V_{½}$ could not be resolved (**Figure 5**). Given the reported small absolute shift in $V_{½}$, the relatively small sample size and the fact that comparisons were made between *separate* cells with either ± cAMP in the pipette, the fact that this shift could not be resolved is perhaps no surprise

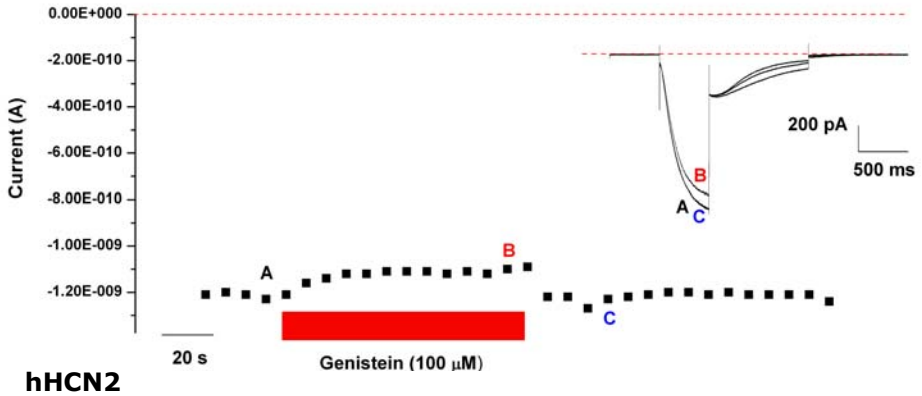
Figure 5. Activation Curve. The instantaneous current on stepping back to either -70 mV (no cAMP, open squares) or -50 mV (cAMP 100 µM, solid squares) from various test potentials was measured for each cell and normalized to the instantaneous current obtained with a pre pulse to -140 mV (no cAMP) or -120 mV (cAMP 100 µM). The control data (no cAMP) is from Figure 2 and the mean data in the presence of cAMP is from 3 cells. The data, in the presence of cAMP, could be described by a Boltzmann equation giving an estimated $V_{½}$ of -84.3 ± 2.2mV and a slope of 7.1 ± 0.12 mV.



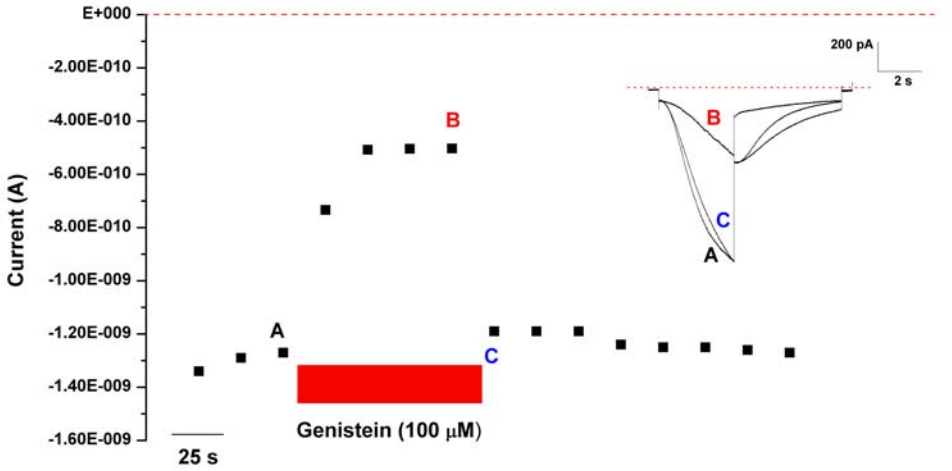
Pharmacology – Genistein:
The tyrosine kinase inhibitor genistein has been reported to inhibit HCN2 currents in *Xenopus* oocytes by reducing the whole cell conductance and causing a hyperpolarizing shift in the $V_{½}$ of activation (Yu *et al.*, 2004). However, the same study reported that HCN1 was unaffected by the same concentration (100 µM) of genistein. Thus genistein appears to be a useful pharmacological tool to distinguish between either type of HCN channel. Data from the hHCN1-HEK293 (CYL3040) and hHCN2-HEK293 (CYL3041) cell lines support this notion since bath application of 100 µM genistein rapidly and reversibly inhibited hHCN2 currents by around 66% (**Figure 6B**) but had a negligible effect on hHCN1 currents inhibiting them by only 9.5 ± 1.7%, n=4 (**Figure 6A**).

Figure 6. Effect of genistein on hHCN1 and hHCN2 currents. The voltage protocol used in the upper panel, to evoke hHCN1 currents, involved stepping from the holding potential of -30 mV to -140 mV then stepping back to -70 mV for 1 s prior to returning to the holding potential. Pulses were applied every 10 s. Peak currents at the end of the -140 mV step were measured before, during and after 100 µM genistein and plotted versus pulse number where the presence of genistein is depicted by the red bar. The inset shows current traces recorded before (**A**) during (**B**) and after (**C**) genistein application. The voltage protocol used to evoke hHCN2 currents in the lower panel was similar although the durations of the pulses were longer and peak current was assessed at -110 mV instead of -140 mV.

hHCN1



hHCN2

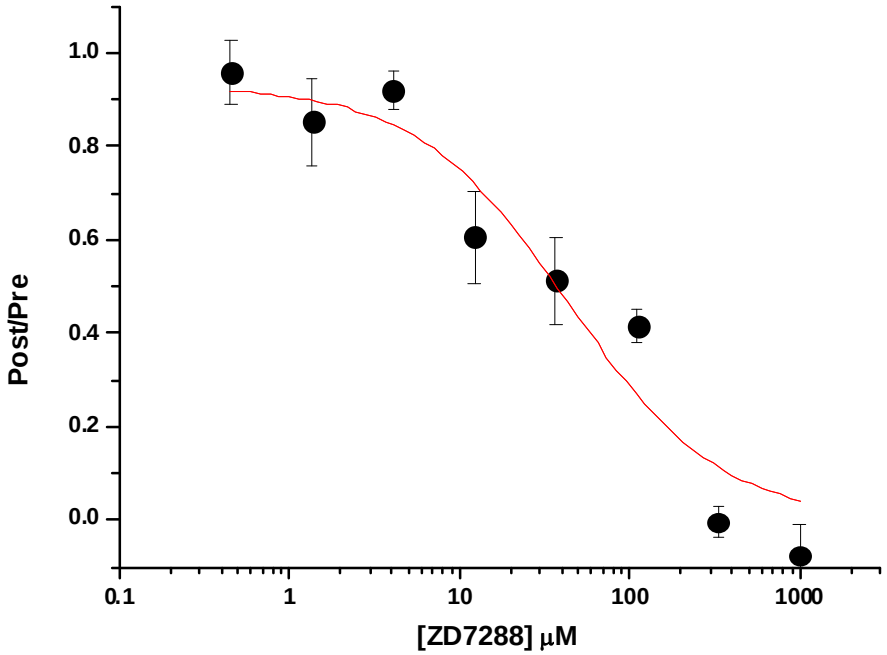


IonWorks™ HT Electrophysiology.

Pharmacology – ZD7288:

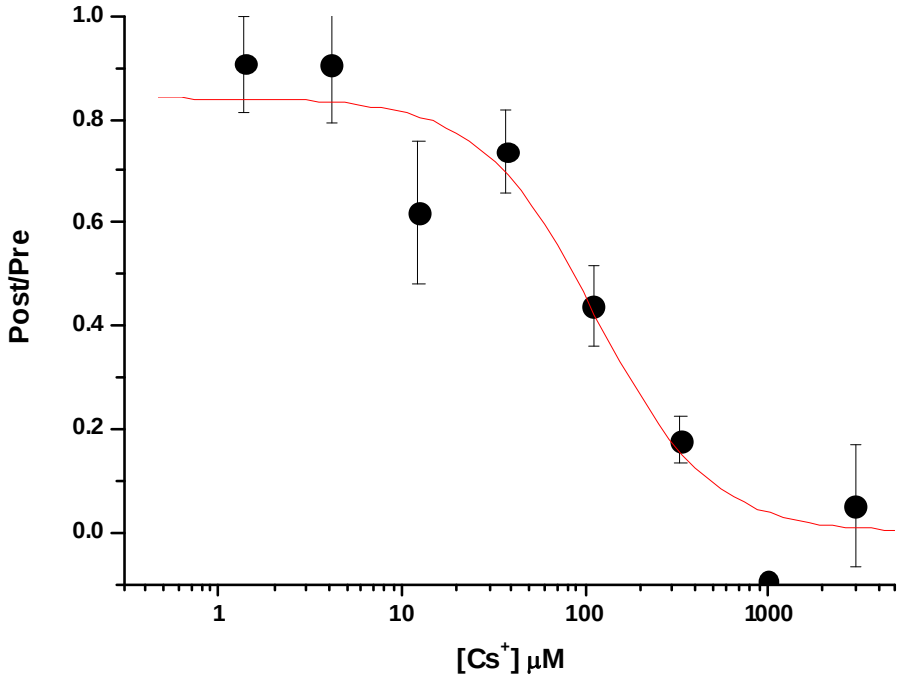
Using IonWorks™ HT the effect of ZD7288 was assessed on the amplitude of hHCN1 currents evoked by a hyperpolarizing voltage step to fully activate the channels. ZD7288 is a relatively specific blocker of HCN channels and the reported IC_{50} value for block of HCN1 channels is around 20 µM (Stieber *et al.*, 2005) that is similar to the IC_{50} value of 44 µM reported here (**Figure 7**).

Figure 7. Effect of ZD7288 on HCN1 currents. The effect of a 10 min incubation of various concentrations of ZD7288 was assessed on the amplitude of hHCN1 currents recorded using IonWorks™ HT automated electrophysiology. Each data point represents the mean of 3-8 cells.



Pharmacology – Caesium (Cs^+):
As well as being a non-specific blocker of K^+ channels, Cs^+ also blocks HCN channels with IC_{50} values of around 200 µM (Stieber *et al.*, 2005). In accordance with this, Cs^+ dose-dependently blocked HCN1 currents with an estimated IC_{50} value of 114 µM (**Figure 8**).

Figure 8. Effect of Cs^+ on hHCN1 currents. The effect of a 10 min incubation of various concentrations of Cs^+ was assessed on the amplitude of hHCN1 currents recorded using IonWorks™ HT automated electrophysiology. Each data point represents the mean of 3-8 cells.

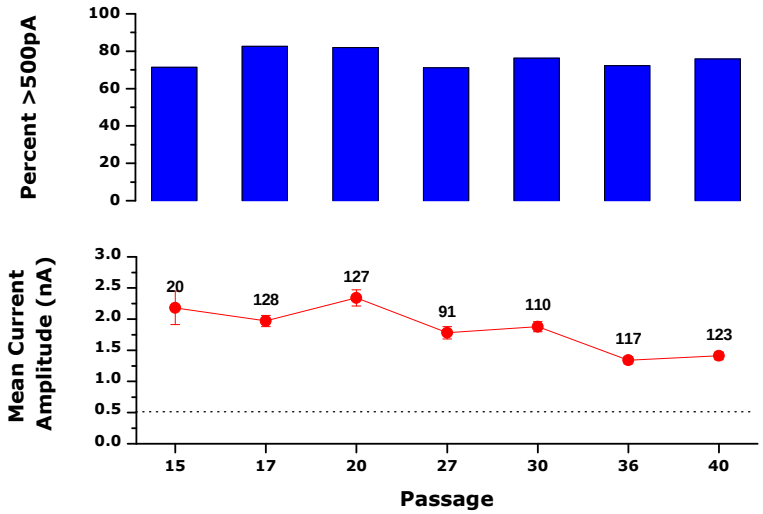


Stability of hHCN1-HEK293 Cell Line.

The hHCN1-HEK293 cell line has stable expression for >40 passages.

Functional channel expression was monitored over 40 passages using IonWorks™ HT and defined as cells producing hHCN1 current of ≥500 pA on stepping from the holding potential of -30 mV to -120 mV. In all cases functional channel expression was >60% and in some cases was >80% (**Figure 9**, upper panel). The mean current amplitude at -120 mV was typically between 1 and 2 nA and therefore ideal for screening assays using automated electrophysiology (**Figure 9**, lower panel).

Figure 9. Stability of expression over passage. The upper panel shows the percentage of cells expressing a mean peak current >500 pA at -120 mV at cell passages 15, 17, 20, 27, 30, 36, and 40. The lower panel shows the mean current amplitude (mean ± SEM, red circles) and the number of these cells (numbers above red circles - out of 32 cells for passage 15 and out of 192 cells for all other passages).



Recommended Culture Conditions:

Cells should be grown in a humidified environment at 37°C under 5% CO_2 using D-MEM/F-12 medium with 1% L-Glutamine, 10% FBS, 1% Non Essential amino acids, plus 400 µg/ml of Geneticin to ensure that the recombinant expression is maintained.

Transfection of HEK293 cells with the hHCN1 ion channel does not appear to have altered the growth characteristics of the host cells which exhibited a typical cell division time of 24 hours.

It is recommended to quickly thaw a frozen aliquot from liquid nitrogen, by agitation in a 37°C water-bath, before transferring into a T75 cm^2 flask containing 20 ml of pre-equilibrated media according to the formulation below. Allow cells to adhere for 4-8 hours at 37°C under 5% CO_2 before gently removing the media and replacing with 20 ml of fresh media.

The cell line should not be allowed to exceed 80% confluency within the culture vessel, to prevent contact inhibition causing senescence and should thus be passaged every 3-4 days using a seeding density of 1-1.5×10^6 cells per T75 cm^2 or 2-3×10^6 cells per T175 cm^2 flask. Pre-washing with phosphate buffered saline before harvesting with Trypsin/EDTA and seeding into new flasks is recommended to passage the cell line. It is essential that the cell line is continually maintained in the presence of Geneticin (400 µg/ml), which should be added to the culture vessel or media immediately prior to use.

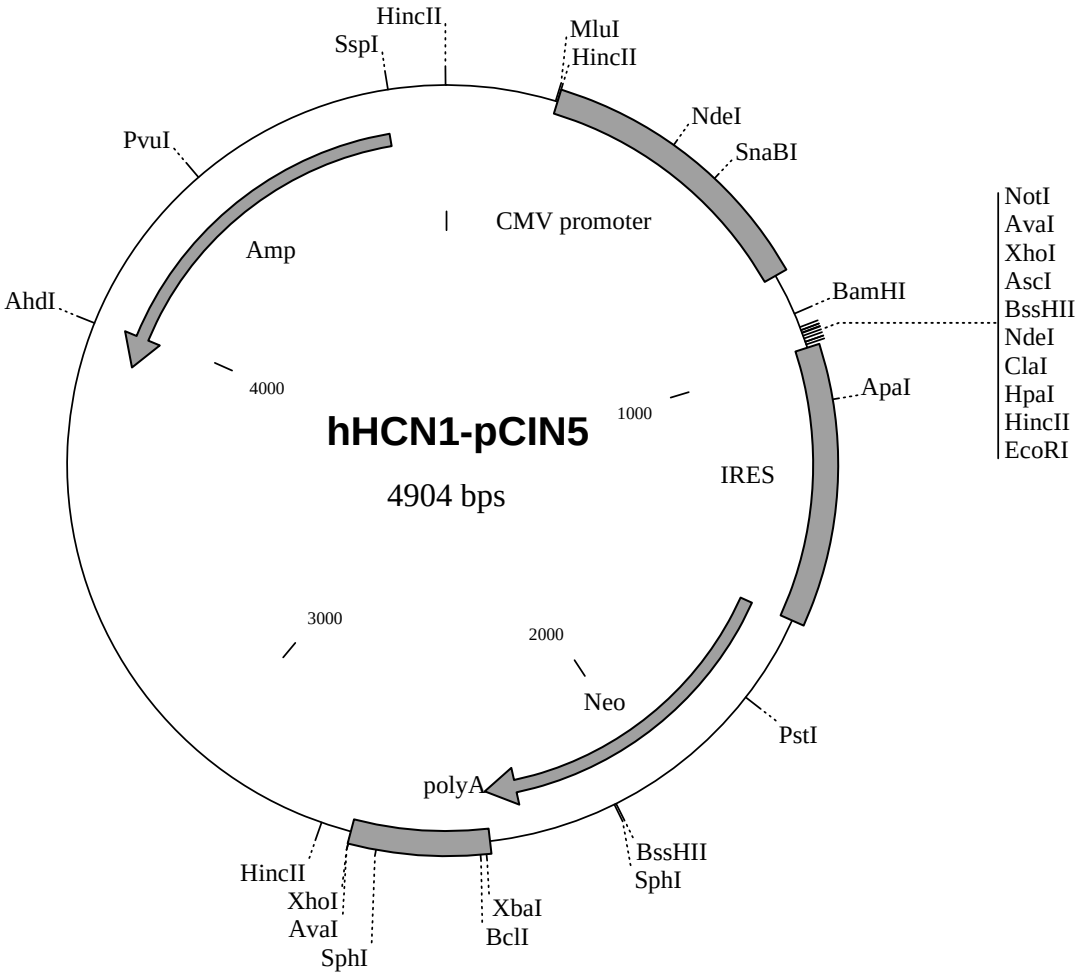
Media Formulation:

D-MEM/F-12 (with L-Glutamine)	(Invitrogen	#11320)
10% Foetal Bovine Serum	(Invitrogen	#16000)
1% Non Essential amino acids	(Invitrogen	#11140)
400 µg/ml Geneticin (G418)	(Invitrogen	#10131)

Other reagents required:

Trypsin/EDTA	(Invitrogen	#25300)
PBS	(Invitrogen	#14190)
Trypan Blue	(Sigma	#T8154)
DMSO	(Sigma	#D2650)

Vector hHCN1-pCIN5:



Polylinker: CMV-NotI-**hHCN1**-AscI-ClaI-HpaI-EcoRI-IRES-*neo*

hHCN1 Sequence (Accession Number NM_021072):

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