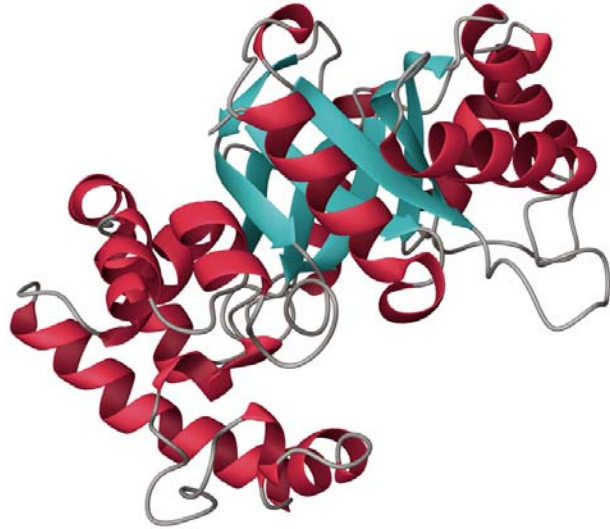


hHCN2-HEK293
Recombinant Cell Line

cat. #CYL3041

Revision 1



Ordering Information and Technical Services:

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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 2 (HCN2).

Format:

2 x 1 ml aliquots containing 1.14×10^6 cells/ml in 10% DMSO at passage 11.

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 hHCN2 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 hHCN2 both in terms of $V_{1/2}$ of activation (-102.6 ± 0.3 mV, $n=7$) and the time course of activation (e.g. 4026 ± 712 ms at -100 mV and 670 ± 140 ms at -130 mV, $n=8$). Under whole-cell recording conditions the mean current amplitude was 5.5 ± 1 nA at -130 mV ($n=7$) and reversal potential measurements indicated permeability to both Na^+ and K^+ ($P_{\text{Na}}/P_{\text{K}}$ around 0.2) typical of HCN channels.

Currents were dose-dependently inhibited by the selective HCN blocker ZD7288 (IC_{50} value of $25 \mu\text{M}$) and the inorganic cation Caesium (Cs^+ , IC_{50} value of $119 \mu\text{M}$) in line with reported literature values. The currents also exhibited a marked sensitivity to the tyrosine kinase inhibitor genistein ($100 \mu\text{M}$) inhibiting the current by approximately 66% in contrast to the effects of the same concentration on hHCN1 currents (<10% inhibition, Millipore PreciION™ HCN1-HEK293 recombinant cell line, CYL3040). These differences in sensitivity have been noted by others and support the notion that this cell line selectively expresses hHCN2 channels. The currents were also sensitive to cAMP ($100 \mu\text{M}$) since intracellular application shifted the $V_{1/2}$ of activation by $+13$ mV again typical of HCN2 channels.

Channel expression, monitored using IonWorks™ HT, is robust over at least 40 passages. For example 68% of cells expressed outward current >500 pA at passage 40 ($n=117$) with a mean current amplitude of 3.12 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 $V_{1/2}$ 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 and may represent a novel drug target for the treatment of seizure-evoked epilepsy (Brewster *et al.*, 2005). HCN 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). In the mammalian heart and specifically relating to HCN2 channels, interference with this type of channel activity can lead to sinus node arrhythmias (Steiber *et al.*, 2004, Ludwig *et al.*, 2003).

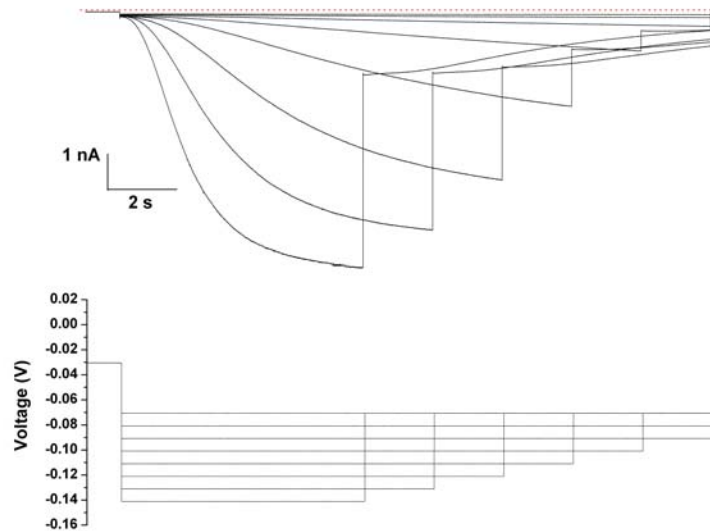
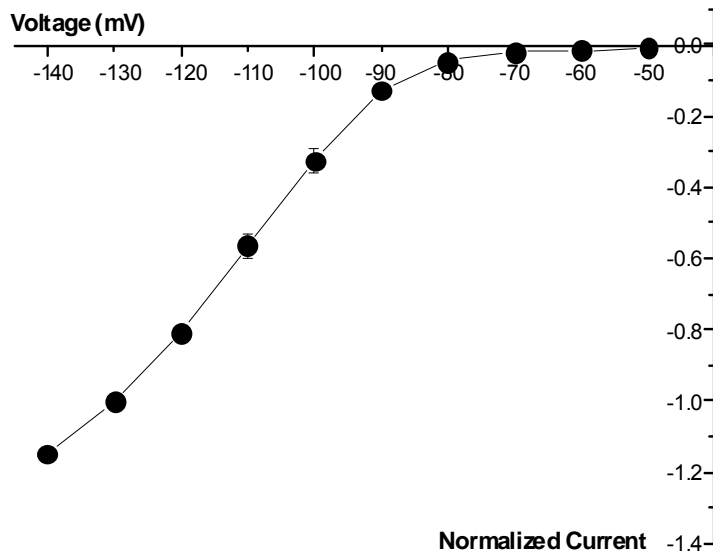
Electrophysiological Properties of the hHCN2 Current.

Conventional Whole-Cell Patch Clamp Electrophysiology.

Current/Voltage Relationship:

The basic electrophysiological properties of hHCN2 were examined using hyperpolarizing steps to various voltages 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 2 s 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 (I/V) relationship (**Figure 1B**), calculate the $V_{1/2}$ 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 -80 mV. Increasing the magnitude of the hyperpolarizing steps from -80 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 HCN2 currents (Chen, Wang and Siegelbaum, 2001). The mean current amplitude at -130 mV was 5.5 ± 1 nA ($n = 7$) and thus the channel is functionally expressed at an appropriate level for manual and automated patch clamp applications.

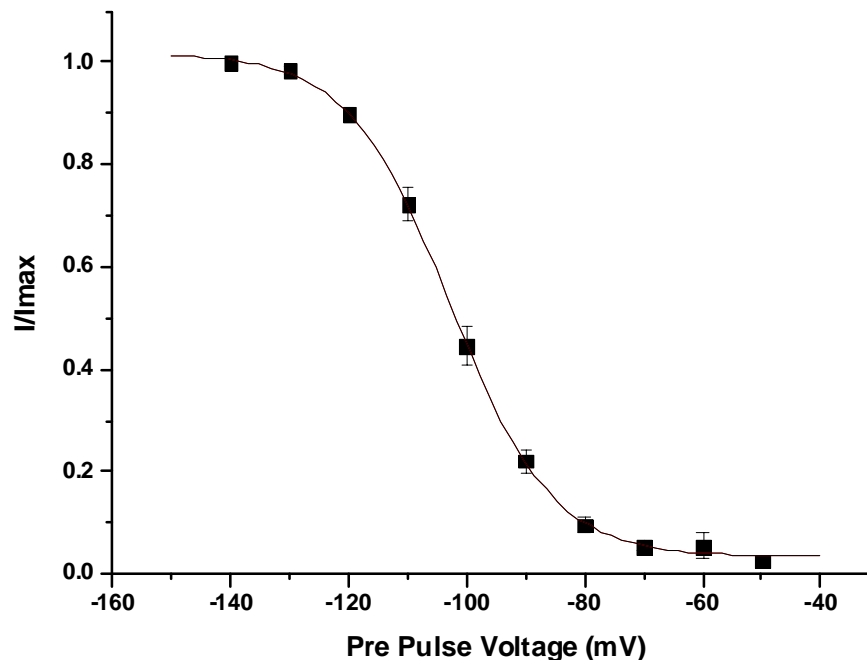
Figure 1. I/V relationship. The upper panel in **A** represents typical current traces obtained by applying the voltage protocol in the lower panel (see text for details). Red dotted line indicates zero current level. The maximum current amplitude was measured on stepping to the various hyperpolarizing potentials and normalized to the maximum current amplitude, obtained at -140 mV. Mean data from 7 cells is plotted in **B**.

A**B**

Voltage-Dependence of Channel Activation:

Using the protocol described in **Figure 1A** it was possible to obtain activation curves for 7 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 $V_{1/2}$ of -102.6 ± 1.3 mV and a slope of 8.4 ± 0.4 mV ($n = 7$). In the majority of studies comparing the voltage dependence of activation of the two channels, HCN2 channels typically begin to activate at voltages approximately 20 mV more hyperpolarized than HCN1 channels and consequently have a more negative $V_{1/2}$ 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 negative than the data obtained for Millipore's PrecisiON™ hHCN1-HEK293 recombinant cell line (catalogue number CYL3040) recorded under identical conditions (86 ± 3 mV and a slope of 7.8 ± 0.6 mV, $n=10$, see datasheet). In addition the absolute value of $V_{1/2}$ falls within the range of reported values using similar methods and recording conditions; around -98.7 mV, Stieber *et al* 2003; -98.6 mV, Chen *et al* 2005 and -91 to -95 mV, Yu *et al.*, 2004.

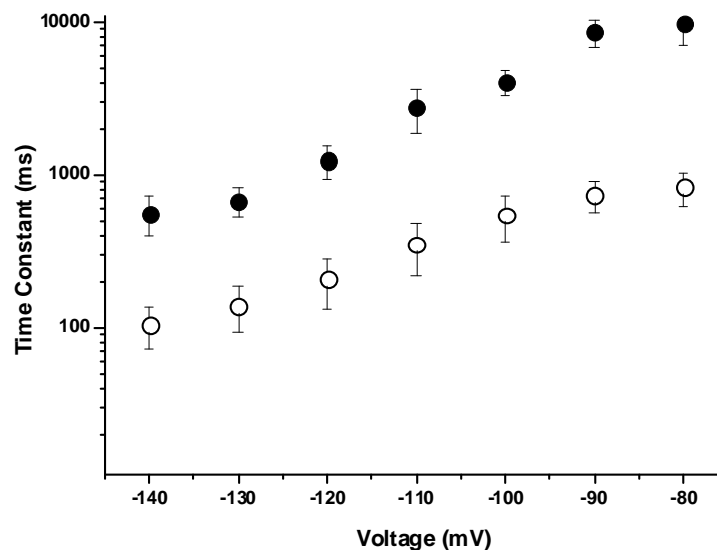
Figure 2. Activation Curve. The instantaneous current on stepping back to -70 mV from various test potentials was measured for each cell and normalized to the instantaneous current obtained with a pre pulse to -140 mV. The mean data from 7 cells is shown that could be described by a Boltzmann equation giving an estimated $V_{1/2}$ of -102.6 ± 1.3 mV and a slope of 8.4 ± 0.4 mV.



Time constant of Activation:

Although at more hyperpolarized voltages the time constant of activation could occasionally be described by two exponentials, the contribution of the slower component was significantly less than the other, accounting for <10% of the total current amplitude. This is consistent with Stieber *et al.*, 2003 who found that the fast component of activation accounted for 90-95% of the current amplitude. In view of this and for simplicity the time constant of the predominant component is plotted in **Figure 3** (solid circles) for comparison with the time constant of the predominant component for hHCN1 (open circles). It is clear that hHCN2 activates 5-10 fold slower than hHCN1 consistent with the difference reported by Wainger *et al.*, 2001 (4-6 fold slower) and Chen, Wang and Siegelbaum, 2001 (10-fold slower). The absolute values between studies at a given voltage vary somewhat but the time constant of activation at -140 mV reported here (559 ± 163 ms $n=3$) is similar to that of Mistrik *et al.*, 2005 (470 ms). In conclusion, currents activated with hyperpolarizing steps activate along time courses reported previously for hHCN2.

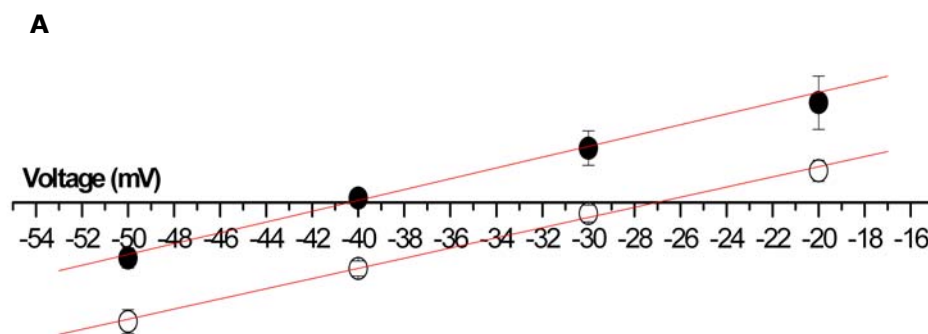
Figure 3. Time Constants of Activation. On stepping to various hyperpolarizing voltages from a holding potential of -30 mV, hHCN currents activated following an exponential time course after an initial delay. hHCN2 time constants obtained by exponential fits decreased with increasing hyperpolarizing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



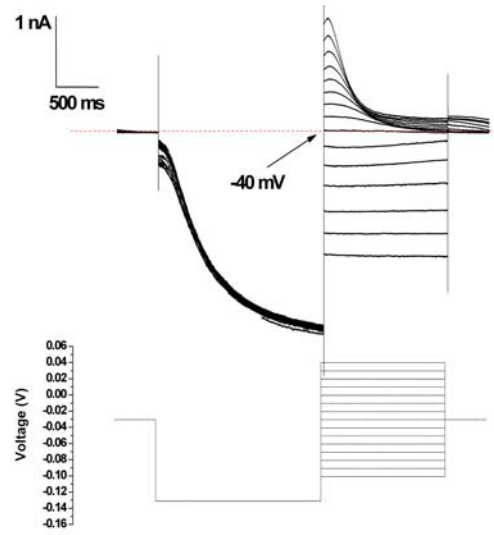
Reversal Potential:

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 (low K^+ external solutions, 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 (high K^+ external solutions, open circles). Under these conditions the reversal potential was around -40 mV and -27 mV respectively (calculated by measuring the instantaneous current on stepping to various test potentials after a pre pulse to activate the channels). These values indicate that the channels are indeed permeable to both cations with a P_{Na}/P_K ratio of roughly 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.

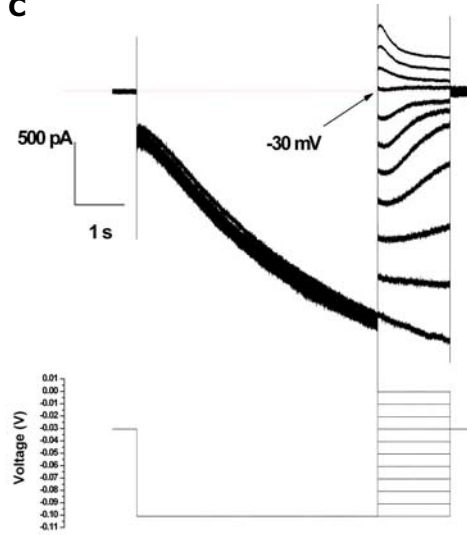
Figure 4. Reversal Potential Measurements. The reversal potential was calculated either in low K^+ external solutions (solid circles in **A**) or high K^+ external solutions (open circles in **A**) by using the voltage protocols in **B** and **C**. The data is the mean of 3 and 4 cells respectively. 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 at -40 mV whereas in high K^+ external conditions in **C**, the current reverses at -30 mV.



B



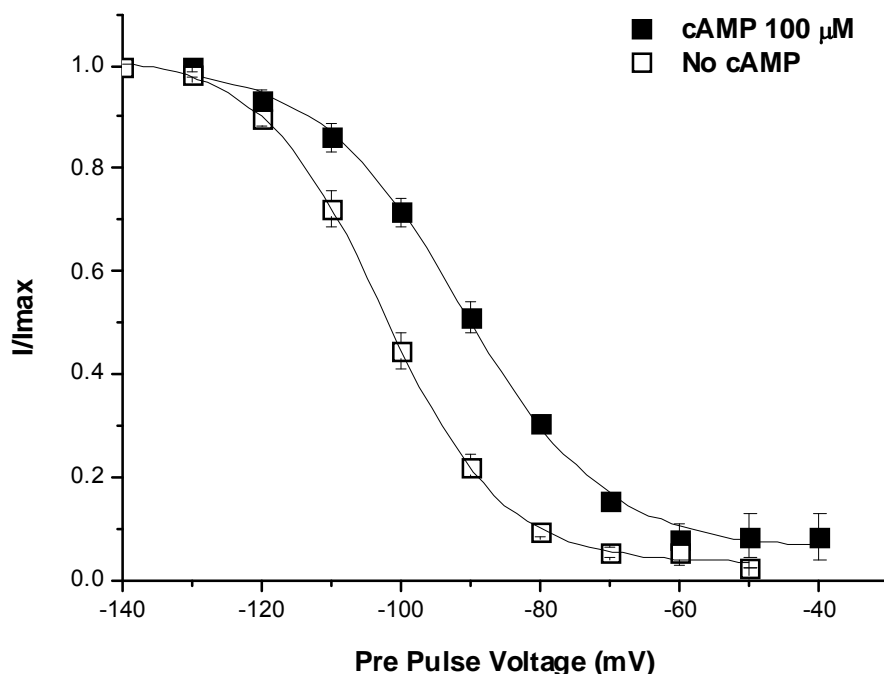
C



Pharmacology – Adenosine 3',5'-cyclic monophosphate (cAMP):

A hallmark feature of HCN2 channels is their sensitivity to cAMP (Wang *et al.*, 2002). Specifically, it has been reported to shift the $V_{1/2}$ of activation to more positive membrane potentials and to increase the rate of activation of these channels (Chen, Wang & Siegelbaum, 2001, Wainger *et al.*, 2001, Stieber *et al.*, 2003). Consistent with these reports inclusion of cAMP (100 μ M) in the patch pipette shifted the $V_{1/2}$ of inactivation by +13 mV from around -103 mV (**Figure 2**, and **Figure 5**, open squares) to -90 \pm 1.8 mV, n =4, in the presence of cAMP (**Figure 5**, solid squares). The degree of shift in $V_{1/2}$ is identical to the shift reported by Stieber *et al.*, 2003 who used the same concentration of cAMP and similar methodologies.

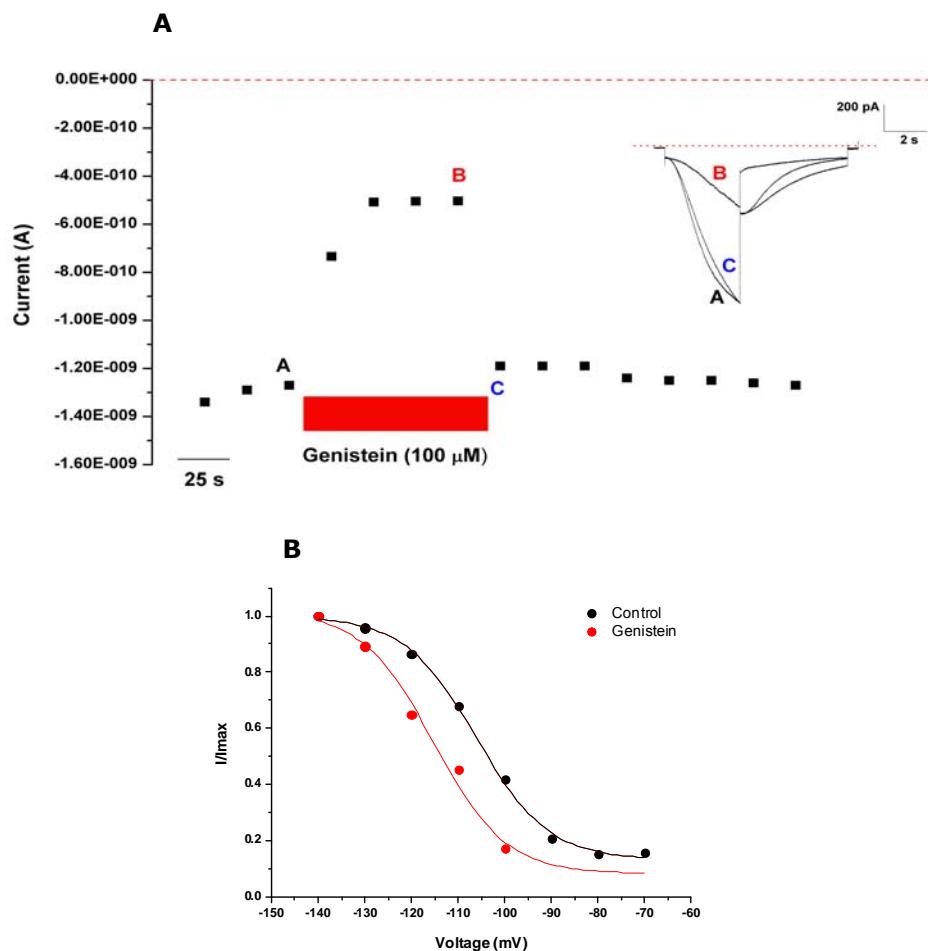
Figure 5. Activation Curve. The instantaneous current on stepping back to either -70 mV (no cAMP, open squares) or -50 mV (cAMP 100 μ M, solid squares) from various test potentials was measured for each cell and normalized to the instantaneous current obtained with a pre pulse to -140 mV (no cAMP) or -130 mV (cAMP 100 μ M). The control data (no cAMP) is from Figure 2 and the mean data in the presence of cAMP is from 4 cells. The data could be described by a Boltzmann equation giving an estimated $V_{1/2}$ of -90.3 ± 1.8 mV and a slope of 11.6 ± 2.2 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 $V_{1/2}$ of activation (Yu *et al.*, 2004). Yu *et al.*, also reported that HCN1 was unaffected by the same concentration (100 μ M) of genistein. Consistent with this, bath application of 100 μ M genistein rapidly and reversibly inhibited HCN2 currents (**Figure 6A**). The mean percentage inhibition of the current evoked by stepping from the holding potential of -30 mV to -110 mV was $66 \pm 6\%$ ($n = 6$). This is in contrast to the effect of the same concentration of genistein on HCN 1 currents (see datasheet CYL3040) where the percent inhibition was only $9.5 \pm 1.7\%$ ($n=4$). Furthermore 100 μ M genistein caused an approximate 9 mV negative shift in the $V_{1/2}$ of activation from -106 mV to -115 mV (**Figure 6B**) that is close to the 8 mV shift reported by Yu *et al.*, 2004.

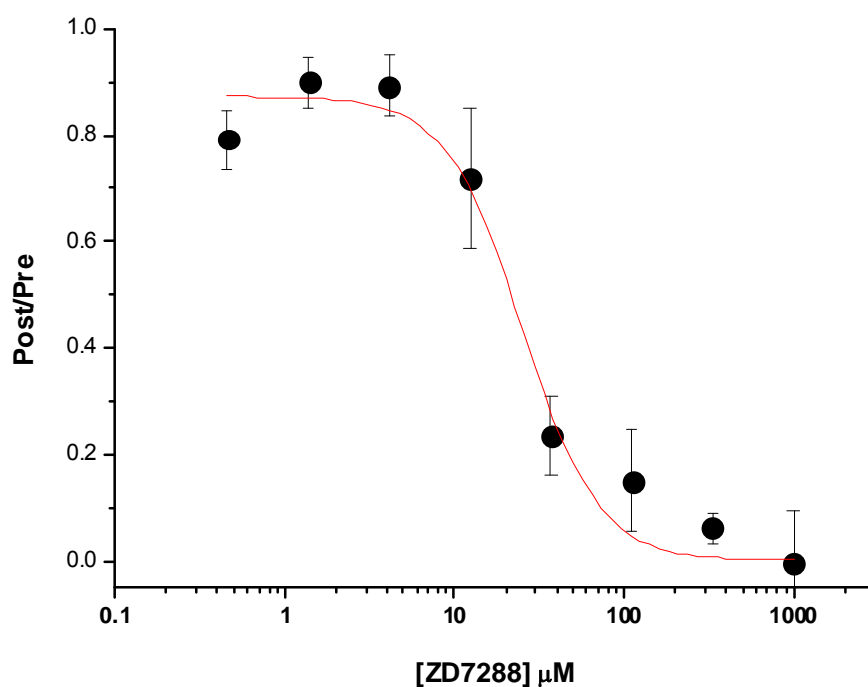
Figure 6. Effect of genistein on hHCN2 currents. The voltage protocol used in upper panel **A**, to evoke HCN2 currents, involved stepping from the holding potential of -30 mV to -110 mV for 3.5 s then stepping back to -70 mV for 5 s prior to returning to the holding potential. Pulses were applied every 20 s. Peak currents at the end of the -110 mV step were measured before, during and after 100 μ M 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. In lower panel **B** Genistein (100 μ M) shifted the $V_{1/2}$ of activation from -106 mV to -115 mV.



IonWorks™ HT Electrophysiology.**Pharmacology – ZD7288:**

Using IonWorks™ HT the effect of ZD7288 was assessed on the amplitude of hHCN2 currents evoked by a hyperpolarizing voltage step to fully activate the channels. ZD7288 is a relatively specific blocker of HCN channels with a reported IC₅₀ value for block of HCN2 channels of around 41 μM (Stieber *et al.*, 2005) which is similar to the IC₅₀ value of 25 μM reported here (**Figure 7**).

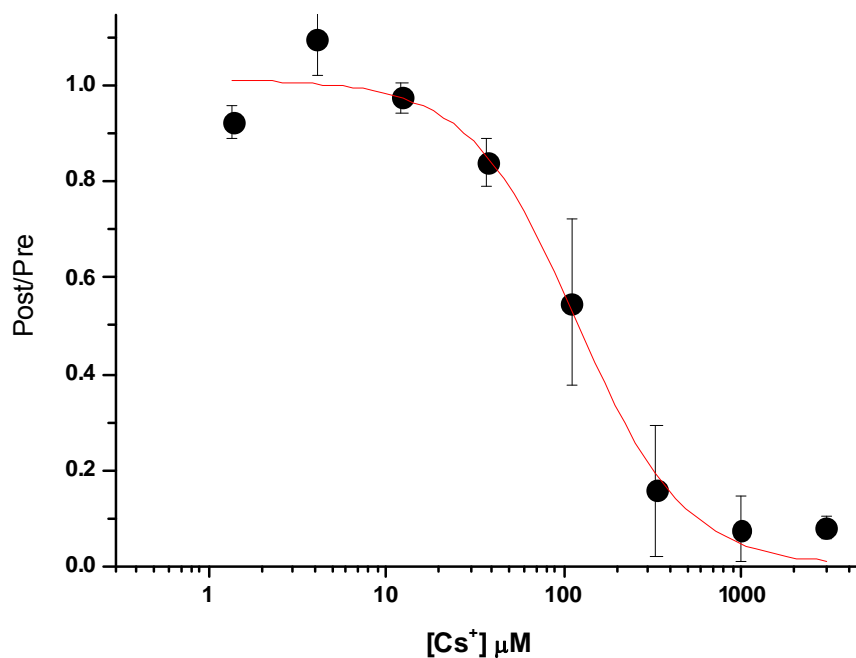
Figure 7. Effect of ZD7288 on HCN2 currents. The effect of a 10 min incubation of various concentrations of ZD7288 was assessed on the amplitude of hHCN2 currents recorded using IonWorks™ HT automated electrophysiology. Each data point represents the mean of 3-8 cells.



Pharmacology – Caesium (Cs⁺):

As well as being a non-specific blocker of K⁺ channels, Cs⁺ also blocks HCN channels with IC₅₀ values of around 200 μM (Stieber *et al.*, 2005). In accordance with this, Cs⁺ dose-dependently blocked hHCN2 currents with an estimated IC₅₀ value of 119 μM (**Figure 8**).

Figure 8. Effect of Cs⁺ on hHCN2 currents. The effect of a 10 min incubation of various concentrations of Cs⁺ was assessed on the amplitude of hHCN2 currents recorded using IonWorks™ HT automated electrophysiology. Each data point represents the mean of 3-8 cells.

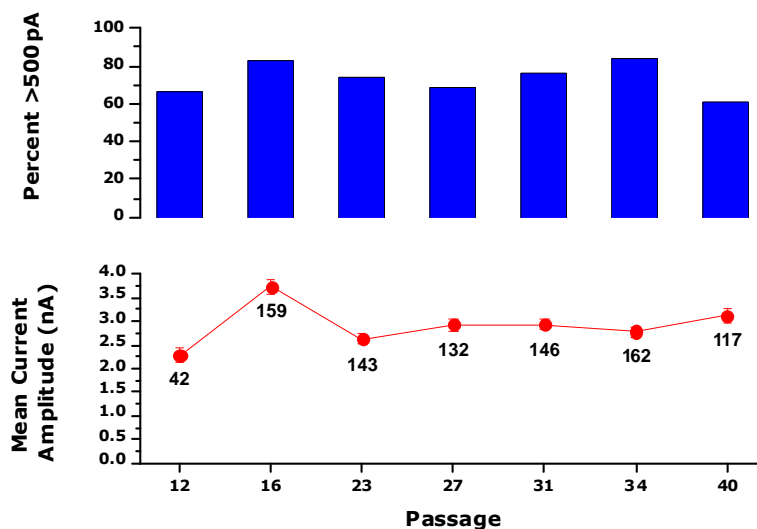


Stability of hHCN2-HEK293 Cell Line.

The hHCN2-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 hHCN2 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 2 and 3 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 12, 16, 23, 27, 31, 34, and 40. The lower panel shows the mean current amplitude (mean \pm SEM, red circles) and the number of these cells (numbers adjacent to red circles - out of 64 cells for passage 12 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% CO₂ 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 hHCN2 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 cm² 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% CO₂ 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 1-1.5×10⁶ cells per T75 cm² or 2-3×10⁶ cells per T175 cm² 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.

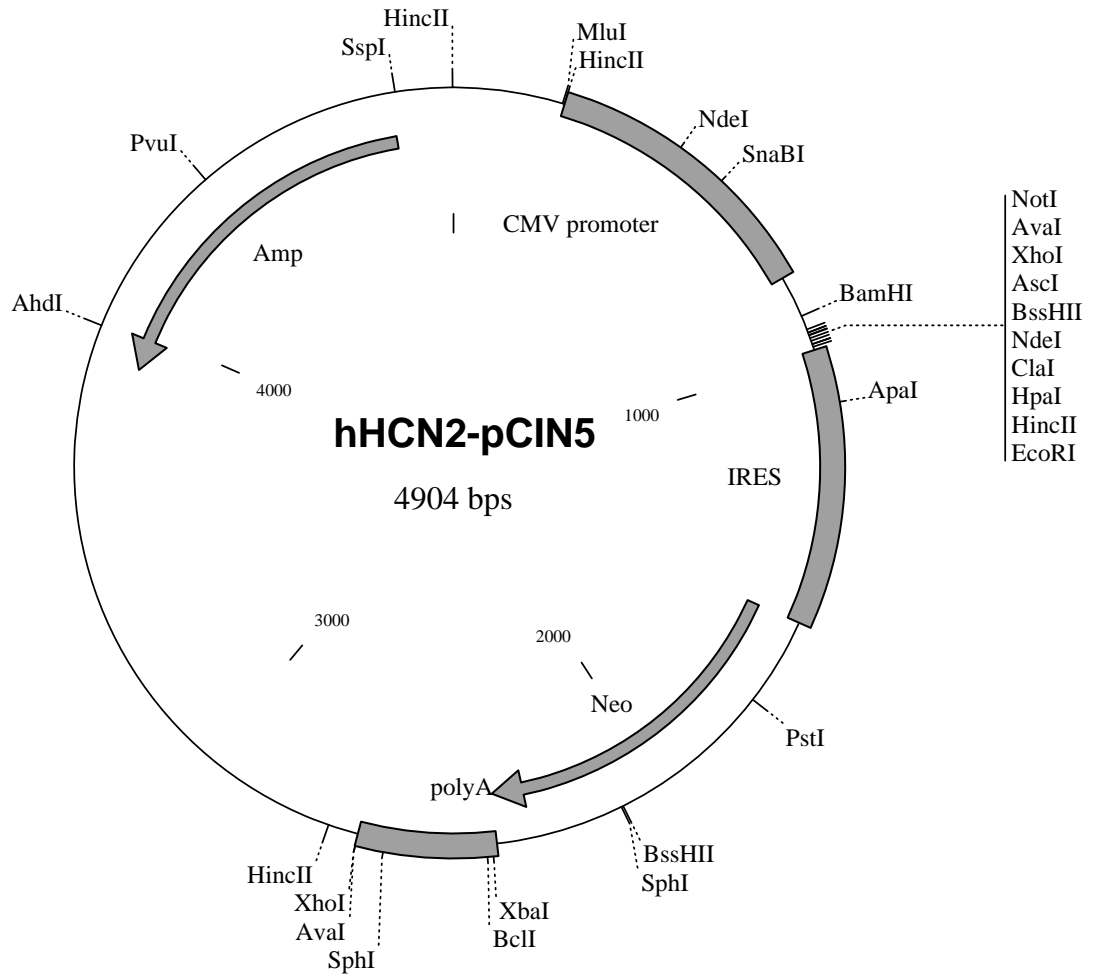
Media Formulation:

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 hHCN2-pCIN5:



Polylinker: CMV-NotI-AscI-ClaI-**hHCN2**-HpaI-EcoRI-IRES-*neo*

hHCN2 Sequence:

The first 527 bases of this sequence were synthetically constructed with an altered codon usage (all silent mutations with respect to the accession number (NM_001194)). There were three further silent mutations (**ATI-ATC** (Ile - 919-921 bp), **CCI-CCC** (Pro - 1165-1167 bp) and **GCC-GCT** (Ala - 1870-1872 bp)) and a single coding mutation **AGG-GGG** (Arg-Gly - 2545-2547 bp) in the rest of the sequence with respect to the accession number (NM_001194).

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