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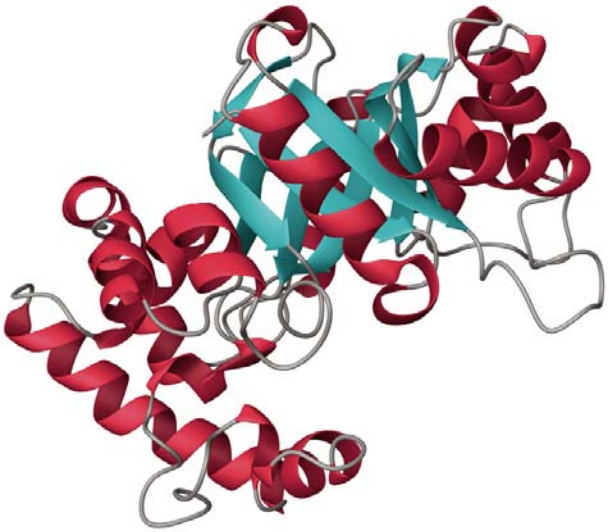
hKir2.1-HEK293 Recombinant Cell Line

cat. #CYL3032

Revision 1



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MILLIPORE (UK) LIMITED
6-7 Technopark
Cambridge
CB5 8PB
UK
Tel: +44 (0) 1223 508191
Fax: +44 (0) 1223 508198

Customer Services UK: 0800 0190 333
US: 800 437 7500

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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. Use, in the USA, of the bgh polyadenylation signal found in screening systems sold by Millipore requires a license from Research Corporation Technologies, Inc. (RCT). After purchasing these materials from Millipore, 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 Kir2.1 [inwardly-rectifying voltage-gated potassium channel, subfamily J, member 2 (KCNJ2), Accession Number NM_00891].

Format:
2 x 1 ml aliquots containing 1.12 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 hKir2.1 were characterised in terms of their pharmacological and biophysical properties using whole-cell patch clamp techniques.

The cell-line was shown to be expressing hKir2.1 current since the current/voltage relationship (I/V) displayed all the expected characteristics such as a region of negative slope conductance between -70 mV and -40 mV and strong inward rectification so that no outward current was detected at voltages more depolarized than -40 mV.

The current was K^+ selective and elevating extracellular K^+ resulted in parallel shifts in the I/V relationship and 'cross-over' of currents; a distinctive feature of hKir2.1.

The currents were blocked by Ba^{2+} in a voltage and time-dependent manner with an estimated IC_{50} value of 1-2 μM, assessed at the end of 1 s hyperpolarizing steps to -120 mV, in agreement with published data.

Functional channel expression over time was monitored using IonWorks™ HT. Channel expression is both consistent and robust over at least 38 passages: For example 88% of cells expressed hKir2.1 currents at passage 38 giving a mean current amplitude at –60 mV of 5.12 ± 0.08 nA (n=156).

IonWorks™ HT is a trademark of Molecular Devices Corporation

Introduction:

The inwardly rectifying potassium channels consist of seven subfamilies (Kir1-7) that have a similar structure consisting of only two transmembrane segments M1 and M2 that are analogous to S5 and S6 that line the pore in Kv channels (Gutman *et al.,* 2003). Whilst they lack the other transmembrane domains found in Kv channels, they similarly form functional channels as tetramers. (see review of Isomoto *et al.,* 1997). Since these channels lack the S4 voltage sensors found in Kv channels their non-linear current-voltage properties are governed principally by intracellular Mg^{2+} and polyamine block at depolarized potentials (inward rectification) and consequently only significantly conduct current at potentials negative to the reversal potential (see review of Lopatin and Nichols, 2001). The seven subfamilies are distinguished by the degree of this rectification and modulation by different messenger systems (see review of Reimann and Ashcroft, 1999).

Kir2.1 (IRK1) subunits exhibit strong inward rectification and are thought to be the principle subunits underlying the cardiac inward rectifier current (I_{K1}). This current is distributed throughout the heart and because it is the predominantly active current at very negative potentials it sets the 'resting' (diastolic) potential close to E_K, the reversal potential for potassium ions (Nichols *et al.* 1997). These channels also contribute to terminal phase 3 of repolarization when the voltage hyperpolarizes to values more negative than -20 mV (Shimoni *et al.,* 1992, Tamargo *et al.,* 2004). Since Kir2.1 have such a fundamental role in controlling the resting potential and shaping the cardiac action potential it is likely that drugs which affect this channel will have a marked effect on cardiac excitability. Indeed, mutations in the *KCNJ2* gene encoding Kir2.1, that reduce functional expression of the channel, cause a form of long QT syndrome (Andersen Syndrome, LQT7) that predisposes individuals to ventricular arrhythmias (Tristani-Firouzi *et al.,* 2002).

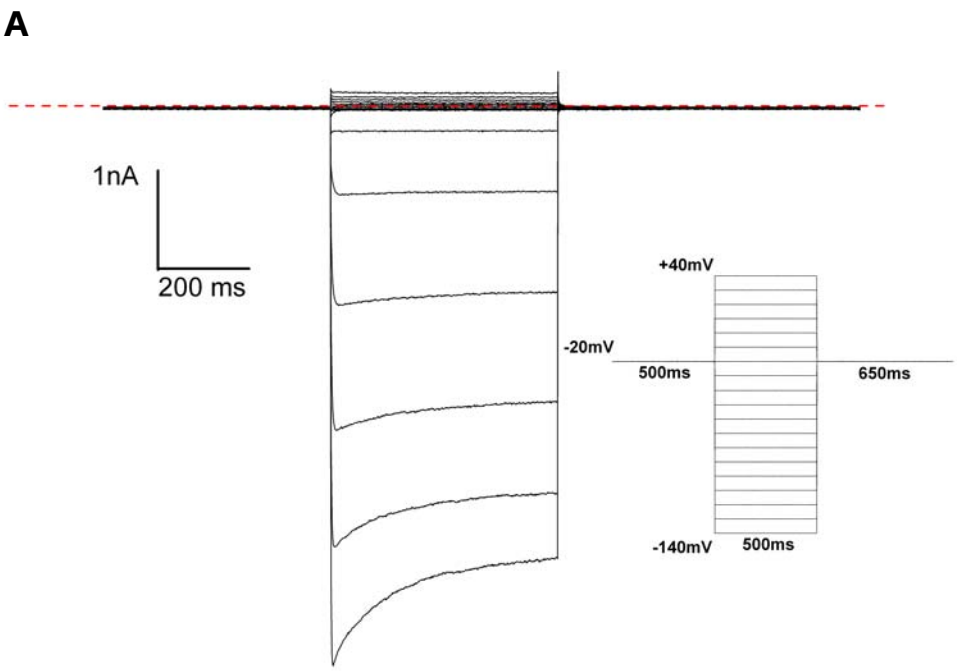
Here we characterize hKir2.1 channels selectively expressed in a HEK293 cell line (CYL3032).

Electrophysiological Properties of the hKir2.1 Current.

Conventional Whole-Cell Patch Clamp Electrophysiology.

Current / Voltage Relationship:
Stepping the membrane voltage from a holding potential of -20 mV, where Kir2.1 channels are non-conducting, to potentials negative to the reversal potential, evokes large relatively sustained inward currents (**Figure 1A**). However at very negative voltages (e.g. -140 mV,-130 mV) there is clearly a time-dependent component. These characteristics are typical of hKir2.1 where the time-dependent component is thought to primarily result from block by extracellular cations, particularly Na^+ (Biermans G *et al., 1987*, Barrett-Jolley *et al., 1999*, Lopatin and Nichols, 2001). Stepping the membrane voltage to potentials more positive than the reversal potential first resulted in small outward currents that were maximal at -70 mV. A subsequent step to -60 mV elicited a much smaller outward current and stepping to even more depolarized voltages (e.g. ≥ -40 mV) evoked no detectible outward current. Again this is a typical feature of Kir2.1 that shows strong inward rectification and a region of negative slope conductance between around -60 mV and -40 mV resulting from polyamine and Mg^{2+} block (Lopatin and Nichols, 2001, Dhamoon *et al.*, 2004, Yan *et al.*, 2005). The mean current-voltage relationship (n=5) illustrating these characteristics is shown in **Figure 1B.** This graph also suggests a reversal potential of between -80 mV and -90 mV, close to the theoretical reversal potential (-89 mV) for a K^+ selective channel under these recording conditions.

Figure. 1 I / V relationship.
A hKir2.1 currents were evoked by 500 ms depolarising voltage pulses stepped in 10 mV increments from +40 mV to -140 mV from a holding potential of –20 mV once every 5 seconds (inset). The red dotted line indicates zero current level. **B** The steady state current amplitudes elicited by the voltage protocol shown in **A** were normalised to the current evoked by the –140 mV voltage step for each cell. The mean data (n=5) is shown plotted against the step potential in mV.



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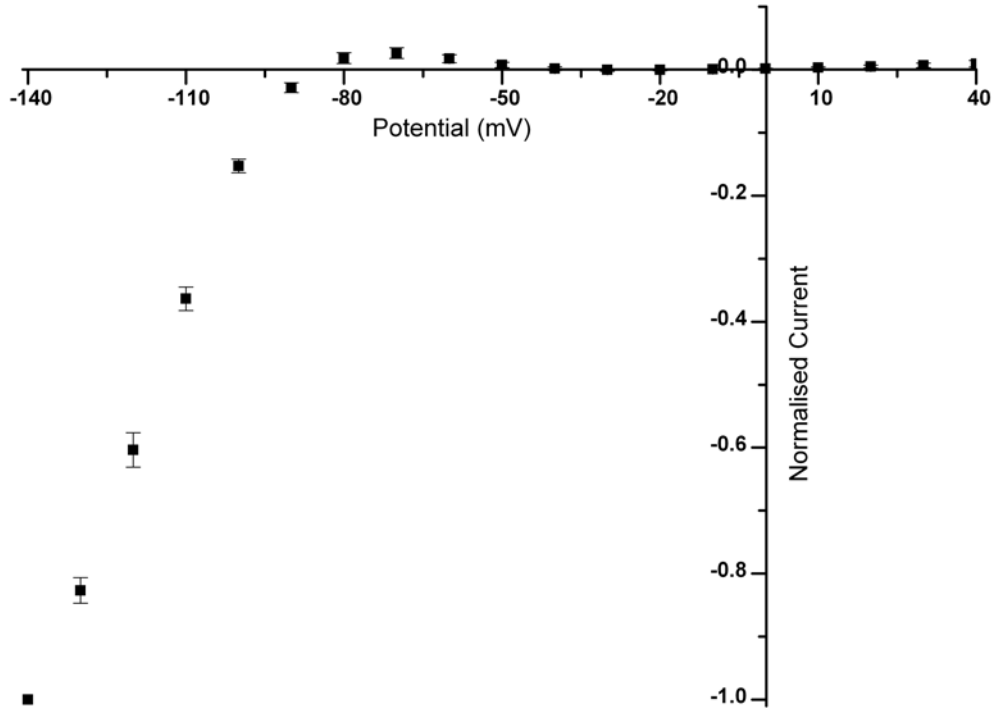
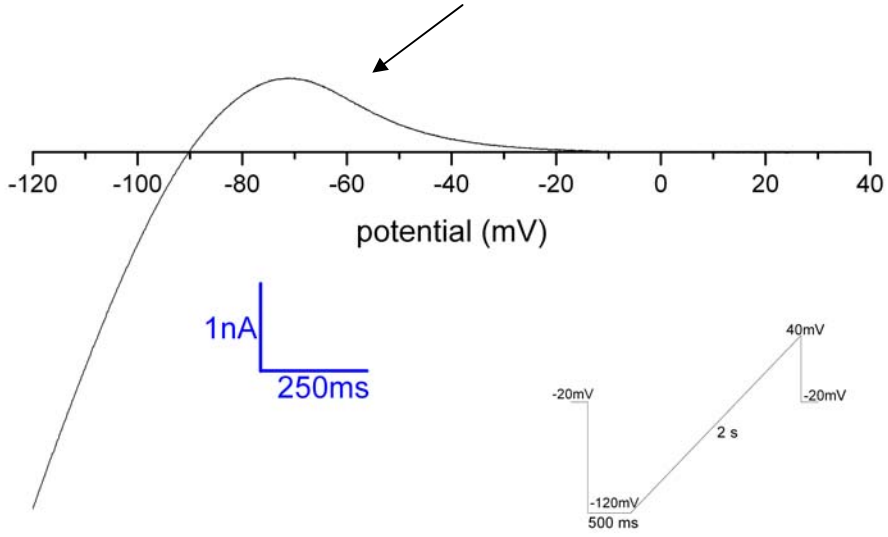


A more convenient way to assess the I/V relationship, especially, as in this case, if the currents are relatively rapidly activating and sustained at a given voltage, is to apply a voltage ramp. Kir2.1 current evoked in such a manner (**Figure 2**) showed an I/V relationship very similar to that obtained with voltage steps and a pronounced region of negative slope conductance (arrow) positive to the reversal potential of -90 mV.

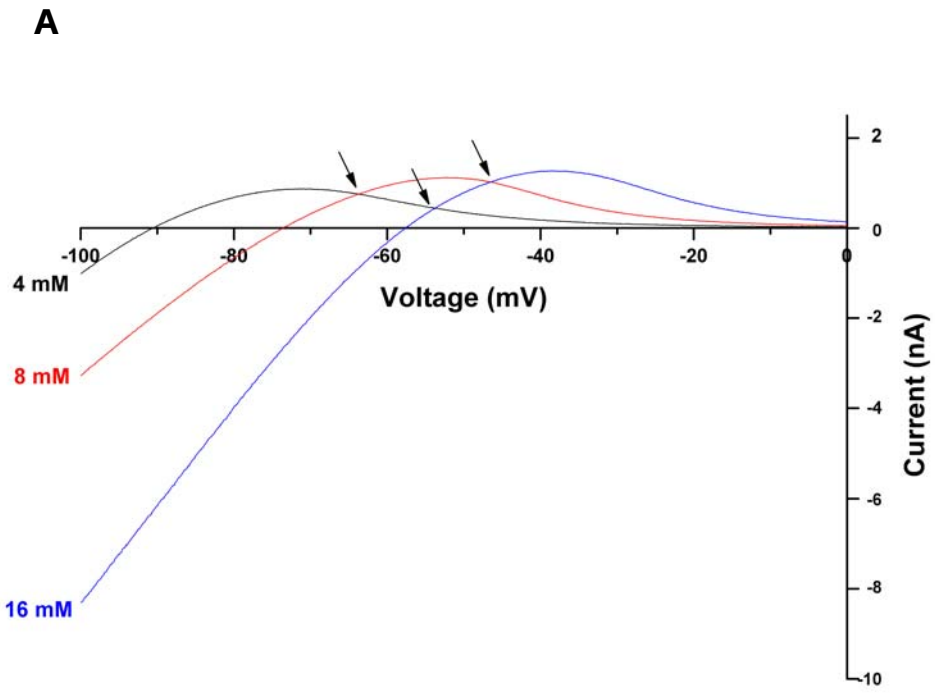
Figure 2. Ramp I/V Relationship.
Cells were held at –20 mV and stepped to –120 mV for 500 ms followed by a 2 second depolarizing ramp to +40 mV.



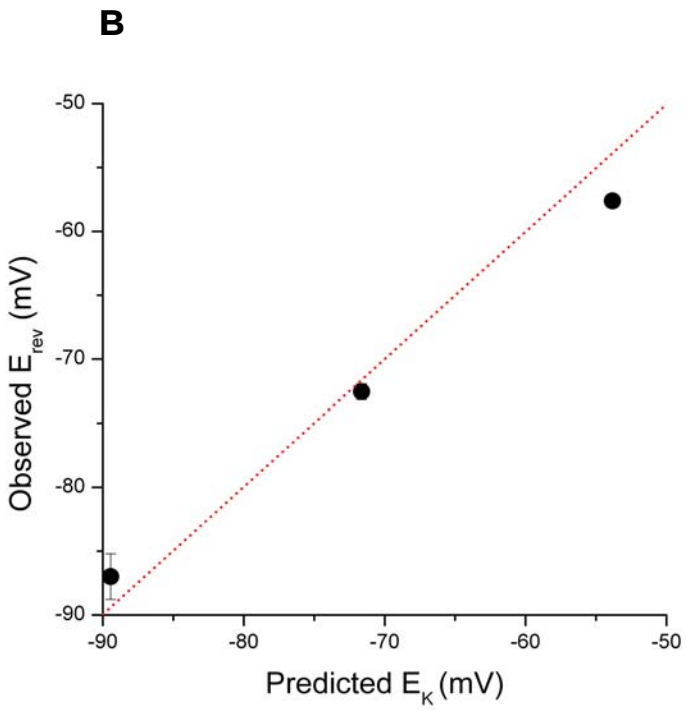
Effect of Elevated External K^+:
Increasing extracellular K^+ has been shown to cause a parallel shift in the inward I/V relationship, a Nernstian shift in the reversal potential and a 'cross-over' of outward currents (Lopatin and Nichols, 2001, Dhamoon *et al.*, 2004, Dhamoon and Jalife, 2005). This latter phenomenon results from the negative slope conductance and is particularly marked in Kir2.1 channels i.e. outward currents through Kir2.1 are increased in a critical voltage region positive to the reversal potential, where these currents 'cross-over'. This behaviour has important physiological consequences since an elevation of extracellular K^+ has been reported to shorten the ventricular action potential via this mechanism (Hume and Uehara, 1985).

Consistent with these reports increasing extracellular K^+ cumulatively from 4 mM to 8 mM and then to 16 mM caused parallel shifts in the ramp I/V relationship (**Figure 3A**), a change in reversal potentials according to the predicted change in E_K (Nernstian behaviour, **Figure 3A** and **B**)) and a 'cross-over' of currents (**Figure 3A**, arrows).

Figure 3. Effect of Extracellular K^+.
A A typical trace illustrating a section of the ramp protocol (as in **Figure 2**). The section shows the ramp from –100 mV to 0 mV carried out in the three different external K^+ concentrations (4, 8 and 16 mM) cumulatively applied to the cell. Note the cross-over points (arrows) and the voltage regions beyond this point where the Kir2.1 conductance is greater in the higher external K^+.



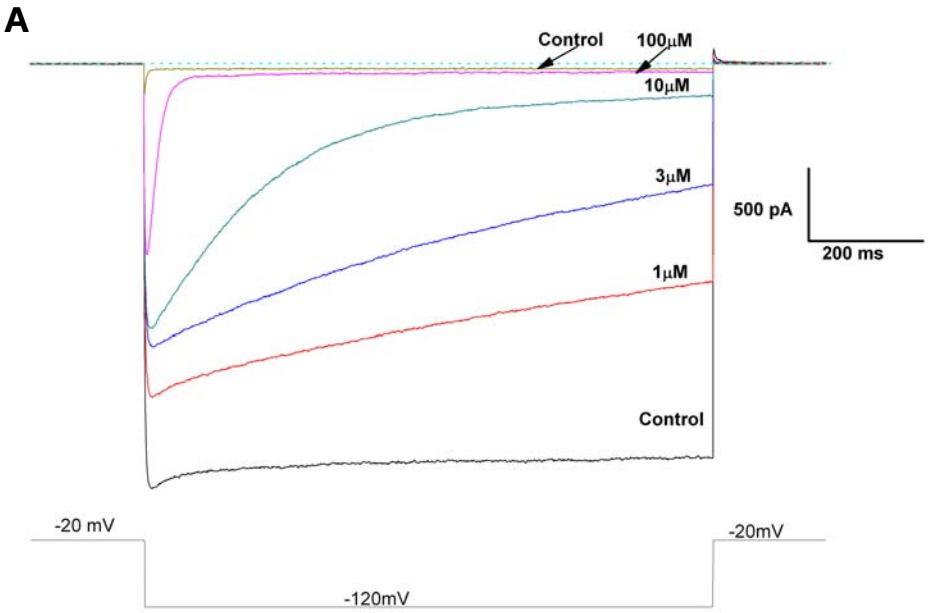
B The predicted mean of E_K (n=3) according to the Nernst equation has been plotted against the observed E_{rev} in **Figure 3A**. the red dotted line illustrates the line of unity.



Pharmacology – Barium:
Although to date there are no selective blockers of hKir2.1 channels, block by Ba^{2+} in the low micromolar range is a distinctive characteristic of inward rectifier channels. Furthermore different Kir2.x subunits show different sensitivities to Ba^{2+} (see for e.g. Liu *et al.*, 2001). Block is not only concentration dependent but also shows true voltage-dependence, blocking with higher affinity at more hyperpolarized potentials within the electrical field (Alagem *et al.*, 2001).

Using the voltage protocol described in **Figure 4A**, increasing concentrations of Ba^{2+} blocked Kir2.1 currents during the 1 s hyperpolarizing step to -120 mV in a dose and time-dependent manner. Clearly, during the inter pulse interval at -20 mV, Ba^{2+} has a marked tendency to unbind from the channel. These properties are consistent with previous reports describing Ba^{2+} block of Kir2.1 (Shieh *et al.*, 1998, Alagem *et al.*, 2001). Although the IC_{50} value for Ba^{2+} block is highly protocol dependent it was possible to estimate an IC_{50} value of around 1-2 µM by measuring the 'quasi' steady state current amplitude at the end of the hyperpolarizing step to -120 mV in various concentrations of Ba^{2+} (**Figure 4B**). This value is very close to the IC_{50} value of 2.2 µM reported by Shieh *et al.*, 1998) measured at the same voltage (-120 mV).

Figure 4. Effect of Barium at –120 mV.
A Cells were pulsed to a potential of –120 mV for 1 second from a holding potential of -20 mV and repeated every 10 seconds. Once a stable current amplitude was achieved under control conditions, increasing doses of Ba^{2+} were cumulatively applied to the cell, allowing each concentration to achieve a stable reduction in current amplitude prior to addition of a subsequent dose. **B** The amplitude of the current at the end of the hyperpolarizing step to -120 mV in various concentrations of $Ba^{2+}$was measured and expressed as relative current remaining compared to the control response, prior to addition. These values were plotted against concentration to obtain the dose-response curve. This could be described by a Hill equation with an estimated IC_{50} value of 1.5 µM.



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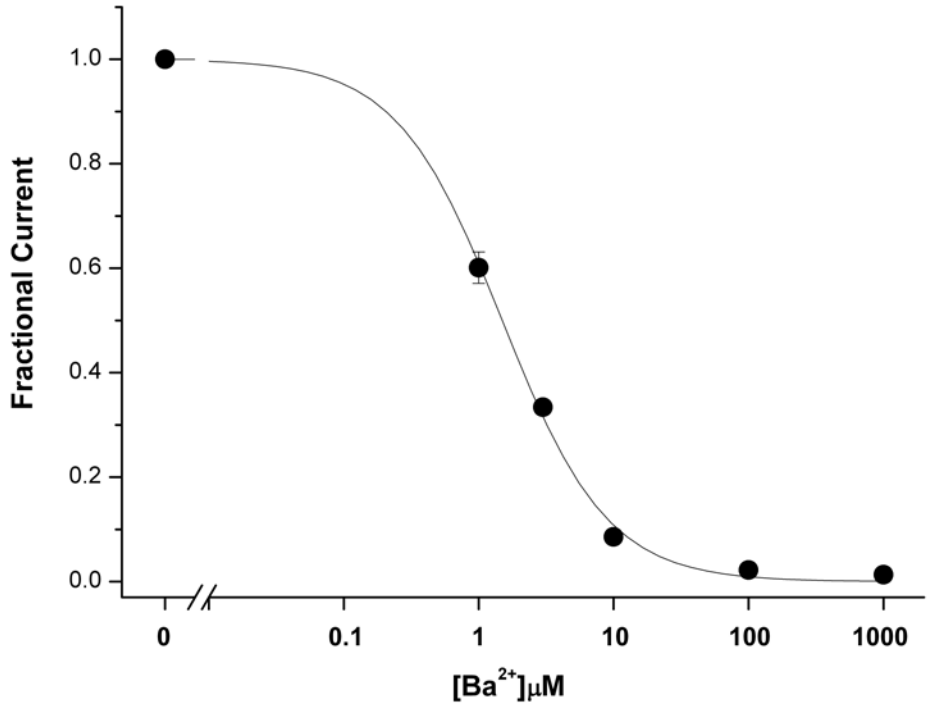
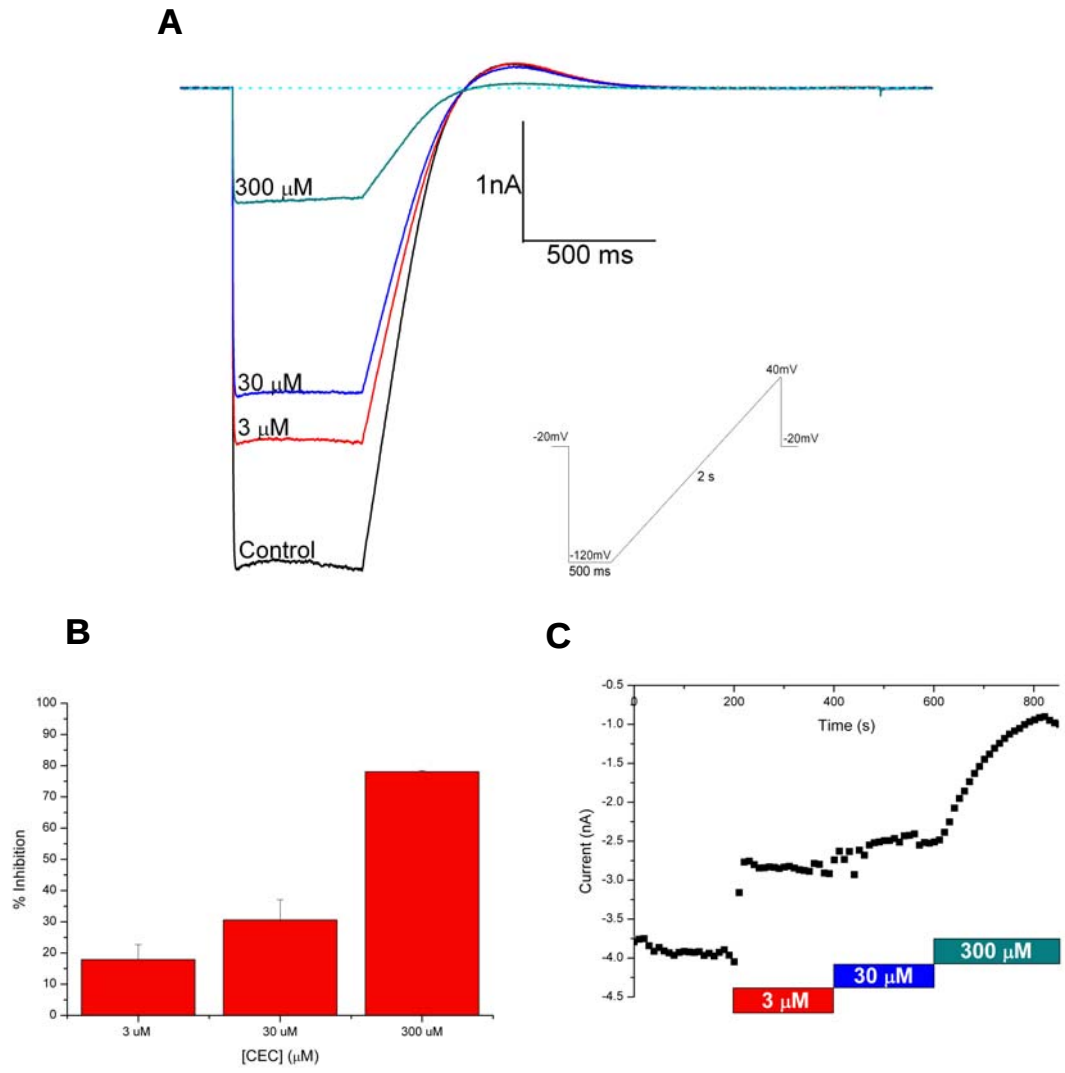


Pharmacology - Chloroethylclonidine (CEC):
Chloroethylclonidine (CEC) is a modulator of α–adrenergic receptors that has also been shown to block I_{K1} in skeletal muscle (IC_{50} around 40 µM at -60 mV) and recombinant Kir2.1 channels in a concentration (1-100 µM) and voltage dependent manner (Barrett-Jolley *et al., 1999*). Increasing concentrations of CEC were applied resulting in inhibition of the current either using voltage ramps (**Figure 5A**) or voltage steps to -120 mV (**Figure 5B**). The half-maximal inhibition of CEC was found to be between 30 µM and 300 µM consistent with the published data.

Figure 5. Effect of chloroethylclonidine (CEC) on hKir2.1 currents.
A. hKir2.1 current traces evoked by stepping to -120 mV for 500 ms from a holding potential of –20 mV followed immediately by a 2 second ramp from –120 mV to +40 mV in the presence of various concentrations of CEC. A pulse interval of 10 seconds was used. **B.** Inhibition values (n=3) obtained during the steady state current at –120 mV have been plotted against CEC concentration. **C.** Time course of block, with stable –120 mV currents plotted against time.



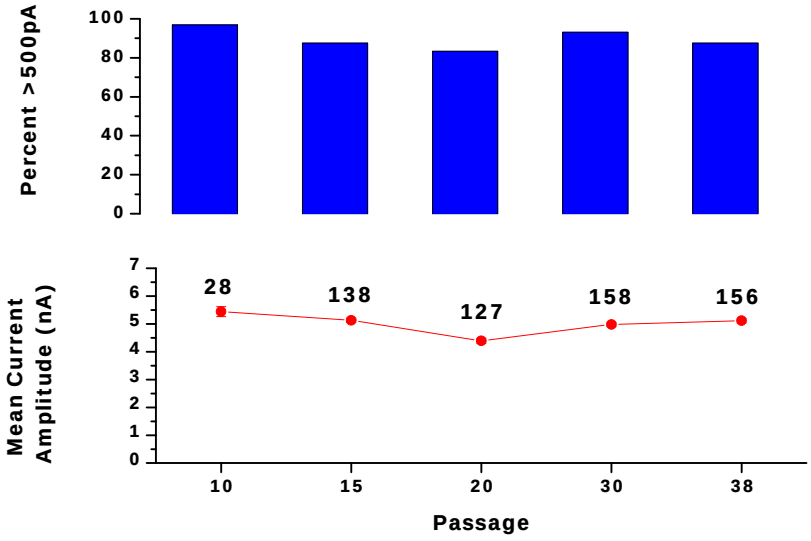
Stability of hKir2.1-HEK293 Cell Line:

IonWorks™ HT Electrophysiology.

The hKir2.1-HEK293 cell line has stable expression for >38 passages.

Functional channel expression, defined as cells expressing potassium current of ≥ 500 pA, was monitored using IonWorks™ HT, stepping to -60 mV from a holding potential of 0 mV in equimolar potassium conditions. This data and the mean current amplitude are shown in **Figure 6**.

Figure 6. Stability of expression over passage.
The upper panel shows the percentage of cells expressing a mean peak current >500 pA at -60 mV at cell passages 10, 15, 20, 30, and 38. The lower panel shows the mean current amplitude (mean ± SEM, red circles) and the number of cells (numbers above red circles - out of 32 cells for passage 10 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 DMEM/F12 medium supplemented 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 hKir2.1 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.

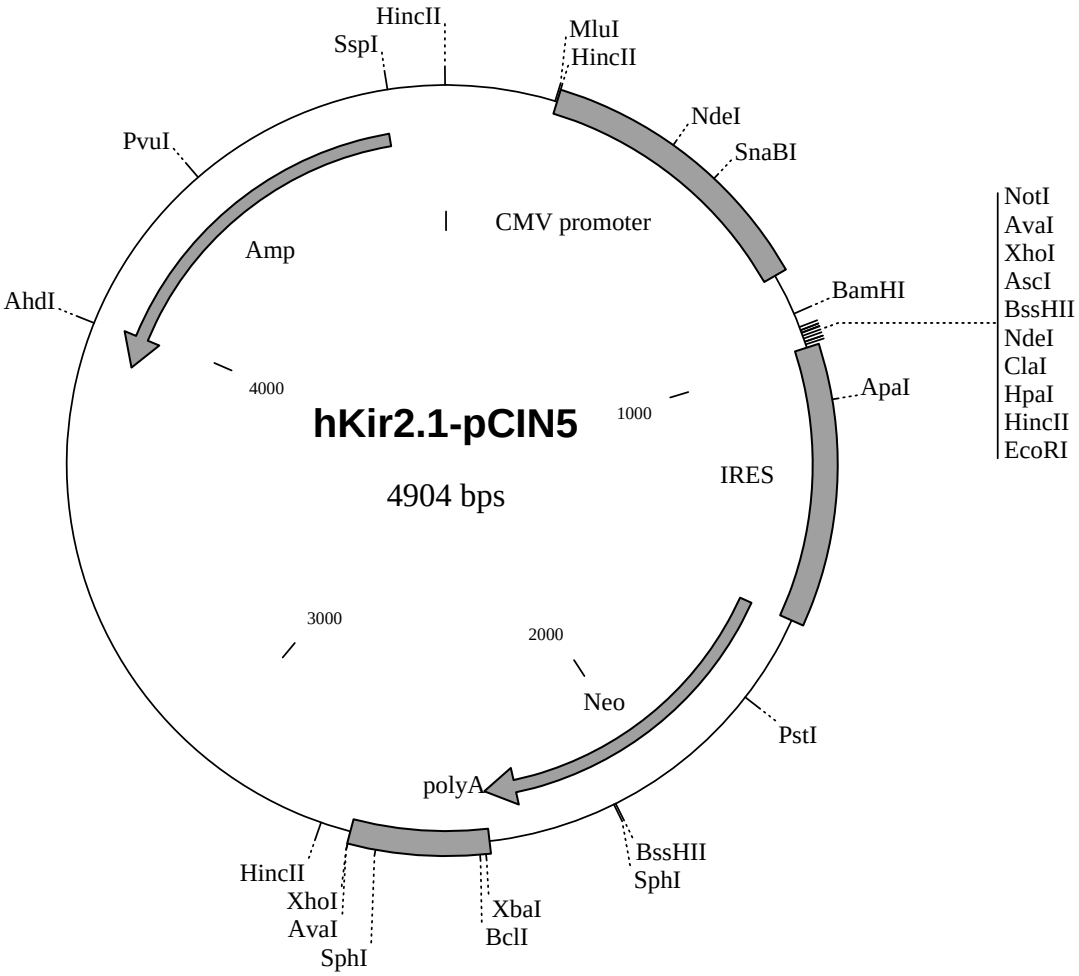
Media Formulation:

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:



Polylinker: CMV-BamHI-NotI-**hKir2.1**-EcoRI-IRES-*neo*

hKir2.1 Sequence:

The sequence of hKir2.1 used to create this stable cell line contains the following silent base change with respect the GenBank accession number NM_000891:

Bases 1063-1065: CTT-CTC (Leu - silent)

ATGGGCAGTGTGCGAACCAACCGCTACAGCATCGTCTCTTCAGAAGAAGACGGTATGAAGTTGGCCACC
ATGGCAGTTGCAAATGGCTTTGGGAACGGGAAGAGTAAAGTCCACACCCGACAACAGTGCAGGAGCCGC
TTTGTGAAGAAAGATGGCCACTGTAATGTTCAGTTCATCAATGTGGGTGAGAAGGGGCAACGGTACCTC
GCAGACATCTTCACCACGTGTGTGGACATTCGCTGGCGGTGGATGCTGGTTATCTTCTGCCTGGCTTTC
GTCCTGTCATGGCTGTTTTTTGGCTGTGTGTTTTGGTTGATAGCTCTGCTCCATGGGGACCTGGATGCA
TCCAAAGAGGGCAAAGCTTGTGTGTCCGAGGTCAACAGCTTCACGGCTGCCTTCCTCTTCTCCATTGAG
ACCCAGACAACCATAGGCTATGGTTTCAGATGTGTCACGGATGAATGCCCAATTGCTGTTTTCATGGTG
GTGTTCCAGTCAATCGTGGGCTGCATCATCGATGCTTTCATCATTGGCGCAGTCATGGCCAAGATGGCA
AAGCCAAAGAAGAGAAACGAGACTCTTGTCTTCAGTCACAATGCCGTGATTGCCATGAGAGACGGCAAG
CTGTGTTTGATGTGGCGAGTGGGCAATCTTCGGAAAAGCCACTTGGTGGAAGCTCATGTTCGAGCACAG
CTCCTCAAATCCAGAATTACTTCTGAAGGGGAGTATATCCCTCTGGATCAAATAGACATCAATGTTGGG
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AGTCCTTTATATGATTTGAGTAAACAGGACATTGACAACGCAGACTTTGAAATCGTGGTCATACTGGAA
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AATGGAGTTCCAGAAAGCACTAGTACGGACACGCCCCCTGACATAGACCTTCACAACCAGGCAAGTGTA
CCTCTAGAGCCCAGGCCCTTACGGCGAGAGTCGGAGATATGA

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