Fluid flow modulates electrical activity in cardiac hERG potassium channels

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Running title: hERG potassium channels respond to laminar shear stress.

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Abstract

Fluid movement within the heart generates substantial shear forces, but the effect of this mechanical stress on the electrical activity of the human heart has not been examined. The fast component of the delayed rectifier potassium currents responsible for repolarization of the cardiac action potential, Ikr, is encoded by the hERG channel. Here, we exposed hERG1a channel–expressing HEK293T cells to laminar shear stress (LSS) and observed that this mechanical stress increased the whole-cell current by 30%–40%. LSS shifted the voltage dependence of steady-state activation of the hERG channel to the hyperpolarizing direction, accelerated the time course of activation and recovery from inactivation, slowed down deactivation, and shifted the steady-state inactivation to the positive direction, all of which favored the hERG open state. In contrast, the time course of inactivation was faster, favoring the closed state. Using specific inhibitors of focal adhesion kinase (FAK), a regulator of mechano-transduction via the integrin pathway, we also found that the LSS-induced modulation of the whole-cell current depended on the integrin pathway. The hERG1b channel variant, which lacks the Per-Arnt-Sim (PAS) domain, and long QT syndrome–associated variants having point mutations in the PAS domain were unaffected by LSS, suggesting that the PAS domain in hERG1a channel may be involved in sensing mechanical shear stress. We conclude that a mechano-electric feedback pathway modulates hERG channel activity through the integrin pathway, indicating that mechanical forces in the heart influence its electrical activity.

Introduction

The beating of the heart is initiated by an elaborate and well-tuned electrical system which generates the cardiac action potential (cardiac AP). The shape and duration of the cardiac AP is held within close tolerances as small variations can lead to cardiac arrhythmias and even catastrophic heart failure. The firing of the Action Potential, in turn, results in contractions of the musculature of the heart and hence blood flow through the body. The effect of mechanical forces at work during the heart beat on the electrical system of the heart have not, surprisingly, been investigated in detail so far. Regulation of ion channel activity plays a central role in controlling heart rate, rhythm, and contractility responses to cardiovascular demands. Dynamic beat to beat regulation of ion channels in the context of hemodynamic shear...
forces is still a grey area. Here we investigate the influence of mechanical shear on a critical component of the electrical system – a voltage-gated ion channel that plays a major role in the repolarization of the cardiac Action Potential.

Voltage-gated ion channels are abundant in the heart, playing pivotal roles in the proper shaping of the cardiac action potential. Several neuronal voltage-gated potassium channels have recently been shown to be mechanosensitive (1). Voltage-gated cardiac channels (cardiac VGC’s) share the same architecture as neuronal VGC’s, raising the possibility that they are also mechanosensitive (2). The ventricles lack specialist stretch activated channels i.e. channels that are relatively insensitive to other stimuli but mediate cation currents in response to stretch (3, 4). The myocardium is continuously exposed to a variety of forces over each contraction and must therefore adapt to the associated mechanical stresses. One such major force is the shear stress which arises from blood flow and the sliding of myocardial layers against each other with each heart beat (5). If cardiac VGCs respond to shear forces (6, 7), they could contribute to both physiological and pathological mechano-electric feedback in the heart. However, it is unclear as to whether mechanosensitivity is an integral part of cardiac voltage gated channel function and, if so, how gating transitions are modified under shear stress.

The human ether-a-go-go related gene (hERG) encodes a K+ channel which is responsible for the Ikr current that contributes to the falling phase of the cardiac action potential. hERG channels, unlike other voltage gated potassium channels, have a unique inactivation mechanism that limits potassium efflux during depolarization but rebounds during repolarization, thereby facilitating a significant outward K+ tail current (8, 9). Perturbation of these repolarizing currents could result in alteration of the cardiac action potential duration (APD) (10, 11). Prolonged APD is seen in disease states like Long QT syndrome (12), while reduced APD results in short QT syndromes (13–15). Mutations in hERG have been associated with both Long QT syndrome and Short QT syndromes as well as life threatening arrhythmias. Chronic heart failure could follow perturbation of hERG channel function (16).

The transmission of external forces acting on cells to stretch-sensitive channels in the plasma membrane requires the intermediary of physical structures. A collection of transmembrane proteins that transmits force and initiates transduction pathways within cells is the integrin family. Integrins are transmembrane glycoproteins forming heterodimers that act as extracellular matrix receptors and physically link the extracellular matrix to the cytoskeleton (17). The β1D splice variant is the major β isoform expressed in adult heart (18, 19). In myocytes, integrins are a component of costameres, which are macromolecular complexes with Z lines specialized for force transmission (19–21) and are activated by clustering of integrin receptors. Focal Adhesion Kinase binds to the cytoplasmic domain of β1 integrin, resulting in autophosphorylation of FAK at Y397. Previous studies using heterologous expression of hERG channels in HEK293T cells to study its interaction with β1 integrins (22), have reported effects on current activation and cell spreading upon interaction of β1 integrins with extra cellular matrix. This, in turn, activates downstream effectors FAK (autophosphorylation at Tyr 397) and Rac1 in HEK293T cells. β1 integrins are highly expressed in cardiomyocytes and shown to vary with age of the myocytes (23). Blockage of hERG channels resulted in down regulation of FAK phosphorylation (24) indicating a two way communication between FAK and hERG. The FAK-integrin system could thus constitute a pathway to transmit forces that may arise both from intracellular (contractile) forces and forces from outside the cell (fluid shear stress) in the heart (25) to the hERG channel (26, 27).

We have expressed hERG channels in HEK293T cells and investigated the response of whole-cell currents to shear stress, reproduced in vitro by laminar flow in a patch clamp recording chamber. Laminar shear stress is correlated with an increase in whole cell currents together with shifts of the V1/2 of activation in the
hyperpolarizing direction, the $V_{1/2}$ of inactivation towards depolarizing potentials, acceleration of the activation kinetics, decrease in deactivation rates and increase in the rate of recovery from inactivation. All of these changes favor the open state. Conversely, shear stress also increased the rate of inactivation favoring a non-conducting state. Shear effects were saturable and reversible. β1 integrins are highly expressed in HEK293T cells (22) and our data suggests that mechano-transduction occurs via the integrin pathway. The cytoskeleton participates in the shear response. hERG1b, a splice variant lacking the PAS domain, as well as Long QT mutants harboring point mutations in the PAS domain were unresponsive to shear stress suggesting a possible role of the PAS domain in mechano-sensation.

Results

Laminar shear stress increases hERG currents

The effect of Laminar Shear Stress (LSS) on hERG transfected HEK293T cells was investigated using the whole cell patch clamp configuration in the voltage clamp mode. Voltage was stepped to a series of potentials from a holding potential of -80mV, and then taken to a test potential of -60mV. The current just prior to the -60mV step is taken as the step current, while that immediately after the step is the tail current. As shown in Fig 1B, increased flow rate caused enhancement in tail current recorded at -60mV from a step potential of 0mV. The data is presented as percentage change from the value of the parameter at a baseline flow rate of 1ml/min, which was taken as pre-shear. We present all data as Mean±SD.

Flow rates between 1ml/min to 15ml/min were tested and tail current at -60mV found to be significantly greater under shear than pre-shear at all flow rates above 1ml/min. The data suggest two states for the whole cell current and fit well to a Boltzmann function with a midpoint at 5.2±0.10ml/min (n=5). Currents at flow rates of 7, 10 and 15ml/min were essentially indistinguishable, indicating that shear-induced changes in hERG physiology had saturated by 7ml/min (Figure 1C). All further studies compared recordings at flow rates of 10ml/min and 1ml/min. The corresponding shear stress to which the cells in the recording chamber were exposed is not precisely known. However, assuming a linear velocity variation in the direction orthogonal to flow, the shear stress is estimated to be 11.3 dyne/cm² at 10ml/min (28).

In addition to enhancement of tail current, the step current also increased on application of shear stress (Fig 1A & 1C). An increase of around 27% from 42.5±10.02pA/pF to 54±16.48pA/pF (n=16, p<0.001, paired T-test) was observed for a step to 0mV. Tail currents in the same experiment increased from 51.2±13.80pA/pF at 1 ml/min flow to 66.8±16.56pA/pF (n=20, p<0.001) at 10ml/min – an increase of 30.4%. Around 88% of the cells recorded showed increase in current in both step and tail current on being subjected to shear stress. However, some cells displayed increase in either step or tail current and not both.

The reversibility of the phenomenon was tested at -60mV by switching flow rates between 1ml/min and 10ml/min (Fig 1D). After acquiring a baseline at 1ml/min, flow was increased to 10ml/min for 390 seconds, then allowed to recover at 1ml/min for 360 seconds before returning to 10ml/min. Tail currents increased by the first measured time point, which was at 20 seconds after switching to 10ml/min on both occasions, and continued to increase with a time constant of 91.4 seconds (Fit shown with a smooth black line). Saturation of the response was seen in about 300 seconds. Decay to baseline was slower, with a time constant of 154.8 seconds (Fit shown with a smooth black line). Recovery saturated at a value that was 13% higher than the original baseline. The second episode of high flow resulted in an increase of current to a value that was 95% of the first cycle (n=6).

Another mechanical stress is membrane stretching by cell swelling. We recorded hERG1a potassium currents in whole cell mode first under iso-osmotic conditions and then under hypo-osmotic conditions for a period of 2.5min to induce cell swelling at a constant flow
rate of 1ml/min (Supplementary Fig 4A). Osmotic strength was modified using sorbitol to maintain the ionic composition. On switching from iso-osmotic to hypo-osmotic solutions, the step currents increased from 56.22±12.40pA/pF to 69.16±13.14pA/pF at 0mV (an increase of 23.2%) and tail currents increased from 80.66±20.33pA/pF to 97.97±17.17pA/pF at -60mV (an increase of 21.4%). Statistical comparison was done using Paired T-test, n=6, p=0.004 and p=0.03 for step and tail currents, respectively. Cell capacitance didn’t change significantly (p>0.05, n=6, paired T-test) between iso-osmotic (15.18±4.87pF) and hypo-osmotic conditions (14.54±4.48pF), which renders the fusion of channel containing vesicles with the plasma membrane an unlikely source of the increased current (Supplementary Fig 4B).

The initial survey of sensitivity to shear stress was restricted to a 0mV step (Fig 2A, 2B & 2C). Analysis of the full range of step potentials at 10ml/min reveals that the step current is enhanced in the presence of shear at all potentials below +10mV (Fig 2C). Additionally, when the I-V plots are normalized, a shift of 10mV to hyperpolarizing potentials can be observed in the presence of shear (Fig 2C inset). This means that normalized current is higher under shear than in its absence at any potential below the peak potential of +10mV. The combination of voltage shift and increased amplitude result in the two curves being indistinguishable above +10mV (Fig 2C).

The tail current seen immediately after stepping to the test potential of -60mV is proportional to the number of channels that had opened during the step pulse. The voltage dependence of channel opening could be fit with a Boltzmann function with a mid point (V_{1/2}) of 0.6±0.23mV at a flow rate of 10ml/min and at -8.8±0.39mV at 10ml/min, a hyperpolarizing shift of -9.4 mV (Fig 2D, n=7). A mid point (V_{1/2}) of -2.4±0.46 was observed after switching back to 1ml/min flow rate which is close to the pre-shear value (Fig 2D dashed lines, n=7). No change in reversal potential was seen (Fig 3A).

**Laminar Shear stress (LSS) affects hERG channel activation and deactivation rates**

The tail current is proportional to the number of channels open at the end of the step pulse. Decay of the tail current is due to the closing of these channels i.e. deactivation. The decay of the tail current could be fit to two exponentials. At -60mV and -100 mV, both the deactivation time constants (\(T_{\text{deact}}\)) (Fig 3B&3C) increased with shear 161.3±38.2ms to 173±38.07ms (P<0.0001, n=5, paired T-test) and 63±18.24ms to 92±39.23ms (p=0.01, n=5, paired T-test) respectively, for the fast component, which comprises 90% of the deactivation, and 1040±161ms to 1137±57.92ms (p=0.01, n=5, paired T-test) and 343.8±93.39ms to 558.9±168.1ms (p=0.02, n=5, paired T-test) respectively for the slow component. Hence the outward current through hERG lasts longer under shear. Examination of kinetic transitions provides more information regarding the influence of shear stress on hERG current activation. Fig 4A illustrates the envelope of tails protocol used to measure the activation time constant uncontaminated by the faster inactivation process (29). Because the inactivation of HERG channels is released at a faster rate than the channels deactivate (30), this is a more specific measure of activation. The tail current at -60mV, which is proportional to the number of channels having transitioned from closed to open during the preceding step to 0mV, is plotted as a function of time in Fig 4B. Peak tail current values at different time points were fit with a single exponential function as shown. The analysis reveals a t_{1/2} of 279.9±5.9ms at a flow rate of 1ml/min and t_{1/2} of155.5±17ms under LSS (Fig 4C) indicating that transitions from the closed to the open state almost double under shear (p<0.0001, n=8, paired T test).

**Homomeric hERG 1b channels do not respond to Laminar Shear Stress**

hERG1b is a splice variant which lacks the PAS domain found in the N-terminal cytoplasmic tail of hERG1a. hERG1b also has a unique N-terminal cap comprising of 20 amino acid
residues (31, 32). It has been reported earlier that hERG1b fails to form functional channels in HEK293T (29). However with 2µg DNA we were able to get measurable currents (step current density: 36.2±11.59pA/pF and tail current density: 11.4±4.396pA/pF). As shown in Fig 5A and Supplementary Fig 1A, laminar shear stress had no effect on hERG1b current activation (p>0.05, n=7, paired T-test).

Since hERG1b lacks the PAS domain and is resistant to shear stress, we tested the role of the PAS domain in the shear response. The PAS domain alone, expressed as an independent polypeptide, interacts directly with hERG1a/1b heteromeric channels and it has been suggested that it occupies sites left vacant by hERG1b subunits (33–35). Expressing the PAS domain together with hERG1b resulted in a large increase in currents (Supplementary Fig 1B). As shown in Fig 5B, increasing flow rates to 10ml/min had little effect in step currents in this system but did increase the tail currents by around 16% (73.1±26.04pA/pF to 87.2±29.43pA/pF, P=0.01, n=5, paired T-test). The PAS domain thus restored some degree of mechanosensitivity.

Both hERG1a and hERG1b are co-expressed in cardiac tissue and the hetero-multimer has been well characterized in heterologous expression systems (29, 34, 36). Cells transfected with 1µg DNA of each construct exhibited substantial currents resembling native Ikr comprising of a large outward current and a fast deactivating tail current which is much larger than in the cells transfected with hERG1a alone (supplementary Fig 1C). As shown in Fig 5C, increasing the flow rate to 10ml/min increased current still further from 116.8±11.15 to 128.4±12.11pA/pF (p=0.01, n=7) for step currents and from 63.52±21.46 to 77.10±20.48pA/pF (p=0.00, n=8, paired T-test) for tail currents. The fractional increases observed were significantly smaller than those observed for hERG1a alone (10% vs 27% for step currents and 21.4% vs 30.4% for tail currents).

**Laminar Shear Stress effects on inactivation and recovery from inactivation.**

We investigated the inactivation properties of the hERG1a channel (Fig 6). A first three step protocol was applied to investigate the voltage-dependent steady state inactivation. After reaching a steady state at +40mV, the channels were subjected to brief (10ms) hyperpolarizing pulses ranging from +30mV to -140mV. The peak current amplitude after return to +40mV was normalized and plotted against the potential of the hyperpolarizing step (Fig 6A). The data was fit to a Boltzmann function with a mid point V_{1/2} at -54.6±3.3mV at a flow rate of 1ml/min and -51.5±3.63mV at 10ml/min, which is not significantly different (P>0.05, n=7, paired T-test).

The kinetics of inactivation were studied by activating the channels with a 200ms depolarizing step to +40mV followed by a brief hyperpolarizing step (10ms) to -120mV to allow the channels to recover from inactivation. The hyperpolarizing step was followed by steps to various potentials from 0mV to +40mV. The onset of inactivation is seen in the decline of the observed current to the steady state (Fig 6B). Fitting the current traces to a single exponential function yields the time constant of inactivation (T_{inact}). LSS decreased the time constant for inactivation (Fig 6B) (13.3±4.2ms to 9.3±2.2ms at 0mV, p=0.005, n=9, paired T-test). Shear thus decreases the dwell time in the open state and effectively suppresses outward current during positive voltage commands (29, 36).

We further determined the time constants for the recovery from inactivation (T_{recovery}). After reaching a steady state at +20 mV, tail currents were evoked at different potentials from -60 to +10mV. Single exponential fits to the rising phase of the tail current yielded time constants for recovery at each test potential (Fig 6C). Recovery was found to be faster during LSS as compared to pre-shear state at all potentials tested. At -50mV, T_{recovery} was found to be 9±2.15ms and 7.7±1.65ms in pre-shear and post shear condition respectively (p=0.001, n=5, paired T-test) consistent with the resurgent
current and larger tail current amplitudes seen in Fig 1A & 1B.

Laminar shear stress requires an intact cytoskeleton

10µM Cytochalasin D was used to depolymerize actin filaments (Fig 7A & A(i) and Supplementary Fig 2A & 2C). After a 40min treatment of hERG1a transfected HEK293T cells, step currents were unaffected by shear (74.5±42.38pA/pF at 1ml/min and 77.1±47.84pA/pF at 10ml/min). A 14.5% increase in tail current, from 59.9±17.71pA/pF to 68.5±18.91pA/pF (p=0.003, n=5, paired T-test) is smaller than the 30.4% increase in tail currents observed with untreated cells, but the response was not completely eliminated.

2 hours after cell plating, we used Nocadazole to disrupt the microtubule network and examined the effect of shear stress. hERG1a transfected HEK293T responded to shear stress after a 2hr treatment with 10µM Nocadazole (Fig 7B&7B(i)). The treated cells didn’t change morphology. Interestingly, tail current amplitudes were lower than the step current amplitudes in these cells. Shear stress increased the step current by 22.6% i.e. from 54.1±13.91pA/pF to 64.4±16.10pA/pF (p=0.01, n=5, paired T test) and the tail current by 33.6% i.e. from 35.3±13.3pA/pF to 47.2±16.6pA/pF (p=0.03, n=5, paired T-test). The increases seen are similar to those observed with untreated cells.

The extent to which disruption of the cytoskeleton affected the stress response was evaluated in terms of the attenuation of the stress response from that seen in untreated cells. Cytochalasin D essentially eliminated the effect of shear on step currents, while halving that on tail currents, as compared to untreated cells (mean±SD, p<0.01 & p<0.05 for step and tail current respectively, n=5, Mann Whitney test). Nocodazole treatment, on the other hand, had no effect on tail current sensitivity to shear while marginally attenuating the step current response (mean±SD, p>0.05, n=5, Mann Whitney Test). (Fig 7C).

Involvement of the Integrin/FAK mechano-transduction pathway

Integrins are central to the mechano-transduction process, conveying mechanical forces into the cell. β1 Integrins and their downstream effector FAK are associated with hERG1a in HEK293 cells (22). Fluid shear forces induce rapid remodeling of Focal adhesions formed by focal adhesion kinases (37). The FAK is tightly coupled to integrins and respond to integrin clustering during fluid shear stresses by autophosphorylation and activation (27). RGD peptides inhibit the binding of integrins to fibronectin (38). 0.1mM RGD peptide incubated for 30min changed the morphology of the cells plated on Fibronectin (Supplementary Fig 2A & 2B) indicating that RGD peptides were able to inhibit the binding of integrins to the ECM. The treated cells responded to Fluid Shear Stress by a small decrease in step and tail currents as shown in Fig 8A. The step currents declined from 38.98±21.95pA/pF to 31.26±18.05pA/pF (p=0.003, n=8, paired T-test) during shear stress and the tail current from 63.11±29.95pA/pF to 53.45±28.25pA/pF (p=0.004, n=8, paired T-test) – decreases of 19% and 15.33% in hERG1a step and tail current respectively.

FAK is an early downstream effector of integrins and has been shown to be activated in stretched cardiomyocytes (39). FAK involvement in the response to LSS was tested by preventing the activation of FAK by inhibiting the autophosphorylation at tyrosine 397. When dialyzed intracellularly with 50µM FAK inhibitor 14 (1, 2, 4, 5-benzenetetramine tetrahydrochloride) for 15mins, the enhancement of hERG1a currents was abrogated. Indeed, the currents declined under shear to an even greater degree than on inhibiting integrin. The step current density declined from 57.77±27.55pA/pF to 45.67±15.86pA/pF (p<0.05, n=8, paired T-test) under LSS and the tail current from 71.14±28.39pA/pF to 53.58±21.52pA/pF (p=0.007, n=8, paired T-test)- i.e. 29.9% and 25% decreases in hERG1a step and tail currents respectively (Fig 8B). Together these results indicate that integrins play a critical role in the mechanosensitivity of hERG channels and form the mechanosensor. The mechanical signal is
then conveyed via the activation of FAK to hERG1a channels.

**Long and Short QT mutants show differential shear sensitivity.**

Mutations in the human *ether-a-go-go-related gene* (*HERG*) cause LQT, an inherited disorder of cardiac repolarization (40–46). Reduction of $I_{Kr}$ causes delayed myocyte repolarization (47) and an increased risk of life-threatening ventricular arrhythmia. As seen in Fig 5C, heterotetrameric hERG1a/1b channels have low mechanosensitivity. This could be due to the presence of hERG1b which is neither sensitive to LSS nor possesses a PAS domain (Fig 5A). The add-back of the PAS domain restores some mechanosensitivity (Fig 5B). This suggests that interactions involving the PAS domain could be crucial for the electrical response of hERG1a to mechanical stress.

Responses of two Long QT type 2 mutants, with mutations in the PAS domain, were analyzed under laminar shear stress. Both N33T and R56Q mutations in the PAS domain of the hERG1a channel have accelerated channel deactivation and altered inactivation gating (48) due to disruption of the interaction of the amino terminal region with the rest of the channel. Laminar shear stress had minimal effect on the N33T mutant (Fig 9A & Supplementary Fig 3A). There was small but significant increase in step current density (43.49±18.38pA/pF pre-shear vs 47.05±20.61pA/pF) and tail current (40.1±17.21pA/pF vs 43.29±18.1pA/pF) observed during LSS (p=0.03, n=7, paired T-test). As shown in Fig 9B & Supplementary Fig 3B, the R56Q mutant showed a non-significant response to LSS (Step current density:152.6±38.03pA/pF pre-shear vs 156.4±38.13pA/pF under shear stress and tail current density: 139.2±36.69pA/pF pre-shear vs 150.3±46.63pA/pF under shear stress (p=0.05, n=7, paired T-test)). Thus both N33T and R56Q mutations in the PAS Domain display reduced shear sensitivity.

The Short QT mutant, N588K, is a gain of function mutation that abolishes hERG rectification and passes more current during the cardiac Action Potential (13–15, 49). N588K showed significant mechanosensitivity to LSS. As shown in Figure 9C and Supplementary Fig 3C, the step current density increased by 22.8% from 160.45±37.77pA/pF to 197±35.4pA/pF under shear stress (p=0.003, n=7) and tail current increased by 31.5% from 39.25±9.3pA/pF to 51.62±8.4pA/pF, (p=0.003, n=7, paired T-test). Hence, the N588K mutation does not affect mechanosensitivity.

**Shear stress increases hERG1a currents during a cardiac Action Potential.**

Our data suggest that hERG channels would open earlier in the course of the cardiac Action Potential under shear stress and also pass more current. To test this prediction we subjected cells expressing hERG1a to a voltage protocol derived from a pre-recorded action potential from a rabbit ventricular myocyte (29). (Figure 10). Current traces before and during shear stress are shown. hERG channels passed little current during phase 0, phase 1 and phase 2 of the action potential and peaked during phase 3, consistent with their role in the repolarizing phase of the cardiac AP. hERG currents increased even more at this phase under the influence of shear stress. Analysis of current traces before and during shear stress indicated that there was a 30.2±1.31% increase in peak current during the action potential voltage command (n=7).

**Discussion**

The issue of the mechanosensitivity of the cardiac electrical system is a crucial one and also one that is difficult to address. The magnitude of shear stress experienced by a single cardiomyocyte *in vivo* in a working myocardium is difficult to determine (5). Myocytes are cyclically deformed by variable diastolic shear and squeeze forces associated with interlaminar fluid flow and myocardial layers sliding against each other (50–52). Recourse then has to be taken to examine components of the electrical system in heterologous systems subjected to...
mechanical stress. The impact of laminar shear stress on ionic currents by bath perfusion has previously been studied in HEK293 cells heterologously expressing cardiac voltage gated sodium and calcium channels by whole cell patch clamp (6, 7, 53). The crucial role played by K channels in the control of cell excitability has gained considerable attention after discovering that among the causative factors of the long Q-T syndrome, a human genetic abnormality of the cardiac action potential repolarization, were mutations in the human ether-a-go-go related gene (HERG). These mutations result in alterations in the electrophysiology of heterologously expressed hERG channels (43–46).

Membrane stretch induced either by pressure using the patch pipette or variations in osmolarity have been reported to affect voltage-gated K+ currents (1). The kinetics reported are unlikely to be compatible with beat-to-beat modulation of cardiac currents (50, 53, 54) . Our experiments demonstrate that hERG channels respond to both osmotically induced stretch (Supplementary Fig 4) and shear stress. We have chosen to study shear because it is most likely to mimic the effects of the hemodynamic shear stress and because of the marked repeatability of its effects (55).

In this study, we have investigated mechano-electrical coupling in hERG channels using whole cell voltage clamp of hERG transfected HEK293T cells under fluid shear stress. Shear increases the observed hERG conductance in both the step and tail currents. The variation of conductance with shear could be well fit by a Boltzmann function suggesting that the channel exists in two states, and that shear facilitates switching between the states: a “low shear” state and a “high shear” state. The two states differ in a number of parameters: voltage dependence of activation and inactivation; deactivation, time constant of channel opening, inactivation and recovery from inactivation.

**Effect on activation properties**

An analysis of the voltage dependence of the tail currents shows that laminar shear stress induces a hyperpolarizing shift of 9mV in the I-V curve for the step current (Fig 2). These shifts are reversible over two cycles of shear and relaxation spread over more than 10mins (Fig 1D and 2D). They are thus unlikely to arise from a time-dependent drift of channel properties as previously reported for Shaker K+ channels (56). The rate of channel opening was faster under shear than in control cells. In addition, deactivation is slowed significantly under shear (Fig 3B & C). These changes in activation properties favor the open state and could result in a significant increase in repolarization current.

**Effect on Inactivation properties:**

Both onset of and recovery from inactivation are influenced by fluid shear. The shift towards depolarizing potentials in the fractional availability curve seen under LSS is qualitatively consistent with faster recovery from inactivation. Enhanced rate of onset of inactivation would reduce outward K+ currents and thereby aid in faster upstroke velocity due to Na+ influx through sodium channels. On the other hand, rapid recovery from inactivation will result in earlier K+ current rebound during repolarization, thus shortening the APD. Due to faster inactivation upon application of LSS, there is a change in rectification voltage which shifts towards hyperpolarized potentials. This property of hERG channel could avert premature beats due to pressure overload in the heart (57–59) thereby serving a protective role during increased fluid shear stress.

**hERG1b channels are insensitive to shear**

Neither step currents nor tail currents observed in hERG1b expressing HEK cells is affected by shear to a significant extent (Fig 5A). hERG1a and hERG1b co-assemble in cardiac cells, although information regarding native stoichiometry is unavailable (29, 34, 60). Co-expression of the two constructs led to significantly larger currents than was observed
in cells expressing either construct alone (Fig 5C). However, these currents were less sensitive to shear than was hERG1a alone. We ascribe this decreased sensitivity to the fact that hERG1b channels are insensitive to shear and hERG1b subunits probably contribute to a significant fraction of the channels under study. hERG1b is shorter than hERG1a primarily due to an N-terminal deletion that removes the PAS domain. Expression of the PAS domain alone together with hERG1b partially restores sensitivity, but only to tail currents (Fig 5B). Heteromultimeric hERG1a/1b channels display sensitivity intermediate between hERG1a alone and hERG1b + PAS.

Amino-terminal region is responsible for shear sensitivity

Genetic alterations in the PAS domain of hERG channels have been linked to LQT syndromes. Homomeric hERG1a channels have relatively slow channel activation and recovery from inactivation, the two major components which determine K+ current amplitude during repolarization. Heteromeric hERG 1a/1b channels have fewer PAS domains and show a significant increase in current amplitude due to faster activation and recovery from inactivation (29). Antibodies directed against the PAS domain also increase current amplitude by modulating hERG channel kinetics. Interaction of the PAS domain with the cyclic nucleotide binding homology domain (CNBh domain) present in the c-terminal region (61) of the hERG channel has been implicated in these changes in current amplitude (62). It is conceivable that shear stress might also modulate the interaction of the PAS domain with the CNBh domain thereby affecting the gating kinetics.

The N33T and R56Q mutations in the PAS domain extend the cardiac Action Potential resulting in a long QT interval. Channels mutant at either position are less sensitive to LSS (Fig 9A, 9B & Supplementary Fig 3).

Loss of function mutations in hERG result in an increase in the QT interval. Shear tends to shorten the QT interval and could have played a protective role. However, long QT mutants do not respond to shear (Fig 9 & supplementary Fig 3), and thus the protective role played by hERG during increased shear stress is lost in patients carrying loss of function mutations. The gain of function mutant N588K carries more charge during action potential clamp experiments (15), consequently shortening the cardiac action potential. Shear stress leads to even more current through an already over activated channel thus predisposing the mutation carriers towards life threatening conditions.

Our data on deletion of the PAS domain (hERG1b), mutations within the PAS domain (N33T and R56Q) and a mutation outside the PAS domain (N588K, which retains mechanosensitivity) implicate the PAS domain in transducing mechanical shear to the modulation of hERG electrical activity.

Integrin pathway and cytoskeletal elements involvement in the mechanosensation of hERG channels.

If the PAS domain is a major contributor to mechanosensitivity, then it is likely that it interacts with elements that transduce mechanical stress at the cell membrane to internal structures. hERG channels are physically associated with β integrins and undergo current activation after adhesion of integrins to fibronectin followed by association of focal adhesion kinase which undergoes tyrosine phosphorylation (22). Integrins are the main receptors which connect the cytoskeleton to the extracellular matrix (ECM) (27, 63, 64). There is bi-directional transmission of force:tractional force from the cytoskeleton is passed onto the ECM via integrins, and stresses applied by the ECM to the cell surface are transmitted and transduced by integrins. The transduction of physical forces into chemical forces by integrins involves the triggering of multiple signaling pathways (65). Autophosphorylation of Focal adhesion kinases on tyrosine 397 is sensitive to the tethering of integrin to a rigid substratum, and is responsible for cell movement in durotaxis (27). Thus FAK
appears to be an important component of the integrin mediated mechanotransduction apparatus. As shown in Fig 8, preventing the binding of Integrins to Fibronectin or blocking of FAK autophosphorylation at Tyr 397, completely altered the effect of fluid shear stress on hERG1a currents, implicating Integrins and their downstream effector FAK in the mechanosensitivity of the hERG1a channels. Disrupting cytoskeleton elements attenuated shear responses of hERG channels but the effect was less pronounced than on disrupting integrin signaling. A fall-out of our findings is that drugs which affect integrin function could in turn affect mechanosensitivity of hERG channels. This would pose a cardiac liability which normally goes unnoticed during regular preclinical screening.

**hERG currents during a Cardiac Action Potential**

Our data indicate that shear shifts hERG channels from a “low shear” state to a “high shear” state that opens more rapidly and at less depolarizing potentials, which inactivates more rapidly and also recovers more rapidly from inactivation. The implication would be that more current would be passed by the “high shear state” of the channel in the course of the cardiac action potential. Our experiment with the Dynamic Clamp (Figure 10) simulates the voltage changes over the course of an actual cardiac AP. hERG currents are, in fact, over 30% larger under shear than in its absence. The caveat here is that the shear was applied continuously and not in a pulsatile manner as in the heart. It is not clear how myocytes, and in particular the integrin signal transduction pathway, integrate the mechanical stresses of a beating heart. Our data demonstrate that hERG currents are sensitive to continuous shear, though the behavior under pulsatile shear remains to be established.

**Experimental procedures:**

**Cell Culture and Transfection:**

HEK293T cells were routinely cultured in DMEM (GIBCO) supplemented with 10% FBS, 0.5% Pen Strep (Sigma) and incubated at 37°C in 5% CO₂. hERG1a and hERG1b in pXOOM vector were gifts from Dr. Nicole Schmitt, University of Copenhagen, Denmark. Long QT mutants, R56Q, N33T and PAS domain (1-131AA) in pCDNA3.1 were kind gifts from Mathew Trudeau, University of Maryland and N588K in pCDNA3.1 was a gift from Jules C Hancox, University of Bristol, UK. Plasmids were transfected using Effectene reagent (Qiagen). 48hours after transfection, cells were dissociated by treating them with 0.05% trypsin plus 0.02% EDTA and seeded on fibronectin (FN) (Sigma) coated coverslips (20). Patch clamp recordings were performed 5-6 hrs after plating.

**Generation of shear stress:**

Shear stress was generated by a gravity fed perfusion system placed 40 cm above the experimental chamber and attached to a flow regulator. The bath chamber incorporated diamond shaped fluidics yielding laminar flow throughout the bath (Warner instruments). A schematic of this chamber is illustrated in Supplementary Figure 5. The liquid surface of the perfusion chamber during experiments was kept flat (neither concave nor convex). Shear stress levels were calculated from the flow rates and tube geometry on the basis of the fully developed Poiseuille flow solution in a tube of circular cross section as previously described (28).

The magnitude of the shear stress at the outlet was calculated using the equation:

\[ \tau = 4\mu Q/\pi r^3 \]

where \( \tau \) is the shear stress in dyn.cm⁻², \( \mu \) is the viscosity of the fluid (dyn.s/cm²), \( Q \) is the volumetric flow rate (cm³.s⁻¹) and \( r \) is the internal diameter of the inlet tube. Most
measurements were made at a flow rate of 10ml/min, which provided maximum activation of the hERG current.

**Electrophysiology:**

Voltage clamp recordings were performed on hERG transfected HEK293T cells in the whole cell patch configuration using a patch clamp amplifier (EPC10, HEKA). Patch pipettes had resistances between 1 and 3 MΩ. Series resistance compensation was typically 60% to 70%, such that voltage errors were less than 5 mV. No leak subtraction was applied; cells exhibiting leak conductance > 10% maximal conductance were excluded from the study. Data were acquired using Patchmaster (HEKA) acquisition software. To study the current voltage relationship, cells were held at -80mV and stepped to a range of potentials for 3s at 10mV increments, followed by a step repolarization to -60mV for 2s to measure the outward tail current. The tail current amplitudes were used for plotting the steady state activation. To study the time constants of activation, deactivation of HERG and inactivation properties, specific pulse protocols were used as described in the figure legends. Changes in the hERG K⁺ channel current amplitude in response to step changes in flow rates were monitored using a single step depolarization to 0mV for 2s from a holding potential of -80mV, followed by a hyperpolarizing step to -60mV for 3s to elicit the tail current. The pipette solution contained (mmol/L) KCl 130, MgCl₂ 2, CaCl₂ 0.5, EGTA 5, Mg-ATP 4 and HEPES 10, adjusted to pH 7.2 with KOH, Osmolarity maintained at 307-310mOsm/kg. External bath solution (in mmol/L) contained NaCl 150, CaCl₂ 1.8, KCl 4, MgCl₂ 1, Glucose 10 and HEPES 10, adjusted to pH 7.4 with NaOH, osmolarity was maintained at 321-327mOsm/kg. For cell swelling experiments the iso-osmotic extracellular solution (in mmol/L) contained 100mM NaCl, CaCl₂ 1.8, KCl 4, MgCl₂ 1, Glucose 10, HEPES 10 and Sorbitol 80 adjusted to pH 7.4 with NaOH. The Hypo-osmotic solution lacked Sorbitol. Experiments were performed in room temperature. Electrical signals were filtered at 2kHz and digitized at 5kHz.

**Materials:**

Colchicine (10μM) and Nocadazole (10μM) were obtained from Sigma. RGD peptide and FAK14 inhibitor (50μM) were obtained from Santacruz Biotechnology.

**Statistics:**

Data were analyzed with Fitmaster (HEKA), Clampfit (Axon) and Origin (OriginLab, Northampton, UK). Conductance and voltage data were fitted to a single Boltzmann function: 

\[ \frac{I}{I_{\text{max}}} = \left( \frac{I_{\text{min}}}{I_{\text{max}} - I_{\text{min}}} \right) \left[ 1 + \exp \left( \frac{V_{1/2} - V}{k} \right) \right]^{-1} \]

where, \( V_{1/2} \) is the half-activation potential, \( V \) is the test voltage, and \( k \) is the slope factor. Current relaxations with repolarizing voltage steps (deactivation) and the rising phase of the repolarizing voltage step (recovery from inactivation) were fit with an exponential function (\( y = Ae^{-t/\tau} \)) where \( t \) is time and \( \tau \) is the time constant of deactivation or recovery from inactivation. The time course of hERG current activation was measured using single exponential fits of the peak tail current envelope recorded at -60mV. The inactivation time constant was determined using a three-step protocol, as described in Fig 6 (legend), to isolate inactivating currents, which were fit with a single-exponential function. Steady-state inactivation was measured using a separate three-step protocol, as described in Fig 6 (legend). The instantaneous currents at 40mV were normalized and fit with a Boltzmann function. Values presented are mean±standard deviation (SD). Paired T test and Mann-Whitney test were used to compare the difference between 2 groups. Significance level was set at a value of \( p<0.05 \). \( p<0.05 \), \( p<0.01 \) and \( p<0.001 \) are represented as *, ** and *** respectively in the figures. \( n \) represents the number of experiments.
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Author contributions: SR and MKM designed the experiments. SR carried out the experiments and analyzed the data. SR and MKM wrote the manuscript. The authors declare no conflict of interest

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Footnotes

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Figure Legends

Figure 1: Laminar shear stress mediated effects on hERG1a currents A and B. Representative current trace in response to a step potential to 0mV(2s) to activate hERG currents followed by a tail current elicited at -60mV(3s) from a holding potential of -80mV. Pre-shear and Shear stress response are pointed with arrows. B, Scatter plot shows current enhancement during shear stress at both step and tail current. (mean±SD; p<0.001, n=20, paired T-test). Scale bars, 2nA and 500ms. C, Dose dependent effect of fluid shear stress (flow rate in ml/min) on hERG1a tail currents. X axis denotes different flow rate and Y axis is the percentage increase during shear stress application (mean±SEM, n=5). D, Time course of the effect of shear stress on hERG1a tail currents. Responses of hERG1a currents between pre-shear and laminar shear stress were reversible (mean±SEM, n=6). Individual data points at each given voltage are represented as mean±SEM.

Figure 2: Laminar shear stress effect on hERG 1a channel activation A and B. Representative current traces in response to a series of membrane depolarizations in a HEK293T expressing hERG1a. Currents were elicited by a series by 2 second depolarization steps (between -70 and +70mV) followed by repolarization step to -60mV. Scale bar, 2nA and 1 second. C, Current-voltage (I-V) relation curve plotted from current measured at the end of depolarizing step potentials (Relative current amplitude, I/I_max, during depolarizing step potentials plotted against the step potential, I_max=maximal current during application of shear stress.). D, Voltage-dependent activation curves plotted from peak tail currents during a repolarization step to -60mV after depolarization to various potentials. Data normalized to the maximum current in the same curve. The solid lines and dashed lines correspond to the fitted Boltzmann functions. The V_1/2 and the slope factor for pre-shear state is 0.6±0.23 mV and 8.26±0.38mV respectively and during laminar shear stress was -8.8±0.39mV and 9.165± 0.34mV respectively. Post shear switch to pre-shear flow rate yielded a V_1/2 of -2.4±0.46 and a slope factor of 5.57±0.040mV (mean±SD, n=7). Individual data points at each given voltage are represented as mean±SEM.

Figure 3: Effect of laminar shear stress on deactivation properties of hERG1a A. Reversal potential measurement from a fully activated I-V. Currents were elicited by a 2 second depolarization step potential to 0mV followed by a 3s repolarization steps (between -120 to +20mV). Scale bar; 2nA and 1 second. (n=4). B and C, Tail currents measured between -120 and -40mV with an interval of 10mV was fitted with a double exponential function. Time constants of fast and slow components at -60mV and -100mV are plotted for comparison between pre-shear and shear stress (p<0.05, n=5, paired T test). Individual data points at each given voltage are represented as mean±SEM.

Figure 4: Time course of activation is faster after application laminar shear stress A. Currents evoked using an envelope of tails protocol to determine the time course of activation. Peak tail current were evoked by a step to -60mV following a pre-pulse of increasing duration to 0mV. Holding was -80mV. B, Apparent activation is faster during shear stress application. Individual data points at each given voltage are represented as mean±SEM.. The peak amplitudes of the tail currents (A) were plotted against test pulse duration and fitted to a single exponential function. C, Time constant of activation (t_{1/2}) was 279.9±58.2ms at a flow rate of 1 ml/min and 155.5±49.2ms under LSS (mean±SD, p=0.0001, n=8 paired T test).

Figure 5: Contribution of PAS domain on hERG mechanosensitivity. A, hERG1b currents shows no response to laminar shear stress (mean±SD, p>0.05, n=7, paired T test). B, hERG1b co-transfected with 1-131AA (PAS domain). Tail currents responded to shear stress (mean±SD, p<0.05, n=5, paired T test). C, Response of Heterotetrameric hERG1a/1b channels to laminar shear stress (mean±SD, p<0.01, n=7-8, paired T test).
Figure 6: Effect of LSS on inactivation properties of hERG1a. A, Currents were measured at +40mV following a series of 10ms steps to range of voltages from +30mV to -140mV in 10mV decrements. The data was fitted to a Boltzman function. Steady state inactivation plot showing shift of $V_{1/2}$ and slope factor from $-54.64\pm 3.3$mV and $-21.01\pm 3.38$mV respectively (Pre-Shear) to $-51.45\pm 3.63$mV and $-20.13\pm 3.66$mV respectively (Shear stress). A right shift $V_{1/2}$ of 3.1mV ($n=7$), Scale Bar, 5nA and 1 second. B, 200ms pulse to +40mV to activate and then inactivate hERG followed by a brief hyperpolarizing step (10ms) to -120mV to allow the channels to recover from inactivation. In the third pulse, carrying the potential between 0mV to +40mV allowed the inactivation time course to be measured as a function of voltage. The time constant of onset of inactivation are estimated by fitting the decay of the currents in the third pulse to a single exponential function and plotted as a function of test potential ($p<0.01$ at 0mV, $n=9$, paired T test) Scale Bar, 4nA and 50ms. C, Tail currents were evoked at different potentials from -60 to +10mV following a 4 second, +20mV pulse. Plot quantifying showing data from recovery from inactivation at -50mV is faster during shear stress. Time constant were measured as the single exponential fit to the rising phase of the tail current ($p<0.001$ at -50mV, $n=5$, paired T test) Scale Bar, 2nA and 1 second. Individual data points at each given voltage are represented as mean±SEM.

Figure 7: Shear stress and Cytoskeleton. Representative current traces from hERG1a channel before (Pre-shear) and during laminar shear stress (shear stress) are pointed with arrows. Currents were elicited by a depolarizing step to 0mV followed by a repolarization to -60mV from a holding potential of -80mV. A(i), 10µM Cytochalasin D was incubated for 40mins to disrupt actin filaments. Scatter plot shows partial mechanosensitivity as seen with increase in the tail current after LSS application (mean±SD, $p<0.05$, $n=5$, paired T test). Scale Bar, 1nA and 500ms. B(i), 10µM Nocadazole incubated for 2hrs to destroy the microtubule network. Scatter plot shows increase in hERG currents during application of shear stress (mean±SD, $p<0.01$, $n=5$, paired T test). Scale bar, 1nA and 500ms. Untreated vs Cytochalasin/Nocadazole. C, 10µM Cytochalasin D was incubated for 40mins to disrupt actin filaments. (mean±SD, $p<0.05$, $n=5$, Mann Whitney test). 10µM Nocadazole incubated for 2hrs to destroy the microtubule network. (mean±SD, $p>0.05$, $n=5$, Mann Whitney Test).

Figure 8: Shear stress and Integrin pathway. Representative current traces from hERG1a channel before (Pre-shear) and during laminar shear stress (shear stress) are pointed with arrows. Currents were elicited by a depolarizing step to 0mV followed by a repolarization to -60mV from a holding potential of -80mV. A, 0.1mM RGD peptide incubated for 30mins to block integrin binding to Fibronectin. Scatter Plot shows complete loss of mechanosensitivity as seen in both step and tail current (mean±SD, $p<0.01$, $n=8$, paired T test) during LSS application. Scale Bar, 1nA and 500ms. B, 50µM FAK inhibitor 14 dialyzed in the cell through patch pipette for 15mins. Scatter plot shows attenuation of mechanosensitivity of hERG currents in step current (mean±SD, $p<0.05$, $n=8$, paired T test) and tail current (mean±SD, $p<0.01$, $n=8$, paired T test) during application of shear stress. Scale bar, 1nA and 500ms.

Figure 9: Shear sensitivity of hERG mutants. A, Scatter plot shows response of N33T mutant to shear stress (mean±SD, $p<0.05$, $n=7$, Paired T test). B, Scatter plot shows response of R56Q mutant to shear stress (mean±SD, $p<0.05$, $n=7$, Paired T test). C, Scatter plot shows response of N588K mutant to shear stress. Both step (mean±SD, $p<0.001$, $n=7$, Paired T-test) and tail current (mean±SD, $p<0.001$, $n=7$, Paired T-test) showed significant increase in current during shear stress.

Figure 10: Enhancement of hERG1a currents during rabbit action potential stimulus. Different phases of the action potential are labelled as 0, 1, 2, 3 and 4. LSS increases total potassium ions conducted by hERG1a channels during an action potential stimulus (Scale bar, 50mV and 200ms). Typical current traces before and during shear stress are shown (Scale bar, 2nA and 200ms). Dashed line represents the zero current level ($n=7$).

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Figure 1

A

mV
-80
-60
-50
0
-60mV
Shear stress
Pre-shear
Tail current
Step current
2nA
500ms

B

Current density (pA/pF)

Step current (pre shear)
Step current (Shear)
Tail current (Pre shear)
Tail current (Shear)

C

Increase in hERG currents (%)

Flow rate (ml/min)

D

I/I_max

Time (s)
Figure 9

A

N33T

B

R56Q

C

N588K

Current density (pA/pF)

Step current (pre shear) | Step current (Shear) | Tail current (pre shear) | Tail current (Shear)

Current density (pA/pF)

Step current (pre shear) | Step current (Shear) | Tail current (pre shear) | Tail current (Shear)
Figure 10

Shear Stress

200ms

2nA

50mV

Pre-Shear

0

1

2

3

4

Figure 10
