Rapid Report

Functional consequences of the arrhythmogenic G306R KvLQT1 K^+ channel mutant probed by viral gene transfer in cardiomyocytes

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1. I_{Ks}, the slow component of the delayed rectifier potassium current, figures prominently in the repolarization of heart cells. The K^+ channel gene *KvLQT1* is mutated in the heritable long QT (LQT) syndrome. Heterologous coexpression of KvLQT1 and the accessory protein minK yields an I_{Ks}-like current. Nevertheless, the links between KvLQT1 and cardiac I_{Ks} are largely inferential.

2. Since the LQT syndrome mutant KvLQT1-G306R suppresses channel activity when coexpressed with wild-type KvLQT1 in a heterologous system, overexpression of this mutant in cardiomyocytes should reduce or eliminate native I_{Ks} if KvLQT1 is indeed the major molecular component of this current. To test this idea, we created the adenovirus AdRMGI–KvLQT1-G306R, which overexpresses KvLQT1-G306R channels.

3. In $> 60\,\%$ of neonatal mouse myocytes, a sizable I_{Ks} could be measured using perforated-patch recordings (8.0 ± 1.6 pA pF^{-1}, $n = 13$). I_{Ks} was increased by forskolin and blocked by clofilium or indapamide but not by E-4031. While cells infected with a reporter virus expressing only green fluorescent protein (GFP) displayed I_{Ks} similar to that in uninfected cells, AdRMGI–KvLQT1-G306R-infected cells showed a significantly reduced I_{Ks} (2.4 ± 1.1 pA pF^{-1}, $n = 10$, $P < 0.01$) when measured 60–72 h after infection. Similar results were observed in adult guinea-pig myocytes (5.9 ± 1.2 pA pF^{-1}, $n = 9$, for control *vs.* 0.1 ± 0.1 pA pF^{-1}, $n = 5$, for AdRMGI–KvLQT1-G306R-infected cells).

4. We conclude that KvLQT1 is the major molecular component of I_{Ks}. Our results further establish a dominant-negative mechanism for the G306R LQT syndrome mutation.

Mutations of the *KvLQT1* (or *KCNQ1*) gene are responsible for many cases of inherited long QT (LQT) syndrome (Wang *et al.* 1996) including the autosomal dominant Romano-Ward syndrome (RWS; Ward, 1964). The LQT syndrome consists of a prolonged QT interval on the electrocardiogram, syncope, ventricular arrhythmias and sudden death. These clinical findings imply that KvLQT1 is essential for proper cardiac repolarization. When coexpressed with the regulatory protein minK in heterologous systems, KvLQT1 produces currents with biophysical and pharmacological properties resembling the slowly activating component (i.e. I_{Ks}) of the delayed-rectifier potassium currents observed in native cardiac cells (Barhanin *et al.* 1996; Sanguinetti *et al.* 1996). Despite these compelling lines of evidence, the links between KvLQT1 and cardiac I_{Ks} remain largely inferential.

In the present study, we sought to confirm KvLQT1 as a critical molecular component of I_{Ks} by overexpressing a *KvLQT1* construct carrying the dominant negative Romano-Ward mutation G306R (Wang *et al.* 1996; Wollnik *et al.* 1997) in cardiomyocytes via viral gene transfer and looking for changes in native I_{Ks}. We found that virally mediated overexpression of this construct results in significantly diminished I_{Ks}, confirming the critical role of KvLQT1 in I_{Ks} and further establishing a dominant-negative mechanism for the G306R disease mutant.

METHODS

The present investigation conformed to the standards of the US National Institutes of Health regarding the care and use of laboratory animals, and was performed in accordance with the guidelines of the Animal Care and Use Committee of the Johns Hopkins University.

Isolation of ventricular myocytes

Neonatal myocytes were prepared as previously described (Nuss *et al.* 1994). Briefly, ventricles of neonatal mice (1–2 days old, CD-1, Charles River) were aseptically removed immediately following decapitation. A single litter of mouse pups (~10) was used for each experiment. Isolated hearts were pooled, minced and digested at room temperature with trypsin. When the digestion was complete, cells were centrifuged, plated at low density (2×10^5 cells ml^{-1}) and incubated at 37 °C in a humidified atmosphere of 95% O_2–5% CO_2. Adult guinea-pigs were anaesthetized with sodium pentobarbital by peritoneal injection. Ventricular myocytes were isolated using Langendorff perfusion and collagenase digestion (Hoppe *et al.* 1999), and similarly cultured at 37 °C.

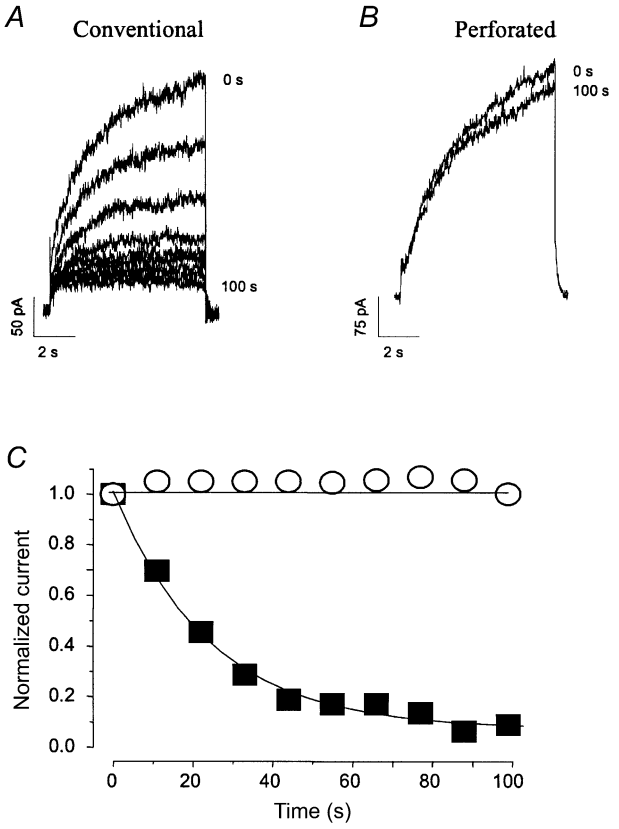

Figure 1. I_{Ks} in uninfected neonatal murine ventricular myocytes

A and *B*, representative raw current traces of slowly activating time-dependent outward currents (I_{Ks}) elicited by long depolarization (8 s) of neonatal mouse myocytes to +70 mV from a holding potential of −40 mV using conventional patch-clamp (*A*) and perforated-patch (*B*) techniques. *C*, current magnitude, measured as the difference in outward current at the beginning and end of the pulse, is plotted against time during these experiments. ■, conventional; ○, perforated-patch recording. Rapid current run-down was observed when I_{Ks} was recorded using the conventional patch-clamp technique but was absent when the perforated-patch technique was employed. Cell capacitance was 42 and 25 pF, respectively, for these cells.

Molecular biology and virus preparation

The human *KvLQT1* gene (provided by Dr Mark Keating, University of Utah) was subcloned into the vector pAdEGI (Johns *et al.* 1999) to make pAdEGI–KvLQT1. Human *minK* was first amplified from purified genomic DNA by PCR and then subcloned into pAdEGI–KvLQT1 making a fusion gene with *minK* at the 5′ end and enhanced green fluorescent protein (EGFP, Clontech, Palo Alto, CA, USA) at the 3′ end, creating pAdEMGI–KvLQT1. When expressed in CHO-K1 cells, this tandem construct directed expression of currents similar to those produced by coexpressing *minK* and *KvLQT1* from separate constructs (data not shown). The point mutation G306R was introduced by PCR with overlapping mutagenic primers. The product pAdEMGI–KvLQT1-G306R was sequenced to confirm that the desired mutation was present. The MGI–KvLQT1 and MGI–KvLQT1-G306R sequences were then spliced into pAdRGI, which carries the Rous sarcoma virus long terminal repeat promoter, to create the final constructs pAdRMGI–KvLQT1 and pAdRMGI–KvLQT1-G306R. Adenovirus vectors were generated by Cre-lox recombination of purified ψ5 viral DNA and shuttle vector DNA as previously described (Hardy *et al.* 1997). The recombinant products were plaque purified, yielding concentrations of the order of 10^{10} plaque-forming units (PFU) ml^{-1}.

Cardiomyocytes were infected by adding purified viruses to the cells at a final concentration of 10 000 particles ml^{-1}. For transfection of HEK 293 cells (human embryonic kidney cell line), the Lipofectamine Plus transfection kit (Gibco-BRL, Gaithersburg, MD, USA) was used. DNA encoding the channel (0.5 μg per well of 6-well plates) was added to the cells with lipofectamine. Transfected or virally infected cells were incubated at 37 °C.

Electrophysiology

Electrical recordings were performed using the whole-cell patch-clamp or perforated-patch technique (Hamill *et al.* 1981; Rae *et al.* 1991) with an integrating amplifier. Pipettes had tip resistances of 1–3 MΩ when filled with an internal solution containing (mM): KCl, 140; ATP (magnesium salt), 4; EGTA, 5; $MgCl_2$, 1; and Hepes, 10; pH 7.4. The external bath solution was composed of (mM): *N*-methyl-D-glucamine, 140; KCl, 5.4; glucose, 10; $MgCl_2$, 1; $CaCl_2$, 0.1; $CdCl_2$, 0.5; 4-aminopyridine, 5; and Hepes, 10; pH 7.4. Clofilium, indapamide, E-4031, forskolin and isobutylmethylxanthine (IBMX) were added to the bath as noted. Perforated-patch recordings were performed by including 100 μM amphotericin B in the pipette solution (Rae *et al.* 1991). All recordings were performed at room temperature (~23 °C) after 12–36 h in culture for uninfected cells and 60–72 h for virally infected or transfected cells. Only successfully infected or transfected cells, as identified by their green fluorescence using epifluorescence microscopy, were selected for experiments. Drug block was assessed from currents elicited at +70 mV (8 s) from a holding potential of −40 mV. Data reported are means ± S.E.M. with $P < 0.05$ indicating statistical significance.

RESULTS

Properties of I_{Ks} in neonatal mouse myocytes

When neonatal mouse ventricular myocytes were depolarized, > 60% of the cells displayed a time-dependent outward current typical of I_{Ks}. Rapid run-down of I_{Ks} was observed when using the conventional patch-clamp technique (Fig. 1*A* and *C*), as previously

reported (Nuss *et al.* 1994). Little current (< 0.5 pA pF^{-1}) remained after complete run-down of I_{Ks}, suggesting that the contribution of the rapid component of the delayed rectifier (I_{Kr}) to the total current was minor under our experimental conditions. In contrast, I_{Ks} recorded with the perforated-patch technique (Rae *et al.* 1991) did not run down (Fig. 1*B* and *C*). Subsequent data presented here were collected using this technique.

Figure 2*A* shows the current–voltage relationship of I_{Ks}. As is characteristic of this current, I_{Ks} increased with progressive depolarization. Current density in these cells was 8.0 ± 1.6 pA pF^{-1} ($n = 13$) at the end of 8 s depolarizing pulses to +70 mV. We next examined the pharmacological effects of indapamide, clofilium, E-4031 and forskolin on I_{Ks} (Fig. 2*B*). Application of 100 μM clofilium, a delayed-rectifier blocker (Arena *et al.* 1988; Colatsky *et al.* 1990) inhibited the current to 55.7 ± 5.6% ($n = 5$) of the control level. Similarly, application of 1 mM indapamide, a putatively selective blocker of I_{Ks} (Turgeon *et al.* 1994), reduced current to 47.6 ± 11.9% ($n = 5$). In contrast, application of 10 μM E-4031, a

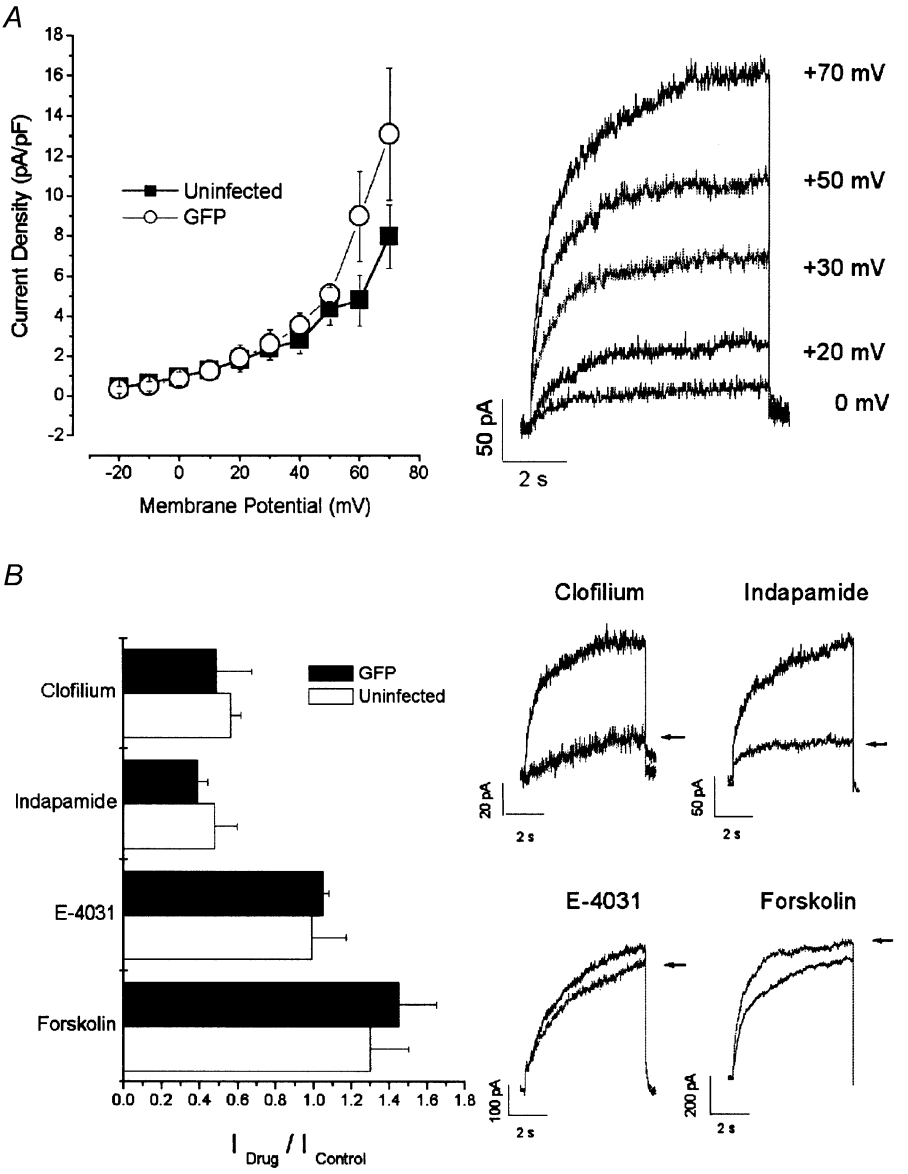

Figure 2. I_{Ks} in neonatal mouse myocytes

A, current–voltage (*I–V*) relationship (left panel, ■) and raw records of a family (right panel) of I_{Ks} recorded from uninfected myocytes using the perforated-patch technique. *B*, left, the effects of 100 μM clofilium, 1 mM indapamide, 10 μM E-4031 and 10 μM forskolin + 100 μM IBMX (Forskolin) on the current amplitude of I_{Ks} were normalized to those measured under drug-free conditions and plotted as bar graphs (□). Right, typical I_{Ks} currents recorded before and after (arrows) application of these drugs. *I–V* relationships (left panel of *A*, ○) and pharmacology (left panel of *B*, ■) of I_{Ks} recorded from AdRGI-infected cells are also shown.

benzenesulphonamide agent which blocks I_{Kr} selectively (Sanguinetti *et al.* 1990), failed to inhibit the current (96.5 ± 3.2%, $n = 3$) consistent with the notion that I_{Ks} was the predominant component. Adrenergic agonists such as forskolin and isoproterenol (isoprenaline) are known to upregulate I_{Ks}, but not I_{Kr}, by elevating the level of intracellular cAMP (Varnum *et al.* 1993). We thus tested the effect of forskolin (10 μM) on I_{Ks} by co-applying it with IBMX (100 μM), a phosphodiesterase inhibitor, which potentiates the cAMP elevation by forskolin. As anticipated, application of forskolin + IBMX enhanced the amplitude of I_{Ks} (130 ± 20%, $n = 2$).

Viral gene transfer of KvLQT1-G306R to cardiomyocytes

Since the Romano-Ward mutation G306R is known to exert a dominant-negative effect on WT KvLQT1 channel activity in a heterologous expression system (Wollnik *et al.* 1997), overexpression of a construct carrying this mutation in cardiomyocytes should reduce or eliminate native I_{Ks} if KvLQT1 is indeed the major molecular component of this cardiac current. To test this hypothesis, we created the constructs pAdRMGI–KvLQT1 and pAdRMGI–KvLQT1-G306R (see Methods). Figure 3*A* shows the experiments that we performed to verify the

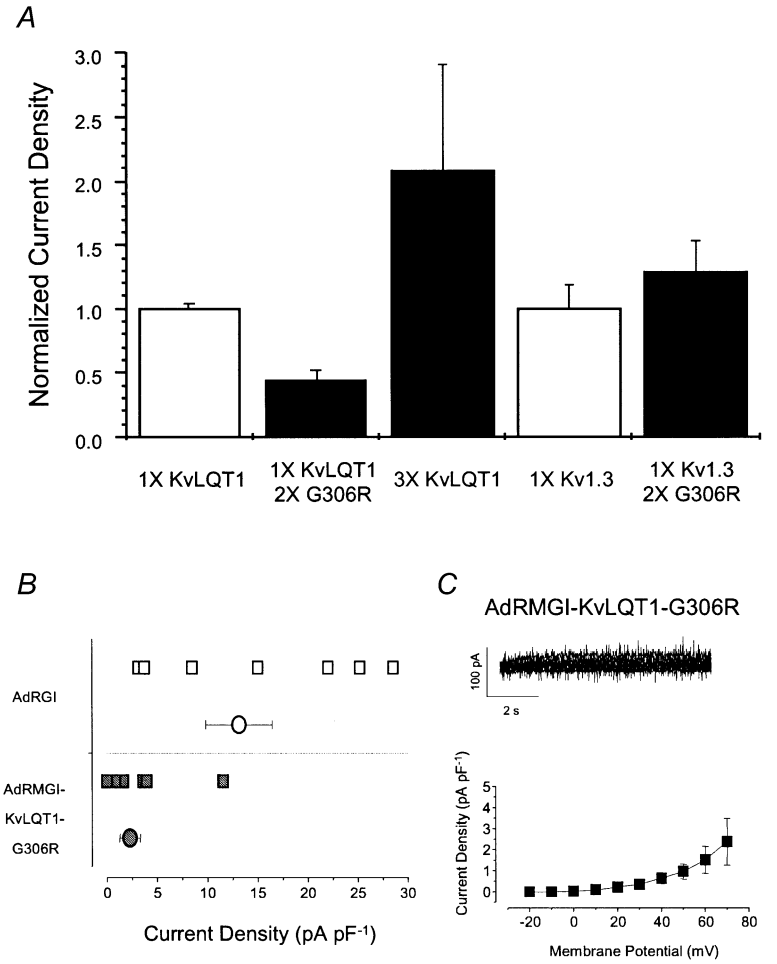

Figure 3. Effects of constructs carrying the dominant-negative mutation G306R

A, bar graphs summarizing the specific inhibitory effects of transfection of pAdRMGI–KvLQT1-G306 on KvLQT1 channels. For each group of channels, current amplitudes were normalized by the mean current of the same channel type (transfected with 1 × DNA) expressed alone. Current through KvLQT1 channels increased when more pAdRMGI–KvLQT1 DNA was used for transfection but was substantially suppressed when it was co-expressed with pAdRMGI–KvLQT1-G306R. In contrast, co-expression of Kv1.3 channels with KvLQT1-G306R did not lead to current suppression compared to expression of Kv1.3 alone. Currents were measured at the end of a 4 s, +70 mV or 1 s, +60 mV pulse from a holding potential of −40 or −100 mV for KvLQT1 and Kv1.3 channels, respectively. *B*, distribution of raw (squares) and averaged (circles) current densities at +70 mV recorded from AdRGI- (open symbols) and AdRMGI–KvLQT1-G306R-infected (filled symbols) neonatal mouse myocytes. The latter was significantly ($P < 0.05$) reduced. *C*, top, typical current traces of I_{Ks} recorded during a family of stepping voltages from AdRMGI–KvLQT1-G306R-infected myocytes. Bottom, *I–V* relationship.

efficacy of our dominant-negative construct. While transfection with 3 times the DNA (by mass) of pAdRMGI–KvLQT1 in HEK 293 cells more than doubled the current magnitude recorded after 72 h, co-transfection of pAdRMGI–KvLQT1 with pAdRMGI–KvLQT1-G306R (DNA ratio of 1:2) reduced current by ~60% compared to expression of WT KvLQT1 alone over the same period. In contrast, co-transfection of a plasmid encoding the Kv1.3 channel with pAdRMGI–KvLQT1-G306R (1:2), did not suppress current relative to expression of Kv1.3 alone. These observations confirm both the specificity and the inhibitory effect of pAdRMGI–KvLQT1-G306R.

Since myocytes are generally resistant to conventional methods of transfection, viral gene transfer techniques were employed to deliver our genes of interest. From the plasmids pAdRMGI–KvLQT1 and pAdRMGI–KvLQT1-G306R, we generated the adenoviruses AdRMGI–KvLQT1 and AdRMGI–KvLQT1-G306R, respectively (see Methods). As a control, we infected myocytes in parallel with AdRGI, which expresses only GFP. Myocytes infected with AdRGI displayed I_{Ks} with a current density (13.1 ± 3.3 pA pF^{-1} at +70 mV, $n = 8$, $P > 0.05$) and pharmacology profile similar to those of uninfected cells (Fig. 2). I_{Ks} measured from AdRGI-infected cells was enhanced by 10 μM forskolin with 100 μM IBMX (145 ± 20%, $n = 2$), blocked by 100 μM clofilium (48.3 ± 18.8%, $n = 2$) or 1 mM indapamide (38.8 ± 5.6%, $n = 3$) but was not affected by 10 μM E-4031 (105 ± 3.2%, $n = 2$; Fig. 2*B*). These observations indicate that infection of cells with an adenovirus reporter construct did not alter the biophysical or pharmacological properties of native I_{Ks}.

In contrast to AdRGI-infected cells (Fig. 3*B*, open symbols), I_{Ks} of AdRMGI–KvLQT1-G306R-infected cells (filled squares) was markedly suppressed (2.4 ± 1.1 pA pF^{-1} at +70 mV, $n = 10$, $P < 0.05$). Figure 3*C* shows the current–voltage relationship of I_{Ks} measured from AdRMGI–KvLQT1-G306R-infected cells. Application of 10 μM forskolin + 100 μM IBMX substantially increased the small remaining current (data not shown) but further pharmacological studies with blockers were not performed because of the small basal current amplitude.

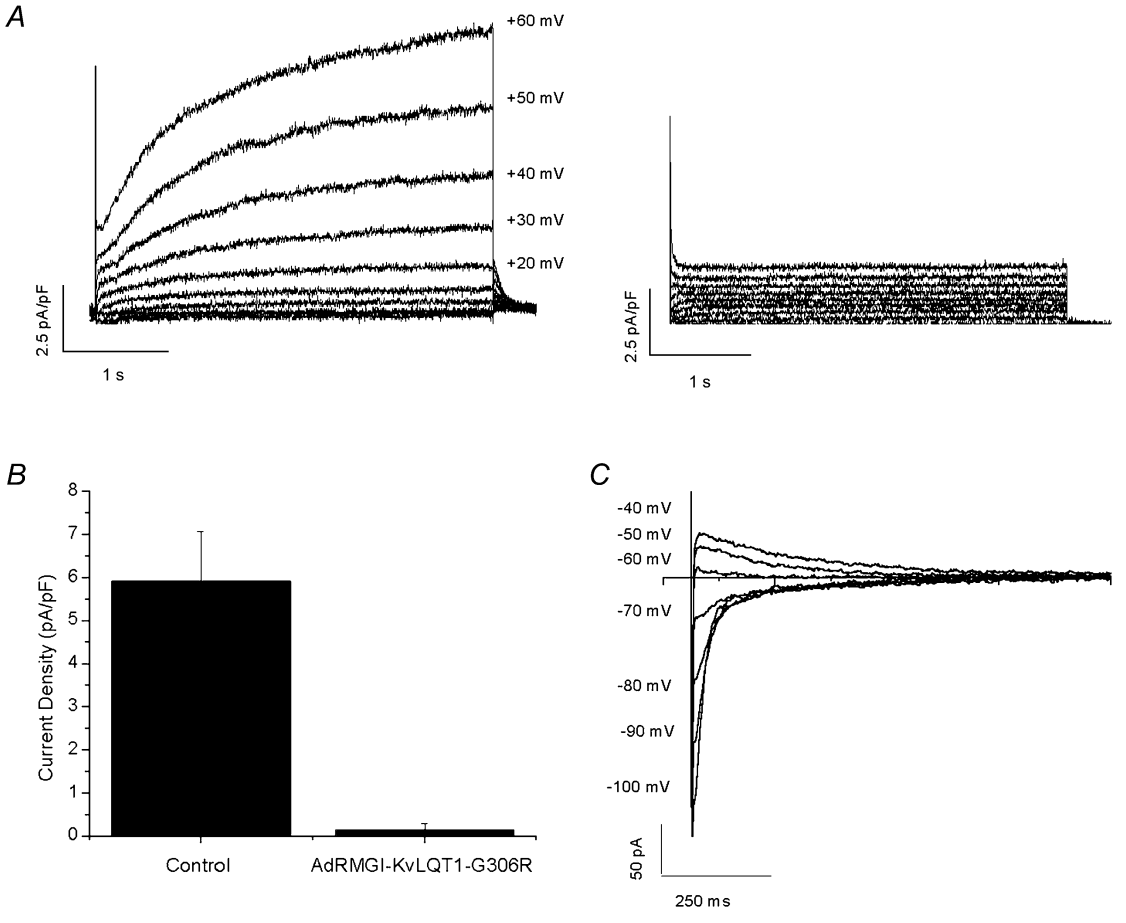

Figure 4. I_{Ks} in isolated adult guinea-pig ventricular myocytes

A, representative raw current traces of I_{Ks} recorded from control uninfected (left) and AdRMGI–KvLQT1-G306R-infected (right) myocytes. *B*, bar graph summarizing the current densities measured at the end of a 4 s depolarizing pulse to +60 mV. *C*, typical tail currents of I_{Ks} recorded from a control uninfected myocyte during a family of stepping voltages after a 4 s prepulse to +70 mV.

As a further confirmation of the identification of KvLQT1 as a critical component of I_{Ks}, we also infected isolated adult guinea-pig ventricular myocytes with our viruses. Similar results were obtained. I_{Ks} density was reduced from 5.9 ± 1.2 pA pF^{-1} ($n = 9$) in control uninfected cells to 0.1 ± 0.1 pA pF^{-1} ($n = 5$) in AdRMGI–KvLQT1-G306R-infected cells ($P < 0.05$) when measured at +60 mV (Fig. 4*A* and *B*). Tail current measurements from uninfected cells further indicate that the current recorded was fairly potassium selective (Fig. 4*C*); although the value of V_{rev} (-68.7 ± 3.1 mV, $n = 6$) was somewhat more positive than the predicted K^+ equilibrium potential (E_K, -86.9 mV under our conditions), our findings are consistent with prior reports of V_{rev} for I_{Ks} in native cardiomyocytes (Sanguinetti & Jurkiewicz, 1990). Tail currents were not studied in neonatal cells owing to the relatively small I_{Ks} amplitude in these cells, resulting from their small size (typically 5–40 pF), despite the sizable I_{Ks} current density.

DISCUSSION

Overexpression of dominant-negative constructs in native cells has been a useful tool to dissect the contributions of various ion channel genes to excitability and different native ionic currents (Johns *et al.* 1997; Barry *et al.* 1998). In the present study, we employed this strategy to confirm the identity of KvLQT1 as the major molecular component of native cardiac I_{Ks} by introducing a dominant-negative construct (G306R) into cardiomyocytes to cripple I_{Ks}. The specific inhibitory effect of the construct on KvLQT1 channels was confirmed by its lack of effect on other channels. Though the exact mechanism of such a dominant-negative effect on channel activity is still unknown, it is generally assumed that coassembly of WT and mutant subunits results in non-functional heteromeric KvLQT1 channels. However, since such stoichiometric combinations are stochastic, some functional homotetramers that are made up of only WT subunits also exist, leading to incomplete elimination of I_{Ks} as observed in AdRMGI–KvLQT1-G306R-infected cells. Alternatively, incomplete elimination may result from competition between expression of the suppressive gene products and turnover of pre-existing functional channel proteins. Regardless of the underlying mechanism, our work demonstrates for the first time that 'poison pill' KvLQT1 constructs suppress I_{Ks} in native myocytes. Such suppression could not be due to dilution of minK since AdRMGI–KvLQT1-G306R coexpresses its own minK (see Methods). Our observation is pathophysiologically relevant to the clinical manifestations of LQT syndrome, since diminished I_{Ks} would lead to prolongation of the action potential and the electrocardiographic QT interval, and subsequently increase the susceptibility of afflicted individuals to malignant arrhythmias. These data support the idea that KvLQT1 is the major molecular component of I_{Ks} and further establish a dominant-negative mechanism for this Romano-Ward mutant. This dominant-negative approach, applied here in cardiomyocytes via gene transfer, is generally useful for dissecting the molecular identity of various native ionic currents.

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