Long-term restitution of 4-aminopyridine-sensitive currents in Kv1DN ventricular myocytes using adeno-associated virus-mediated delivery of Kv1.5

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Abstract Overexpression of a dominant-negative truncated Kv1.1 (Kv1DN) polypeptide in the mouse heart resulted in marked attenuation of a 4-aminopyridine (4-AP)-sensitive current, $I_{K,slow1}$. We used recombinant adeno-associated virus (rAAV) as a vector for direct delivery of Kv1.5 into the mouse myocardium in order to normalize the action potential duration (APD) 6 months after injection. The injection of rAAV-Kv1.5 reconstituted the 4-AP-sensitive outward potassium currents, shortened the APD, and eliminated spontaneous early afterdepolarizations. Immunoblots detected the FL-Kv1.5 polypeptides only in rAAV-Kv1.5-infected hearts. These data demonstrate long-term expression of 4-AP-sensitive potassium currents in ventricular myocytes by gene transfer using rAAV vector encodes Kv1.5.

Key words: Adeno-associated virus; Delayed rectifier K^+ channel; 4-Aminopyridine

1. Introduction

Long QT syndrome is a genetically heterogeneous disease caused primarily by marked reduction in the repolarizing currents due to mutations of the α or β subunits of voltage-gated potassium channel genes [1,2]. These mutations reduce the repolarizing potassium currents by abnormal trafficking of the α subunit or through the formation of non-functional complexes in the membrane [3–5]. The overall effect is prolongation of the action potential duration (APD), which triggers the early afterdepolarizations (EADs), dispersion of repolarization, and refractoriness that underlie reentrant ventricular arrhythmias such as *torsade de pointes*, syncope, and sudden death [6].

We have created a mouse model for long QT phenotype by overexpression of a truncated Kv1.1 polypeptide (*Kv1N206Tag*) in the hearts of transgenic mice (Kv1DN). Ventricular myocytes derived from these mice have prolonged APD, while the mice themselves exhibit prolonged QT interval in the surface ECG and spontaneous and inducible polymorphic ventricular tachycardias [7,8]. Studies of Kv1DN ventricular myocytes showed the attenuation of a 4-aminopyridine (4-AP)-sensitive outward potassium current $I_{K,slow1}$ encoded by Kv1.5 [9]. Analysis of the cellular mechanism of the dominant-negative effect of *Kv1N206Tag* established that the truncated polypeptide trapped both Kv1.4 and Kv1.5 polypeptides in the endoplasmic reticulum [10]. The downregulation of $I_{K,slow1}$ leads to electrical modulation, with the upregulation of Kv2.1-encoded $I_{K,slow2}$ at the apex of the left ventricle (LV) [11]. This electrical remodeling leads to the formation of an apex-to-base dispersion of repolarization that underlies the reentrant arrhythmias [12].

Myocardial gene therapy represents a promising approach to the treatment of inherited heart diseases. Previous studies have shown efficient adenovirus (AV)-mediated gene transfer of numerous genes into the myocardium of several species, including mice [13–15]. Recently, we have shown that direct injection of adenoviral vectors expressing Kv1.5 into the heart resulted in reconstitution of 4-AP-sensitive outward potassium currents and shortening of the APD and the QT intervals [16]. However, the expression of AV-encoded proteins is only transient, due at least in part to a marked response of the immune system to the vector and the lack of integration of the viral vector with the host genome. Therefore, except for proof-of-concept studies, AV-vectors are of limited use for the long-term correction of somatic gene defects. Derived from a non-pathogenic human parvovirus, recombinant adeno-associated viral vectors represent an alternative to AV vectors for long-term expression of genes in the myocardium. Several studies have already established that these vectors can mediate long-term expression of polypeptides in the heart [17–19]. In this study we show for the first time that targeted regional infection with adeno-associated viral vectors encoding a voltage-gated potassium channel, Kv1.5, induced expression of these polypeptides 6 months after injection. This led to the restitution of the 4-AP-sensitive current, $I_{K,slow1}$, shortening of the APD, and elimination of the EADs in recombinant adeno-associated virus (rAAV)-Kv1.5-infected Kv1DN ventricular myocytes.

2. Materials and methods

2.1. Viral vectors

All animal studies were performed in accordance with the guidelines

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Abbreviations: APs, action potentials; APD, action potential duration; rAAV, recombinant adeno-associated virus; 4-AP, 4-aminopyridine; TEA, tetraethylammonium; EADs, early afterdepolarizations; LV, left ventricle

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of the Harvard Medical Area Standing Committee on Animals after approval by the Institutional Animal Care and Use Committee. Kv1DN mice (age: 3–10 months) were infected with a single intramyocardial injection into the free wall of the LV of 25 μl of either vehicle (sterile, pyrogen-free 0.9% NaCl solution) or 25 μl of an rAAV-vector containing approximately 5×10^8 plaque-forming units of a FLAG-tagged Kv1.5 under the control of a cytomegalovirus (CMV) promoter.

2.2. Surgical procedure

After ketamine/xylazine anesthesia, mice were intubated and ventilated with 21% O_2 and their body temperature was maintained with a heating lamp. A midline skin incision was made, and a lateral thoracotomy was performed in the 4/5th intercostal space. After visualization of the lateral free wall, 25 μl of the solution (either rAAV or 0.9% NaCl) was injected intramyocardially using an operating microscope. Efficacy of injection was controlled by observing the brief paling and swelling of the myocardium. After closure of the thorax and the skin, mice were extubated and recovered on heated bedding.

2.3. Animals and cell isolation

Single myocytes were isolated from the LV free wall of transgenic mice (Kv1DN) injected either with rAAV or 0.9% NaCl using the Langendorff technique. Low Ca^{2+} tyrode solution containing 20–30 mM 2,3-butanedione monoxime was used to suppress the contractile activity of the myocytes during the isolation procedure.

2.4. Solutions and chemicals

In all experiments, the following extracellular physiological solution was used (in mM): 135 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, and 10 glucose (pH was adjusted to 7.35 with NaOH). The L-type Ca^{2+} and Na^+ channels were blocked by the application of 2 mM $CoCl_2$ and 20 μM tetrodotoxin (TTX), respectively. The pipette solution contained (in mM): 140 KCl, 1 $MgCl_2$, 10 HEPES, 5 EGTA, 5 Mg_2ATP, 0.1 GTP (pH = 7.2 with KOH). The contamination by ATP-sensitive potassium current was avoided by using a high concentration of ATP in the intracellular solution. Stock solutions of TTX, 4-AP, and tetraethylammonium (TEA) in 2–10 mM concentration were prepared.

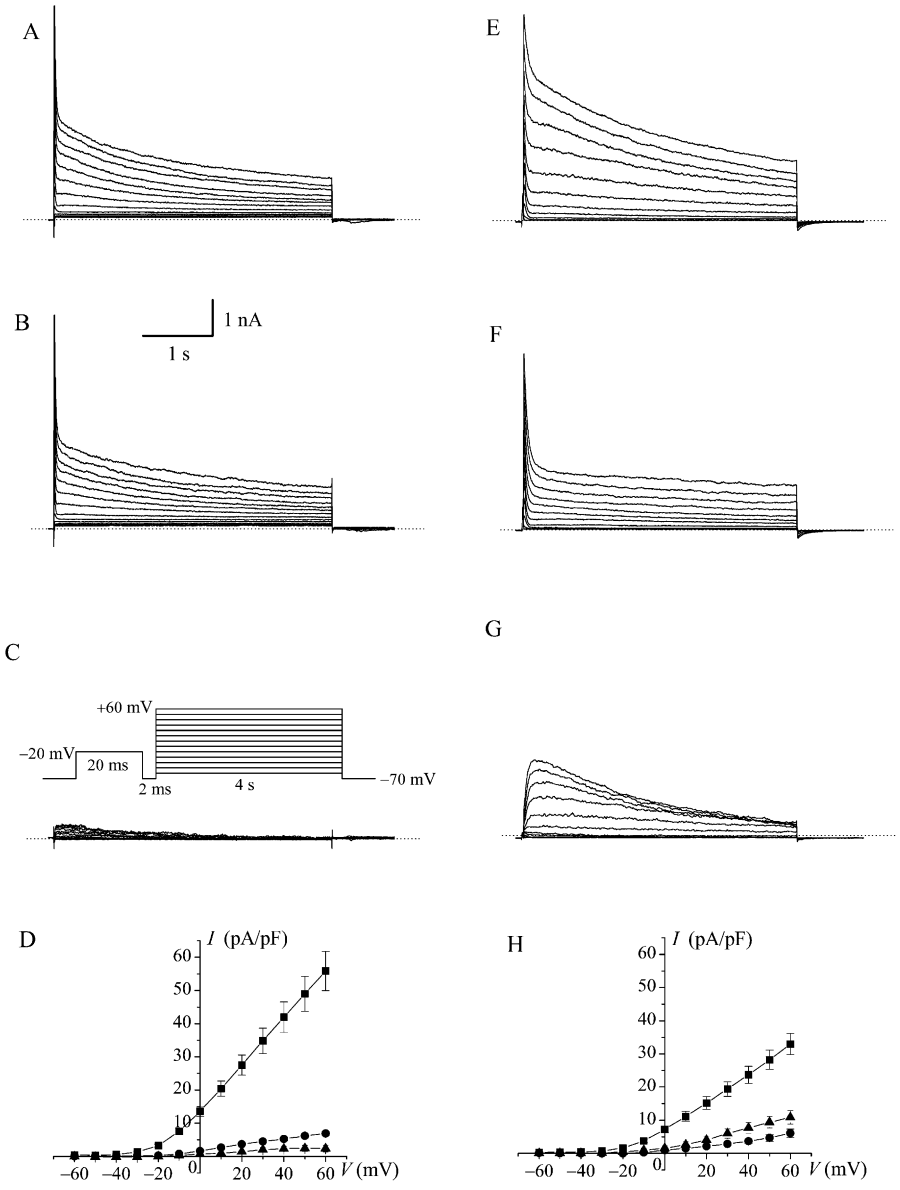

Fig. 1. Infection with rAAV-Kv1.5 restitutes 4-AP-sensitive currents. A family of outward potassium currents in myocytes derived from vehicle-injected (A–D) or rAAV-Kv1.5-injected (E–H) hearts. Current traces were recorded before (A,E) and after (B,F) application of 50 μM 4-AP. The 4-AP-sensitive currents are shown in C and G. D and H depict the *I–V* relationships of peak currents (■), 4-AP-sensitive (▲) and TEA-sensitive currents (●) observed in myocytes derived from vehicle or rAAV-Kv1.5 ($n = 8$–10), respectively. Note the small 4-AP-sensitive currents in vehicle-treated myocytes. Scale bars identically refer to all current traces.

2.5. Electrophysiology and data analysis

Membrane currents were recorded using the whole-cell mode of the patch-clamp technique [20]. The patch electrodes were pulled (P87; Sutter Instruments, USA) from 1.5-mm diameter borosilicate glass capillaries (WPI). Their resistances were 0.8–2 MΩ when filled with a standard pipette solution. An Axopatch 200B amplifier in combination with pClamp 8.1 software and DigiData 1322A (Axon Instruments, USA) were used for data acquisition. In this study, cell capacitances of vehicle- and rAAV-Kv1.5-injected myocytes were 166 ± 17 pF ($n = 10$) and 198 ± 31 pF ($n = 8$), respectively ($P > 0.05$). Raw traces are shown without correction for leakage currents. Experiments showing a significant leak (recognized after depolarizing steps) were discarded. Data analysis and graphs were obtained using Clampfit 8.1 (Axon Instruments), Excel 2000 (Microsoft), and Origin 7.0 software (Microcal). The data are presented as the mean ± S.E.M., and n corresponds to the number of experiments. Student's paired t-test was used to examine the difference between data groups; $P < 0.05$ was considered significant.

3. Results

3.1. Restitution of the 4-AP-sensitive current

The rAAV-Kv1.5 or vehicle was injected into the free wall of the LV. Six months after the injection, the mice were sacrificed and ventricular myocytes derived from this region were studied. In both vehicle- and rAAVKv1.5-treated hearts, we observed significant pericardial fibrosis secondary to the procedure (data not shown). We first characterized myocytes derived from vehicle-injected hearts (Fig. 1A–D). Similar to the findings of previous studies [7], vehicle-treated Kv1DN myocytes (Fig. 1A) exhibited a transient outward current (I_{to}) followed by a slowly inactivating current (mainly $I_{K,slow2}$) [11]. The I–V relationship of the peak current of myocytes from vehicle-injected hearts was linear, with a mean value of 54.5 ± 8 pA/pF at +60 mV (Fig. 1D). Application of 50 μM 4-AP did not significantly reduce the amplitude of outward current in control myocytes (Fig. 1B), and the density of 4-AP-sensitive current at +60 mV (Fig. 1C,D) was 3.1 ± 0.3 pA/pF ($n = 8$). This current accounted for 5.8 ± 1% of the total current.

We next compared the outward currents detected in these cells to those in rAAV-Kv1.5-injected hearts (Fig. 1E–H). On the basis of our studies in vehicle-injected hearts (Fig. 1A–D), cells displaying 4-AP-sensitive current ≥ 15% of the total outward potassium current at +60 mV were considered infected with rAAV-Kv1.5 (Fig. 1G). Of 30 cells isolated from virus-injected LV free wall, 10 met these criteria (Fig. 1E–H). These rAAV-Kv1.5-infected cells appeared to express a new component that reconstituted the total outward currents to a pattern similar to that recorded from the wild-type adult murine ventricular myocytes [7]. The I–V relationship of the peak current amplitude of rAAV-Kv1.5-infected cells was linear, with a mean value of 31.4 ± 3.5 pA/pF at +60 mV, significantly lower than that of vehicle-treated hearts ($P < 0.05$). The density of the 4-AP-sensitive current in rAAV-Kv1.5-infected myocytes ranged from 3.1 to 18.4 pA/pF, with a mean value of 9.8 ± 2 pA/pF, which represents 27.5 ± 4.3% of the total outward current, significantly higher than that observed in control myocytes ($P < 0.01$). The inactivation of the 4-AP-sensitive current was best fitted with a single exponential function, with a time constant of 2.1 ± 0.5 s ($n = 4$), which differed from that of wild-type 4-AP-sensitive current, revealing that these currents are different from the endogenous ones. The reduction in peak current despite the upregulation in the 4-AP-sensitive currents correlated with a significant reduction in $I_{to,f}$ from 43.8 ± 8.3 pA/pF ($n = 10$) in control myocytes to 23.3 ± 3.5 pA/pF ($n = 8$) in rAAV-Kv1.5 myocytes ($P < 0.01$). Of note, the TEA-sensitive current (6.8 ± 0.5 pA/pF) did not differ between the two groups and represented 15% of the total current (Fig. 1D and H). Thus, the Kv2.1-encoded current was not modified in these groups [11].

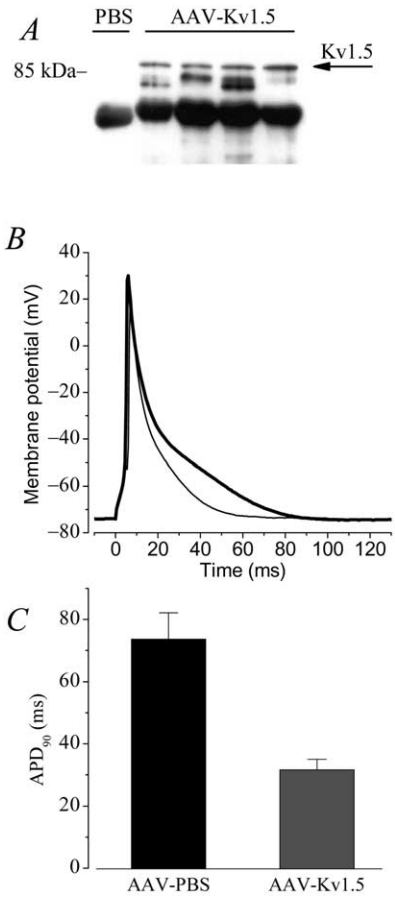

Fig. 2. Expression of exogenous Kv1.5 polypeptides and properties of evoked APs. A: Immunoblot analysis detected the expression of Kv1.5 polypeptide only in rAAV-Kv1.5-infected hearts (the four lanes represent four different animals), not those injected with vehicle. The multiple bands likely reflect posttranslational modifications of the channel polypeptides. B: Morphology of APs in ventricular cells isolated from both hearts injected with vehicle (thick line) and rAAV-Kv1.5 (thin line). C: APD at 90% repolarization in two groups ($n = 14$, $P < 0.001$).

To further prove that the rAAV-Kv1.5-infected hearts express the FLAG-tagged Kv1.5 polypeptides, we used anti-FLAG antibodies to precipitate the FL-Kv1.5 polypeptides from these hearts. The immunoprecipitates were then subjected to SDS–PAGE and blotted with anti-FLAG antibodies. Fig. 2A depicts the precipitates obtained from four different hearts infected with rAAV-Kv1.5. All these hearts express multiple bands, with an apparent molecular mass of approximately 85 kDa. These polypeptides are absent from vehicle-injected hearts. The multiple bands likely reflect post-translational modifications such as glycosylation. Collectively, our data reveal that injection of rAAV-Kv1.5 induced long-term expression of the Kv1.5 polypeptides associated with the restitution of 4-AP-sensitive currents.

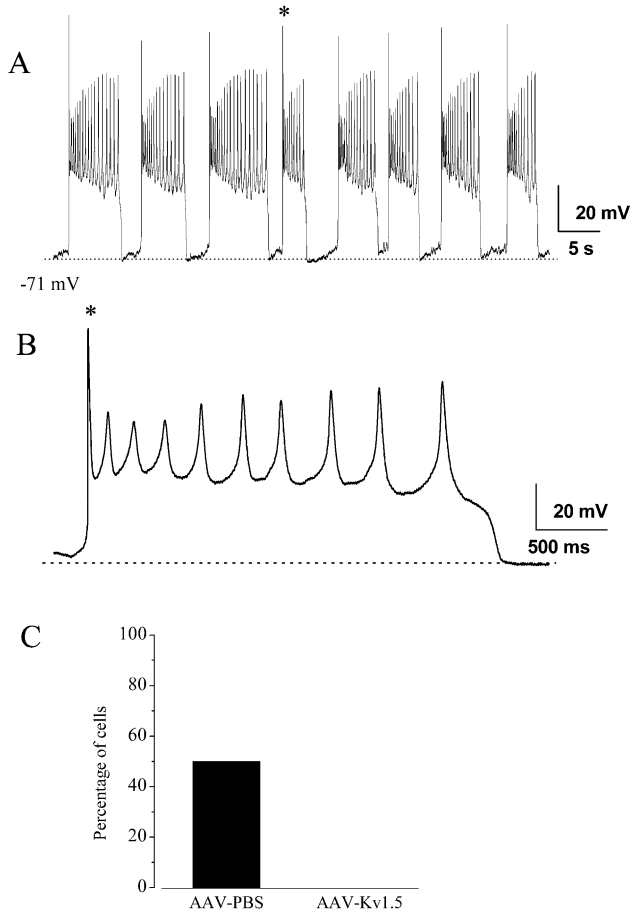

Fig. 3. Spontaneous EADs. A: Abnormal APs recorded from single left ventricular cell of vehicle-injected Kv1DN mouse heart. B: Randomly chosen AP (*) in an expanded time scale. Note the triggered EADs (in this example there are nine EADs). Scale bars are shown. C: Incidence of EADs in vehicle vs. rAAV-Kv1.5 infected myocytes.

3.2. Properties of APs

The functional consequences of Kv1.5 restitution were investigated by examining AP waveforms in myocytes from both vehicle- and rAAV-Kv1.5-injected hearts. Fig. 2B depicts a typical AP of control cardiomyocyte versus that of a rAAV-Kv1.5-infected cell. The APD_{90} of myocytes derived from rAAV-Kv1.5-infected mice was significantly shorter (31.6 ± 3.4 ms, $n = 14$) than that of myocytes from hearts injected with vehicle (70.4 ± 9.2 ms, $n = 14$, $P < 0.001$) (Fig. 2C). The cells from vehicle-injected mice had a resting membrane potential (RMP) similar to that of cells from hearts infected with rAAV-Kv1.5 (−67.3 ± 1.9 mV, $n = 14$ vs. −72.9 ± 2.2, $n = 14$, $P > 0.05$). The AP amplitudes also did not differ on average between the vehicle-injected (−86.5 ± 3.1 mV, $n = 23$) and rAAV-Kv1.5-infected myocytes (−94.6 ± 4.2 mV, $n = 14$, $P > 0.05$).

3.3. rAAV-Kv1.5 abolishes EADs in Kv1DN mice

We next studied whether the treatment with rAAVKv1.5 modified the arrhythmogenic phenotype of Kv1DN ventricular myocytes. During the entire study, most experiments were conducted in the standard physiological solution in order to record both the RMP and the APs. Fig. 3A depicts the spontaneously firing APs recorded from a cardiomyocyte derived from a vehicle-injected heart. These APs exhibited a slow rate of eight cycles per min. The repolarization phase was dramatically delayed, and at −40 mV, oscillations in the membrane potential could subsequently be observed; these are clearly illustrated in an expanded time scale (Fig. 3B). By contrast, we could not detect any EADs in 14 cells infected with rAAV-Kv1.5 (Fig. 3C). Thus, the long-term expression of rAAV-Kv1.5 in myocytes located near the injection site shortened the APD and abolished the EADs.

4. Discussion

We investigated the effect of rAAV-Kv1.5-mediated gene transfer into a small area of the myocardium of transgenic mice that overexpress a truncated potassium channel, Kv1N206Tag. We hypothesized that the potent induction of Kv1.5 under the regulation of the CMV promoter would shift the balance from non-functional heterotetramers of Kv1.5 and Kv1N206Tag polypeptides towards functional Kv1.5 homotetramers. Here we demonstrate for the first time the long-term expression of a voltage-gated potassium channel in the heart using an adeno-associated viral vector. Similar to our previous observations, at one week after infection with adenoviral vector encoding Kv1.5 [16], we demonstrate that the AAV-encoded Kv1.5 also enhanced the 4-AP-sensitive current $I_{K,slow1}$ 6 months after infection, whereas in myocytes derived from vehicle-treated hearts, the 4-AP-sensitive current remained attenuated. It is interesting that this current had a slower inactivation rate, suggesting that the functional complexes in the membrane lack additional subunits.

The enhancement of this current caused a significant shortening of the APD_{90} and the elimination of EADs. We observed fewer cell-to-cell variations in the density of the 4-AP-sensitive currents in the present study than in our previous study using adenoviral vectors encoding Kv1.5. Although Western blot analyses were sufficient to detect the abundance of FL-Kv1.5 polypeptides in AV-treated hearts, here we had to combine immunoblots with immunoprecipitation to detect the markedly smaller amounts of rAAV-induced channel polypeptides. The reduction in $I_{to,f}$ compared with vehicle-treated hearts likely reflects either the effect of long-term expression of Kv1.5 in the myocytes or, more likely, the fibrosis and inflammation induced by treatment with rAAV vectors. To discern the role of the adeno-associated viral vector from that of Kv1.5, we injected mice with AAV vector encoding green fluorescence protein (GFP) as an additional control. Myocytes derived from these mice were harvested 6 months after injection, but we could not detect the expression of the GFP (as detectable by green fluorescence above background) in these cells. Thus, we could not study AAV–GFP-infected cells as an additional control. This contrasts with our experience with AV–GFP, in which we could detect substantial GFP expression 7–10 days after injection [16]. Thus, we cannot discern whether the vector rAAV itself or the rAAV-Kv1.5 was the cause of the downregulation of $I_{to,f}$.

In summary, we demonstrate that in vivo infection of Kv1DN myocardium with rAAV-Kv1.5 restitutes the 4-AP-sensitive current and shortens the APD. The shortening of the APD abolished the EADs. Thus, targeted expression of functional channels may lead to new approaches for therapeutic interventions in patients with cardiac arrhythmias and long QT syndrome.

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