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Differential contribution of sialic acid to the function of repolarizing K^+ currents in ventricular myocytes

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Ufret-Vincenty, Carmen A., Deborah J. Baro, and L. F. Santana. Differential contribution of sialic acid to the function of repolarizing K^+ currents in ventricular myocytes. *Am J Physiol Cell Physiol* 281: C464–C474, 2001.—We investigated the contribution of sialic acid residues to the K^+ currents involved in the repolarization of mouse ventricular myocytes. Ventricular K^+ currents had a rapidly inactivating component followed by slowly decaying and sustained components. This current was produced by the summation of three distinct currents: I_{to}, which contributed to the transient component; I_{ss}, which contributed to the sustained component; and $I_{K,slow}$, which contributed to both components. Incubation of ventricular myocytes with the sialidase neuraminidase reduced the amplitude of I_{to} without altering $I_{K,slow}$ and I_{ss}. We found that the reduction in I_{to} amplitude resulted from a depolarizing shift in the voltage of activation and a reduction in the conductance of I_{to}. Expression of Kv4.3 channels, a major contributor to I_{to} in the ventricle, in a sialylation-deficient Chinese hamster ovary cell line (lec2) mimicked the effects of neuraminidase on the ventricular I_{to}. Furthermore, we showed that sialylated glycolipids have little effect on the voltage dependence of I_{to}. Finally, consistent with its actions on I_{to}, neuraminidase produced an increase in the duration of the action potential of ventricular myocytes and the frequency of early afterdepolarizations. We conclude that sialylation of the proteins forming Kv4 channels is important in determining the voltage dependence and conductance of I_{to} and that incomplete glycosylation of these channels could lead to arrhythmias.

glycosylation; Kv4.3; arrhythmias; mouse ventricular myocytes; transient outward currents

IN THE MOUSE VENTRICLE, membrane depolarization activates a Ca^{2+}-insensitive outward current that has a rapidly inactivating component followed by a slow inactivating sustained component. Three kinetically distinct currents have been proposed to underlie this current in the mouse ventricle (9, 34). The transient component is produced by a rapidly inactivating, transient outward current (I_{to}), while a noninactivating current (I_{ss}) contributes to the sustained component (9, 34). A slowly inactivating current ($I_{K,slow}$) is thought to contribute to both the transient and the sustained components of the ventricular outward K^+ current.

Much progress has been made recently in determining the molecular identity of the channels underlying repolarizing K^+ currents in the mouse heart. K^+ channels of the Kv1 (*Shaker*), Kv3 (*Shaw*), and Kv4 (*Shal*) subfamilies, all of which produce rapidly inactivating currents, have been found in the ventricle of different species and were thus initially considered as possible contributors to I_{to} in this region of the heart (3, 6, 11, 12, 21–23, 37). In mouse ventricular myocytes both Kv4.3 and Kv4.2 are thought to produce the rapidly inactivating I_{to} found in these cells (35). Recent work has also provided clues on the molecular identity of the channels responsible for $I_{K,slow}$ and I_{ss} in heart. In this regard Kv1.5 (20) and Kv2.1 channels (5, 33) are proposed to produce $I_{K,slow}$ in the mouse ventricle. While the search for a molecular correlate of the ventricular I_{ss} has been less fruitful, a recent report suggests that Kv2.1 channels underlie I_{ss} in mouse atria (5).

Reductions in the amplitude of repolarizing K^+ currents have been found to increase action potential (AP) duration and the probability of arrhythmias (24). Changes in K^+ current amplitude could occur due to a reduction in the number of functional channels in the membrane because of a lower number of Kv4 transcripts, as has been suggested during heart failure (17) and arrhythmias (38). However, K^+ channel expression could also be controlled at the posttranscriptional level (25). Posttranscriptional modulation of glycoproteins includes incorporation of sugar residues to appropriate sites in the protein. K^+ channels (31), like Na^+ channels (4, 39), are heavily glycosylated proteins that contain unusually high levels of posttranslationally attached sialic acids on the external side of the channel. Work on human ether-à-go-go-related gene (HERG) (25) and Kv1.1 channels (31) suggests that K^+ channel glycosylation could help to determine the voltage dependence and the surface expression of these channels. Although many of the K^+ channels involved in the repolarization of the ventricle have consensus sites for protein glycosylation, the functional role of these negatively charged particles in the function of these channels is not completely understood.

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The goal of the present study was to investigate the role of sialic acid residues in the function of K^+ channels involved in the repolarization of the mouse ventricle. We have presented evidence suggesting that the channels underlying I_{to}, I_{ss}, and $I_{K,slow}$ in the mouse ventricle vary in regard to the number of sialic acid residues attached to them during posttranslational processing. Furthermore, we have shown that incorporation of sialic acid residues onto Kv4 channels during posttranslational processing of the channel protein is important in determining the voltage dependence and conductance of I_{to}. Finally, our data suggest that improper glycosylation of Kv4 channels could lead to arrhythmogenic I_{to} currents.

METHODS

Isolation of cardiac myocytes. Adult animals (25 g) were euthanized with an intraperitoneal injection of pentobarbital sodium (100 mg/kg) in strict accordance to the guidelines established by the Institutional Animal Care and Use Committee, which follow all applicable state and federal laws. Single mouse ventricular myocytes were isolated as previously described (27) and stored at room temperature (22–25°C) in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical, St. Louis, MO) until used.

Cell culture and transfection. Cultures of Chinese hamster ovary (CHO) clones k1 (American Type Culture Collection, Manassas, VA) were maintained in nutrient mixture F-12 Ham Kaighn's modification (Sigma) supplemented with fetal bovine serum (10%; Life Technologies, Rockville, MD), L-glutamine (2 mM), and a streptomycin/penicillin (S/P; 1%) solution. Lec2 and pro5 cells were maintained in minimum essential medium (alpha modification; Sigma) supplemented with fetal bovine serum (10%), L-glutamine (2 mM), and a 1% S/P solution. Cells were transiently transfected with the pcDNA clones of the α-subunit of the rat Kv4.3 channel (Kv4.3; a generous gift from Dr. Jeanne Nerbonne) and the enhanced green fluorescent protein (EGFP; Clontech Labs, Palo Alto, CA) using Lipofectamine 2000 (Life Technologies) as suggested by the manufacturer. For electrophysiological and imaging experiments, cells were plated at low density on 25-mm coverslips the day before experiments were performed.

Electrophysiology. CHO cells expressing Kv4.3 channels were identified on the basis of EGFP fluorescence. During experiments, cells were continuously superperfused with a solution (*solution A*) containing the following constituents (in mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose; pH 7.4. Cells were patched in this solution, and after a gigaohm seal was formed, a small amount of negative pressure was applied through the patch pipette to break the membrane and achieve the whole cell configuration of the patch-clamp technique. To measure Kv4.3 currents in CHO cells, we filled patch pipettes with a solution that had the following constituents (in mM): 110 K-aspartate, 30 KCl, 10 HEPES, 5 EGTA, and 4 Mg-ATP; pH 7.3. With this solution, the patch electrodes had resistances that ranged from 1.5 to 2.5 MΩ. In the experiments involving cardiac myocytes, the external solution (control solution) in which K^+ currents were measured had the following composition (in mM): 140 *N*-methyl-D-glucamine (NMG), 5.5 KCl, 0.0005 nifedipine, 10 HEPES, 0.1 $CaCl_2$, 2 $MgCl_2$, and 10 glucose; pH 7.4. To this solution we added 4-aminopyridine (4-AP; 50 μM) and/or tetraethylammonium chloride (TEA; 25 mM) to pharmacologically isolate the K^+ current(s) of interest. After the 4-AP and/or TEA was added, the pH of the control solution was verified to be 7.4.

The AP of cardiac myocytes was recorded while cells were continually superfused with *solution A*. APs were evoked by a brief (2 ms) injection of depolarizing current (2 nA) at a frequency of 1 Hz. For these experiments, patch pipettes were filled with the solution used to measure K^+ currents (see above). Membrane currents and APs were recorded at room temperature (22–25°C) with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) controlled by a personal computer running Clampex 8 software (Axon Instruments). Electrophysiological signals were filtered online at 5 kHz with the four-pole Bessel filter of the Axopatch 200B. Respectively, currents and membrane potentials were digitized at a sampling frequency of 10 and 5 kHz.

Analysis of APs and K^+ currents was performed using Clampfit 8 software (Axon Instruments). Normalization of ventricular K^+ currents was performed by dividing current amplitudes by the capacitance (in pF) of the cell from which they were recorded. Cell capacitance was measured with the membrane test module of Clampex 8.0. K^+ current conductance was determined according to the formula

$$G = \frac{I}{(V_{test} - V_{rev})}$$

where G is the conductance of K^+, I is the measured current, V_{test} is the test potential at which the current was measured, and V_{rev} is the calculated Nernst equilibrium potential of K^+ (−84 mV).

Cell fusion. Lec2 and k1 cells were fused using methods similar to those described by Hoppe and colleagues (14) but with some minor modifications. Briefly, the day of experiments, k1 cells expressing Kv4.3 and GFP were dissociated into single cells and plated at very low densities in 25-mm coverslips. One hour after the k1 cells were plated, lec2 cells were stained with the potentiometric dye 8-di-ANEPPS (Molecular Probes, Eugene, OR). To stain lec2 cells with ANEPPS, we incubated these cells in a solution containing a 5 μM concentration of the dye for 30 min. Once lec2 cells had been labeled with ANEPPS, they were dissociated into single cells, resuspended in culture medium, and laid onto k1 cells at a ratio of 1 k1 to 5 lec2 cells per coverslip. One hour after lec2 cells had been added, the culture medium was removed and substituted with a 50% polyethylene glycol (PEG; molecular weight ≈ 1500) solution (Roche Diagnostic) in which cells were maintained for 4 min. At this point the PEG was removed and substituted with a high-K^+ solution with the following constituents (in mM): 125 K-aspartate, 25 KCl, 10 HEPES, 10 glucose, 1 $MgCl_2$, and 1 EGTA; pH 7.4. Cells were maintained in this high-K^+ solution for 10 min and then returned to their normal medium.

Confocal microscopy. Confocal images were collected with a Bio-Rad MRC 600 confocal system coupled to an inverted Nikon diaphot microscope (Nikon, Melville, NY) equipped with a Nikon ×60 oil-immersion lens (NA 1.4). The confocal microscope was operated using a personal computer running COMOS software. Images were analyzed using custom software written by one of the authors (L. F. Santana) in IDL language (RSI, Boulder, CO). EGFP and ANEPPS were excited with the 488-nm line of a krypton-argon laser. The light emitted by EGFP (520 nm) and ANEPPS (>565 nm) was separated with appropriate filter cubes, and each was acquired through one of the two acquisition channels.

Desialylation of ventricular K^+ channels. Enzymatic removal of sialic acid residues from glycoproteins in mouse ventricular myocytes was performed by using a protocol

similar to that described by Yee and colleagues (36). Briefly, freshly dissociated mouse ventricular myocytes were incubated for 3 h in a DMEM solution to which 0.3 U/ml neuraminidase (Fluka, St. Louis, MO) was added. A similar protocol has been shown to desialylate Ca^{2+} (36), Na^+ (39), and Kv1.1 (31) channels.

Statistics. Data are presented as means ± SE. Two-sample comparisons were performed using Student's *t*-test. When multigroup comparisons were necessary, they were made using a one-way analysis of variance (ANOVA) followed by Tukey's test. In all statistical tests a P value <0.05 was considered an indicator of a significant difference.

RESULTS

Sialidase neuraminidase reduces I_{to}, but not $I_{K,slow}$ and I_{ss}, in mouse ventricular myocytes. To investigate the role of sialic acid residues in the function of K^+ channels in the ventricle, we incubated freshly dissociated mouse ventricular myocytes in the presence of 0.3 U/ml sialidase neuraminidase for 3 h at room temperature (22–25°C). Neuraminidase has been shown to hydrolyze sialic acid residues from glycoproteins and has been used by others to study the role of glycosylation in Ca^{2+} (36), Na^+ (4, 39), and Kv1.1 channel (31) function. After ventricular myocytes were incubated with neuraminidase, cells were used for either electrophysiological experiments or biochemical analysis of Kv4 channels (see below).

Our first set of experiments examined the properties of K^+ currents in control and neuraminidase-treated (NT) mouse ventricular myocytes (Fig. 1). K^+ currents were evoked in these cells by step depolarizations (1.2 s) from the holding potential (HP) of −100 mV to potentials from −80 to 60 mV. This protocol evoked outward K^+ currents that activated rapidly and then decayed with time. We could not detect complete K^+ current inactivation in either control or NT myocytes, even when the cells were subjected to longer test pulses (4.5 s; data not shown). Recently, Dubell et al. (9) and Xu et al. (34) have shown that the transient and sustained component of these currents is produced, respectively, by I_{to} and I_{ss}, while $I_{K,slow}$ contributes to both components.

As others have shown under similar experimental conditions (20, 21), the decaying phase of the K^+ currents recorded during step depolarizations to 60 mV could be well fit by the sum of two exponential functions. Analysis of the decaying phase of these composite K^+ currents evoked at 60 mV showed that neuraminidase had no effect on the rate of inactivation of these currents. Respectively, composite K^+ currents (60 mV) in control and NT myocytes had a fast (τ_{fast}) and slow (τ_{slow}) time constant of inactivation of 58.99 ± 5.80 and 55.97 ± 2.12 ms and 757.97 ± 53.74 and 706.13 ± 52.25 ms, respectively ($n = 6$; $P = 0.50$).

We found, however, that the amplitude of the K^+ currents recorded in control myocytes was larger than in NT myocytes. Closer analysis of these K^+ currents revealed that the fast-inactivating, transient component, or I_{to} (defined here as the difference between the peak and the sustained current measured at the end of the 1.2-s pulse) was significantly smaller in NT than in control cells ($P = 0.02$) at most potentials examined. There was no statistical difference between the current-voltage (*I-V*) relationship of the slow-inactivating and sustained component of these currents ($I_{sustained}$) between control and NT myocytes ($P = 0.55$). Thus these data suggest that sialic acid residues modulate

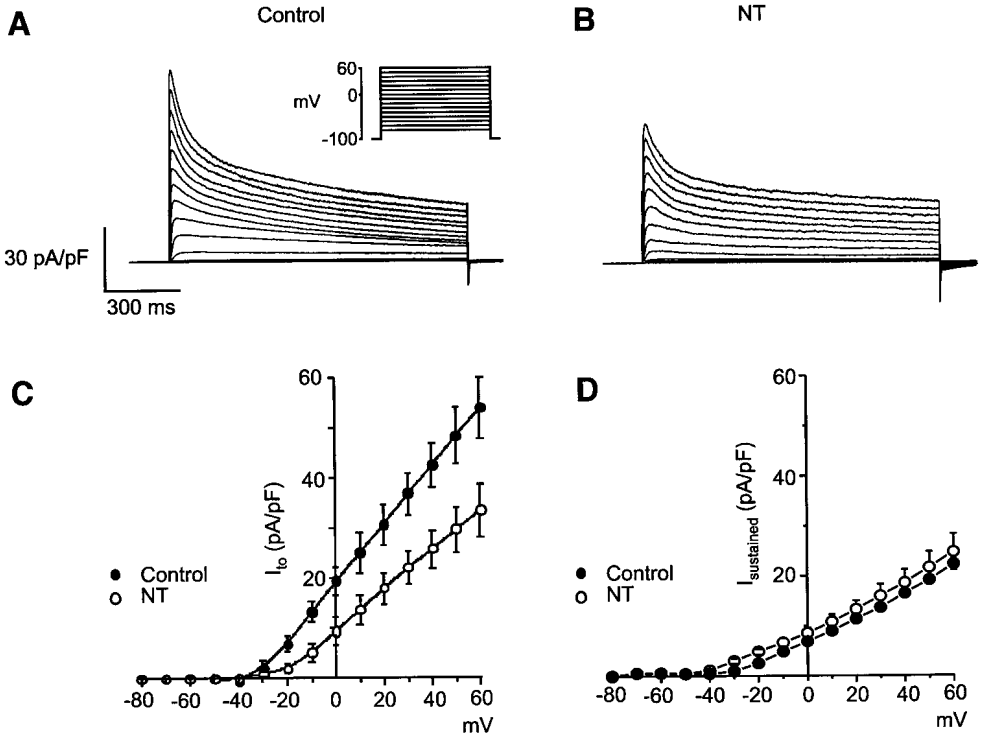

Fig. 1. Neuraminidase reduces the amplitude of rapidly inactivating, transient outward current (I_{to}), but not slowly inactivating, sustained currents ($I_{sustained}$) in mouse ventricular myocytes. Representative K^+ current traces recorded from a control (*A*) and neuraminidase-treated (NT; *B*) mouse ventricular myocyte. *Inset*: voltage protocol used to generate currents. Briefly, currents were evoked by 1.2-s step depolarizations from the holding potential (HP) of −100 mV to voltages ranging from −80 to 60 mV. The current-voltage (*I-V*) relationships of I_{to} (*C*) and $I_{sustained}$ (*D*) are shown in control ($n = 6$) and NT ($n = 6$) myocytes.

the function of I_{to}, but not $I_{sustained}$, in the mouse ventricle.

To provide further support for the conclusion that the K^+ currents giving rise to $I_{sustained}$ (i.e., $I_{K,slow}$ and I_{ss}) are not affected by the sialidase neuraminidase, we decided to isolate, using pharmacological tools, these currents from I_{to} and examine their properties in both control and NT ventricular myocytes. First, we examined $I_{K,slow}$. To isolate $I_{K,slow}$ from the other currents, we measured K^+ currents before and after the application of a solution containing 50 μM 4-AP, which blocks this current but leaves I_{to} and I_{ss} spared (9). Subtraction of the currents recorded in 50 μM 4-AP from those recorded in control solution would therefore result in the 50 μM 4-AP-sensitive currents, or $I_{K,slow}$. Figure 2 shows the results of these experiments. Note that the 50 μM 4-AP-sensitive currents ($I_{K,slow}$) are similar ($P = 0.77$) in control ($n = 8$) and NT ($n = 6$) cells, thus suggesting that $I_{K,slow}$ is unaltered by the sialidase neuraminidase. We then proceeded to investigate the effects of neuraminidase on I_{ss}. In these experiments K^+ currents were measured in the absence and presence of 25 mM TEA^+, which blocks I_{ss} and $I_{K,slow}$ but has no effect on I_{to} (34). The TEA^+-sensitive component of these currents therefore represents $I_{K,slow}$ and I_{ss} (9, 34). Note that the amplitude of the TEA^+-sensitive component of K^+ currents in both control ($n = 4$) and NT ($n = 5$) myocytes is similar at all voltages examined ($P = 0.45$), a finding that provides further support for the conclusion that $I_{K,slow}$ and I_{ss} are unaffected by neuraminidase.

Neuraminidase reduces I_{to} by reducing its conductance and shifting its voltage dependence of activation in ventricular myocytes. Next we investigated the possibility that neuraminidase reduces I_{to} in ventricular myocytes because it modifies the voltage dependencies of activation and/or inactivation of this current. Because I_{ss} and $I_{K,slow}$ are unaffected by neuraminidase, in these experiments these currents were blocked by a combination of 50 μM 4-AP and 25 mM TEA^+, which blocks $I_{K,slow}$ and I_{ss} but has little effect on I_{to} (9). Figure 3 shows two representative families of I_{to} currents from a control and NT ventricular myocyte under these experimental conditions. Again, note that the NT cell had much smaller I_{to} than control cells. Indeed, at 60 mV, I_{to} was 35 ± 5% smaller in NT than in control

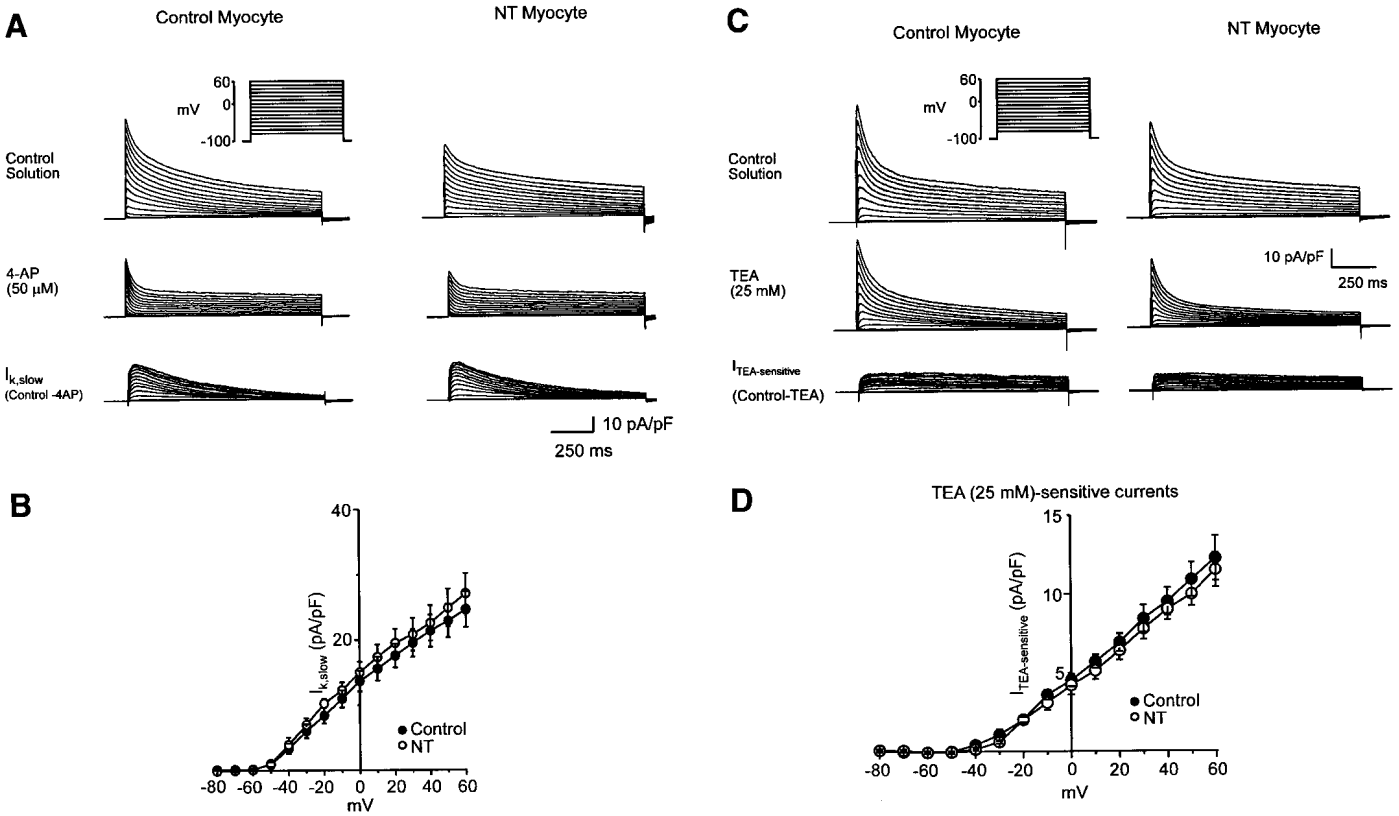

Fig. 2. Neuraminidase does not alter the slowly inactivating K^+ current ($I_{K,slow}$) and noninactivating current (I_{ss}) in mouse ventricular myocytes. *A*: control (*left*) and NT (*right*) myocyte currents recorded while cells were bathed by the control solution (*top*) and after the application of 50 μM 4-aminopyridine (4-AP; *middle*). 4-AP (50 μM)-sensitive currents ($I_{K,slow}$; *bottom*) were obtained after subtracting the K^+ currents recorded in the presence of 50 μM 4-AP from those recorded without 4-AP. *Inset*: voltage protocol used in these experiments, which is similar to that described in Fig. 1. *B*: *I-V* relationship of $I_{K,slow}$ in control ($n = 8$) and NT ($n = 6$) myocytes. *C*: tetraethylammonium (TEA^+; 25 mM)-sensitive currents (i.e., $I_{K,slow} + I_{ss}$) recorded from a representative control and NT myocyte. As in *A*, these TEA^+-sensitive currents ($I_{TEA\text{-}sensitive}$; *bottom*) are digital subtractions of the K^+ currents recorded in the presence of 25 mM TEA (*middle*) from those recorded while cells were bathed by the control solution (*top*). *D*: *I-V* relationship of the 25 mM $I_{TEA\text{-}sensitive}$ in control ($n = 4$) and NT ($n = 5$) myocytes.

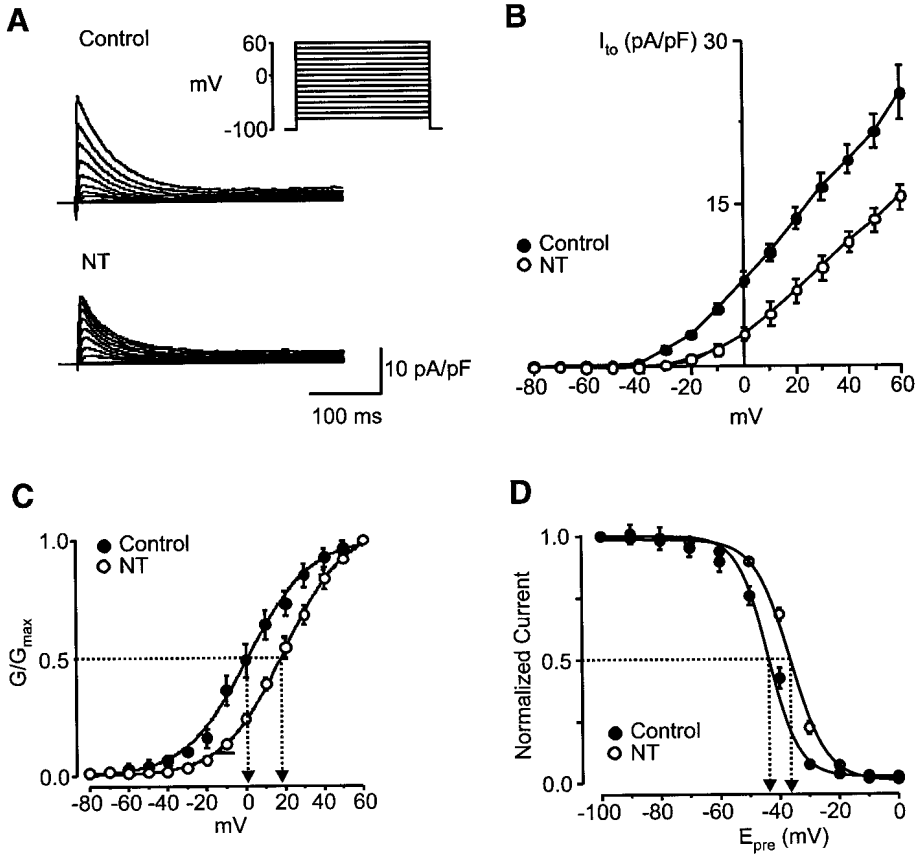

Fig. 3. Neuraminidase modifies the voltage dependence of I_{to} in mouse ventricular myocytes. *A*: representative I_{to} traces recorded from control (*top*) and NT (*bottom*) ventricular myocytes. These currents were generated using the protocol shown in the *inset*, which is similar to that described in Fig. 1. Currents were recorded in the presence of 50 μM 4-AP and 25 mM TEA. *B*: voltage dependence of I_{to} in control ($n = 7$) and NT ($n = 9$) cells. *C*: voltage dependence of the normalized conductance (G/G_{max}) of I_{to} in control ($n = 7$) and NT ($n = 9$) cells. Smooth lines represent best-fit curves to the data determined by a least-squares method using a Boltzmann equation, $y = \{(A_1 - A_2)/[1 + e^{(V - V_{½})}/dV]\}$, where A_1, A_2, $V_{½}$, and dV are the initial value, final value, the voltage at which 50% of the current was observed, and the slope factor. For control myocytes, $A_1 = 0.01$, $A_2 = 1.03$, $V_{½} = 1.91$ mV, and d$V = 16.05$; for NT cells, $A_1 = 0.00$, $A_2 = 1.05$, $V_{½} = 19.51$ mV, and d$V = 15.11$. *D*: voltage dependence of the steady-state inactivation kinetics of I_{to} currents in control and NT cells. Smooth lines represent best-fit curves to the data determined by a least-squares method using a Boltzmann equation similar to that described in *C*. For control cells, $A_1 = 0.99$, $A_2 = 0.02$, $V_{½} = -42.43$ mV, and d$V = 5.08$; for NT cells, $A_1 = 0.98$, $A_2 = 0.01$, $V_{½} = -35.54$ mV, and d$V = 5.21$. Dashed lines in *C* and *D* mark $V_{½}$. E_{pre}, preconditioning potential.

cells. We found that this neuraminidase-induced reduction in amplitude of I_{to} was accompanied by an ~18-mV depolarizing shift of the conductance (G/G_{max})-voltage relationship of NT cells ($V_{½} = 19.51 \pm 1.11$ mV, $n = 9$; $P < 0.001$) relative to control ($V_{½} = 1.14 \pm 0.37$ mV, $n = 7$), where $V_{½}$ is the voltage at which 50% of the current was observed (Fig. 3). Note also that neuraminidase reduced the conductance of I_{to}. In fact, the conductance of I_{to} at 60 mV was nearly 40 ± 4% lower in NT cells than in control cells ($P < 0.05$). Furthermore, the steady-state activation of I_{to} was shifted in the depolarizing direction by neuraminidase. The $V_{½}$ of the voltage dependence of the steady-state inactivation of I_{to} was, respectively, -43.82 ± 0.67 ($n = 7$) and -33.32 ± 0.45 mV ($n = 9$) in control and NT myocytes ($P < 0.001$).

Voltage dependence of activation and steady-state inactivation of Kv4.3 channels expressed in a sialylation-deficient cell line are shifted toward more positive potentials. Previous studies have shown that the ventricular I_{to} is produced by channels of the Kv4 (*Shal*) subfamily (11). Therefore, we investigated whether the effects neuraminidase had on the ventricular I_{to} could be reproduced by the removal of sialic acid residues from Kv4 channels. To test this hypothesis, we expressed the Kv4.3 channel in CHO-k1 (k1) and the sialylation-deficient CHO cell line lec2. The lec2 cells have extremely low levels (<2%) of CMP-sialic acid transport into the trans-Golgi compartments, which results in a dramatic reduction in sialylation during posttranslational processing of glycoproteins (8, 29, 30). As an additional control we also expressed Kv4.3 in CHO-pro5 cells (pro5), the nonmutant parental cell line from which lec2 cells were cloned.

Figure 4 shows three representative families of Kv4.3 currents that were evoked in k1, pro5, and lec2 cells by the protocol described. Similar to ventricular myocytes, there was no significant difference ($P = 0.42$) in the rate of inactivation of transient outward currents in lec2 ($\tau_{slow} = 446.35 \pm 43$ ms and $\tau_{fast} = 51.95 \pm 5.06$ ms, $n = 11$), k1 ($\tau_{fast} = 47.61 \pm 5.93$ ms and $\tau_{slow} = 416.38 \pm 48.81$ ms, $n = 16$), and pro5 ($\tau_{fast} = 51.77 \pm 3.41$ ms and $\tau_{slow} = 414.88 \pm 34.68$ ms, $n = 19$). Using records like those in Fig. 4*A*, we also obtained the conductance-voltage relationship of Kv4.3 currents in k1, pro5, and lec2 cells. Our analysis showed that Kv4.3 currents in lec2 cells ($V_{½} = 11.72 \pm 1.57$ mV, $n = 11$; $P < 0.001$) required a significantly stronger depolarization for activation than in k1 ($V_{½} = -6.81 \pm 1.71$ mV, $n = 16$) and pro5 cells ($V_{½} = -6.06 \pm 1.92$ mV, $n = 19$). The voltage dependence of steady-state inactivation of Kv4.3 currents was also significantly ($P < 0.001$) more depolarized in lec2 (-48.06 ± 0.98 mV, $n = 16$) than in pro5 (-56.64 ± 0.42 mV, $n = 19$) and k1 cells (-58.19 ± 0.34 mV, $n = 16$). Thus expressing Kv4.3 in a glycosylation-deficient cell line mimics the effects of neuraminidase treatment on I_{to} in cardiac myocytes and suggests that sialylation can influence the voltage dependencies of Kv4 channels.

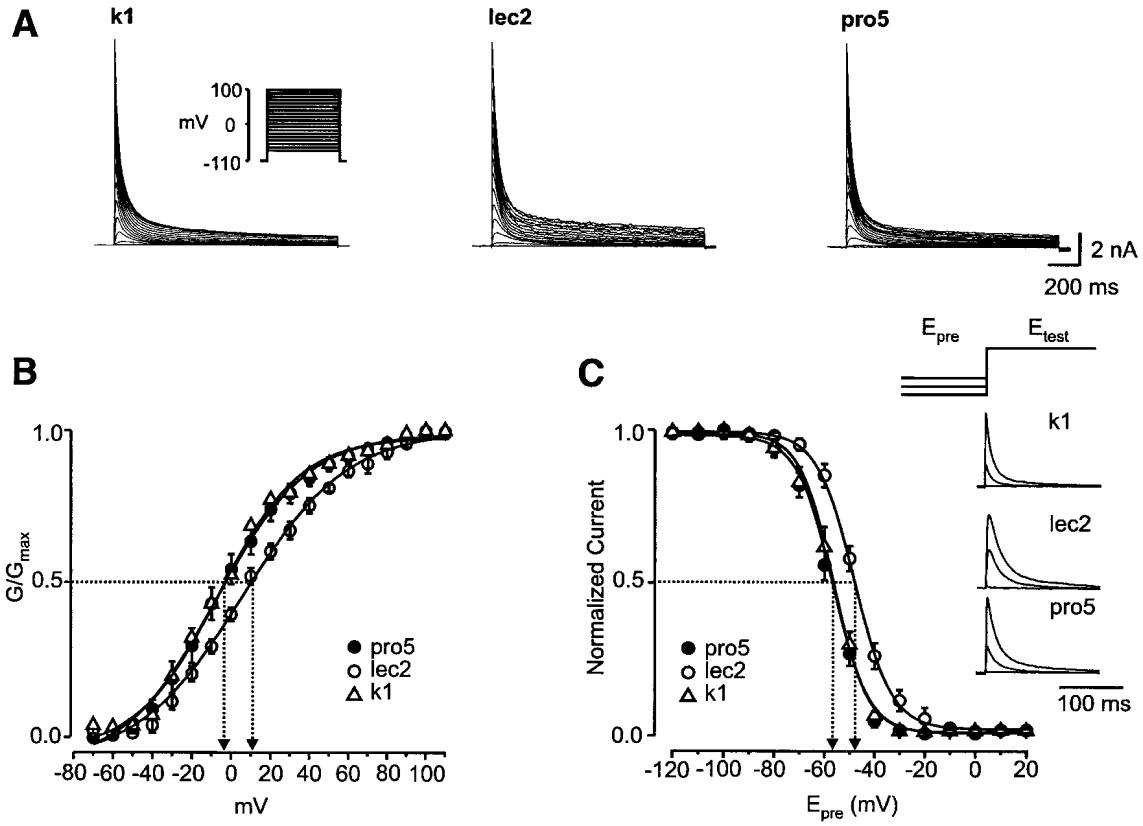

Fig. 4. Sialic acid residues contribute to the voltage dependence of Kv4.3 currents. *A*: representative Kv4.3 current traces recorded from k1 (*left*), lec2 (*middle*), and pro5 cells (*right*), using the protocol illustrated in the *inset*. Briefly, Kv4.3 currents were evoked by 1.6-s step depolarizations from the HP of −110 mV to voltages ranging from −70 to 110 mV. *B*: voltage dependence of the normalized conductance of Kv4.3 currents in k1 (n = 16), lec2 (n = 11), and pro5 cells (n = 19). Smooth lines represent best-fit curves to the data determined by a least-squares method using the Boltzmann equation described in Fig. 3. For k1, A_1 = 0.00, A_2 = 1.01, $V_{½}$ = −6.57 mV, and dV = 22.58; for lec2, A_1 = −0.01, A_2 = 1.01, $V_{½}$ = 12.03 mV, and dV = 18.03; for pro5, A_1 = 0.01, A_2 = 1.01, $V_{½}$ = −6.05 mV, and dV = 20.03. *C*: voltage dependence of the steady-state inactivation kinetics of Kv4.3 currents in k1 (n = 16), lec2 (n = 11), and pro5 cells (n = 19). Smooth lines represent best-fit curves to the data determined by a least-squares method using the Boltzmann equation. For k1, A_1 =1.01, A_2 = 0.01, $V_{½}$ = −58.48 mV, and dV = 7.18; for lec2, A_1 = 0.99, A_2 = 0.02, $V_{½}$ = −48.06 mV, and dV = 7.42; for pro5, A_1 = 0.98, A_2 = 0.02, $V_{½}$ = −56.47 mV, and dV = 7.15. *Inset*: diagrammatic representation of the voltage protocol (*top*) used in these experiments. Briefly, cells were submitted to a voltage step to an E_{pre} from −120 to 20 mV for 5 s, after which cells were depolarized to the test potential (E_{test}) of 60 mV. *Bottom*: Kv4.3 currents evoked by a voltage step to 60 mV from E_{pre} of −110, −50, and 20 mV from k1, lec2, and pro5 cells. Dashed lines in *C* and *D* mark $V_{½}$.

Kv4.3 currents in the sialylation-deficient lec2 cells have voltage dependencies of activation and steady-state inactivation more depolarized than those of pro5 and lec2 cells, suggesting that Kv4.3 channels have a significant number of sialic acid residues linked to them and that these negatively charged components of the channel contribute significantly to the voltage dependence of the channel. On the basis of the surface potential theory, this positive shift in the voltage dependence of sialic acid-deficient Kv4.3 channels results from a reduction in the number of charged particles near the voltage sensor of the channel, which would increase the voltage "felt" within the membrane by the channel voltage sensor. One prediction of the surface potential theory is that increasing external divalent concentration shifts the voltage dependence of K^+ channels because it screens negatively charged particles associated with the membrane and the channel, thus increasing the voltage within the membrane (10). If, as the surface potential theory would predict, sialic acid-deficient Kv4.3 channels gate at more depolarized potentials because they have fewer negatively charged particles, and thus their voltage sensors experience a larger voltage within the membrane, one would expect that the voltage dependence of activation of these channels is less sensitive to external Ca^{2+} than Kv4.3 channels expressed in pro5 and k1 cells. The next series of experiments addressed this particular issue.

Voltage dependence of activation of sialic acid-deficient Kv4.3 channels is less sensitive to external Ca^{2+} concentration. To test the hypothesis that sialic acid-deficient Kv4.3 channels are less sensitive to changes in external Ca^{2+} concentration ($[Ca^{2+}]_o$) than control channels, we recorded Kv4.3 *I-V* relationships in the presence of varied $[Ca^{2+}]_o$ (2, 5, 10, and 20 mM). The results of these experiments are summarized in Fig. 5. We found that increasing external Ca^{2+} concentration shifts the conductance-voltage dependence of Kv4.3

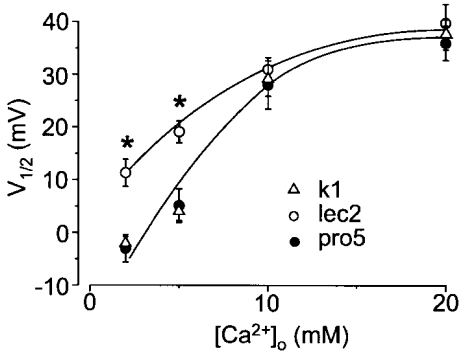

Fig. 5. Sialic acid-deficient Kv4.3 channels produce currents whose voltage dependence of activation is less sensitive to changes in external Ca^{2+} than control channels. The effects of changing external Ca^{2+} concentration ($[Ca^{2+}]_o$) from 1 to 20 mM on the $V_{½}$ of activation of Kv4.3 currents in k1 ($n = 5$), lec2 ($n = 6$), and pro5 cells ($n = 5$) are shown. *Significant difference among groups: $P = 0.02$, 0.01, 0.82, and 0.76 for $V_{½}$ of activation at 2, 5, 10, and 20 mM Ca^{2+}, respectively.

toward depolarized potentials in k1, pro5, and lec2 cells. However, the shift was much less pronounced in lec2 cells, such that an order of magnitude in $[Ca^{2+}]_o$ caused a 39- and 38-mV shift in pro5 and k1 cells, respectively, and only a 28-mV shift in lec2 cells. It is also interesting to note that as the $[Ca^{2+}]_o$ reaches 10 mM or higher, the voltage dependencies of activation of Kv4.3 currents in k1, lec2, and pro5 cells have a similar $V_{½}$ of activation. These findings are consistent with the surface potential theory discussed above. Furthermore, our data suggest that glycosylation of Kv4.3 channels is an important step in the posttranslational processing of the channel protein.

Sialic acids on proteins forming Kv4.3 channels have a much larger impact on the voltage dependence of I_{to} than sialic acids on glycolipids. In the experiments described above, we used two strategies to investigate the role of sialic acid in the voltage dependence of I_{to}. With one strategy, ventricular myocytes were treated with the sialidase neuraminidase, and with the other, Kv4.3 channels were expressed in a cell line (lec2) unable to incorporate sialic acid residues into the channel proteins. However, one difficulty with these experiments is that both the channel and glycolipids could have been deficient in sialic acids. This raises the following question: What is the contribution of sialic acids in glycolipids to the voltage dependence of Kv4.3 currents? The next series of experiments was designed to address this important issue.

To investigate the role of sialylation of glycolipids on the voltage dependence of Kv4.3 channel, we expressed EGFP and Kv4.3 channels in k1 cells and then fused them to lec2 cells. To differentiate lec2 from k1 cells, we stained the surface membrane of lec2 cells with the potentiometric dye ANEPPS. Figure 6*A* shows a set of confocal images taken from a k1 cell expressing EGFP and Kv4.3 and from a lec2 cell stained with ANEPPS before fusion. We chose these fluorescent markers for these experiments because they would be found in different regions of the cells (i.e., cytosol vs. surface membrane) and because of their spectral properties, which allow good separation of the emission signals. Note that in the k1 cell, the EGFP fluorescence is homogeneously distributed in cytosol of the cell, with virtually no signal detected in the ANEPPS acquisition channel. The ANEPPS-labeled lec2 cell, in turn, had the "donut-like" confocal fluorescent pattern typical of a cell that has a fluorophore located in its surface membrane. Note that there is very little (<10%) ANEPPS light "spilling over" into the EGFP channel.

To minimize the probability of fusion between k1 cells, they were dissociated into single cells and then plated at very low densities. ANEPPS-stained lec2 cells were later added to these k1-containing coverslips at a ratio of approximately five lec2 cells to one k1 cell to ensure a high probability of lec2/k1 fusion. After fusion, Kv4.3-k1/lec2 heterokaryons could be easily identified with the confocal microscope because they had both EGFP and ANEPPS fluorescence (Fig. 6*B*). The average capacitance, an indicator of surface area, of k1/lec2 heterokaryons was 36.5 ± 3.5 pF ($n = 12$), a value that was ~3.2-fold higher ($P < 0.001$) than in single k1 (11.4 ± 1.2 pF; $n = 19$) and lec2 cells (12.9 ± 1.3 pF; $n = 25$). These data suggest that, on average, one k1 cell fused with two lec2 cells, which resulted in an ~66% reduction in the number of sialic acid-containing glycolipids in the heterokaryons.

If removal of sialic acids from glycolipids is causing the shifts in the voltage dependence of Kv4.3 currents that we observed in lec2 and NT ventricular myocytes, then fusing a k1 cell (which produces sialic acid-containing Kv4.3 channels) with several lec2 cells (which produce sialic acid-deficient glycolipids) should reduce the number of sialic acid-containing glycolipids and, hence, produce a shift in the voltage dependence of this current. We found that the $V_{½}$ of activation of the k1/lec2 heterokaryon was −2.75 ± 0.94 mV ($n = 12$), a value very close to that obtained when Kv4.3 channels are expressed in k1 (−6.81 ± 1.71 mV) and pro5 cells (−6.06 ± 1.92 mV). Thus fusion of k1 and lec2 cells produced a small (~4 mV) shift in the voltage dependence of activation of Kv4.3 currents as opposed to the 18-mV shift caused by the treatment of myocytes with neuraminidase or the Kv4.3 expression in the sialylation-deficient cell line lec2. These data suggest that sialic acids in glycolipids contribute little to the voltage dependence of Kv4.3 currents.

Neuraminidase increases AP duration in ventricular myocytes. One testable prediction of the experiments presented above is that the smaller I_{to} produced by incompletely glycosylated Kv4 channels would lead to longer APs in the mouse ventricle. To test this hypothesis, we recorded APs from control and NT ventricular myocytes (see METHODS). We found that NT cells had an AP that was significantly longer than control cells at both 50 and 90% repolarization (APD_{50} and APD_{90}; Fig. 7). Indeed, in control and NT cells, APD_{50} was 12.89 ± 2.24 ms ($n = 25$) and 30.69 ± 7.03 ms ($n = 20$) ($P = 0.01$), respectively. Neuraminidase increased APD_{90} from 42.53 ± 8.96 ms (control; $n = 25$) to 199.26 ± 23.86 ms (NT; $n = 20$) ($P < 0.001$). Because

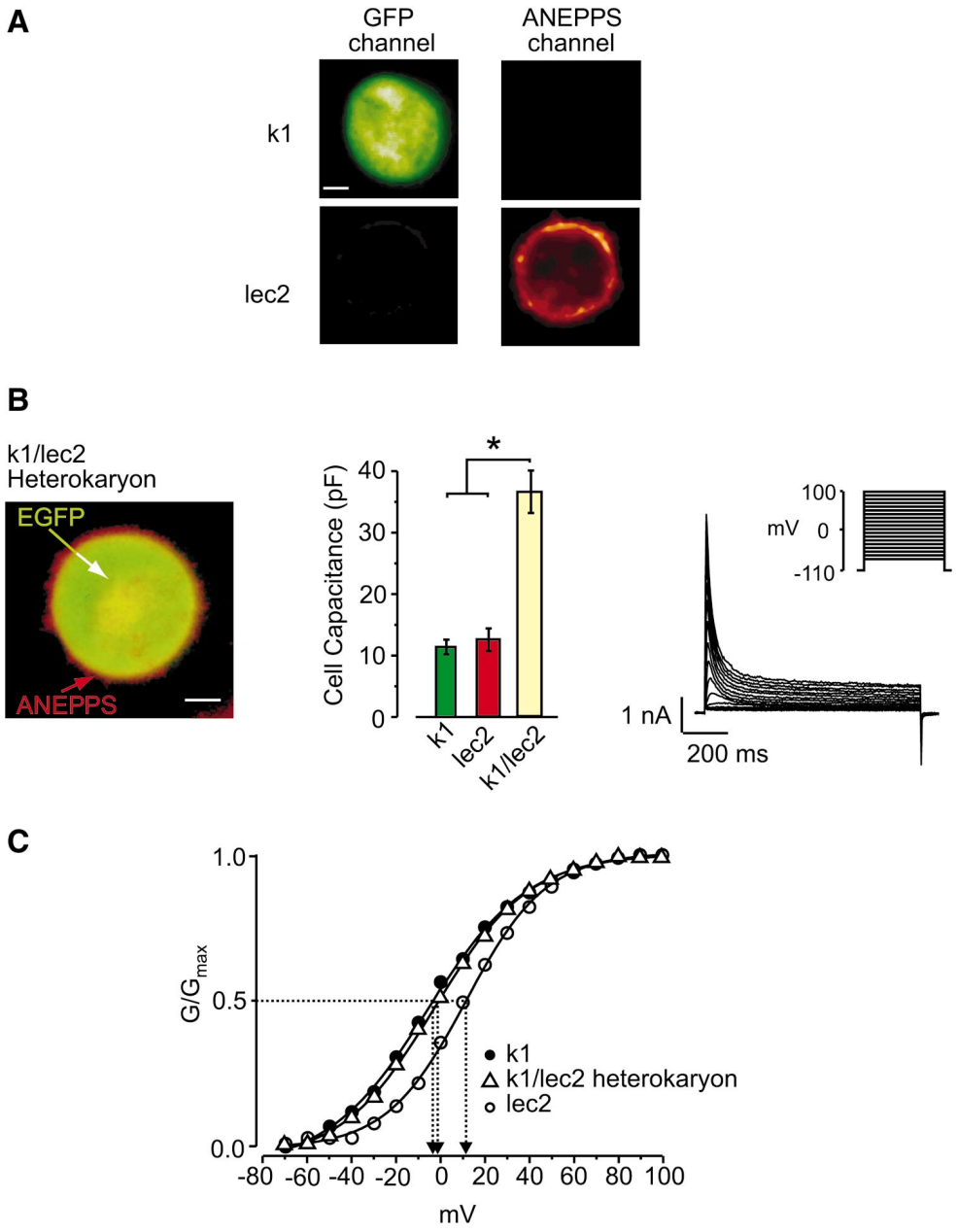

Fig. 6. Sialic acids on Kv4.3 channels have a much larger impact on the voltage dependence of I_{to} than on glycolipids. *A*: set of confocal images of k1 cells (*top*) expressing Kv4.3/enhanced green fluorescence protein (EGFP) and lec2 cells (*bottom*) stained with the potentiometric dye ANEPPS. Scale bar, 10 μm. *B, left*: confocal image of a k1/lec2 heterokaryon in which the images collected through the EGFP and ANEPPS channels have been merged. Scale bar, 10 μm. *Middle*: average capacitance of k1 (n = 19), lec2 (n = 25), and k1/lec2 heterokaryons (n = 12). *Right*: a family of Kv4.3 currents recorded from a representative k1/lec2 heterokaryon. *C*: voltage dependence of the normalized conductance of Kv4.3 currents in k1 (n = 16), lec2 (n = 11), and k1/lec2 heterokaryons (n = 12). Smooth lines represent best-fit curves to the data determined by a least-squares method using the Boltzmann equation described in Fig. 3. For k1, A_1 =1.00, A_2 = −0.07, $V_{½}$ = −6.05 mV, and dV = 21.56; for lec2, A_1 = 0.01, A_2 = 1.01, $V_{½}$ = 11.04 mV, and dV = 18.83; for k1/lec2 heterokaryons, A_1 = 1.00, A_2 = −0.06, $V_{½}$ = −2.60 mV, and dV = 21.30. *P < 0.05. Dashed lines in *C* mark $V_{½}$.

AP prolongation has been found to be a major cause for arrhythmias, we investigated whether the increase in AP detected in NT cells was accompanied by a higher probability of arrhythmogenic voltage fluctuations, such as early afterdepolarizations (EADs), in these cells. For these experiments, NT and control cells were stimulated at a low frequency (1 Hz), to increase the likelihood of arrhythmias, for a period of 5 min. Indeed, we found that cells exposed to neuraminidase were more likely to develop EADs than control cells. While EADs were observed in only 1 ± 1% (n = 25) of the control cells examined, 35 ± 6% (n = 20) of the NT cells had EADs (P < 0.001).

DISCUSSION

In this paper we have examined the role of sialic acids in the function of the K^+ currents involved in the repolarization of the mouse ventricle. We found that the sialidase neuraminidase modified I_{to} but left $I_{K,slow}$ and I_{ss} unaltered. The lack of effect of neuraminidase on $I_{K,slow}$ and I_{ss} suggests that sialic acids are not linked to these channels during posttranslational processing. In parallel experiments we showed that the effects of neuraminidase on I_{to} could be reproduced if Kv4.3, a major contributor to I_{to} in the ventricle, was expressed in a sialylation-deficient cell line. These experiments also showed that sialic acid incorporation onto the K^+ channel protein during posttranslational processing is not an absolute requirement for the surface expression of functional K^+ channels. Furthermore, we found that sialic acids linked to glycolipids contribute little to the voltage dependence of Kv4 channels. Finally, we found that sialic acid removal produces arrhythmogenic changes in I_{to}.

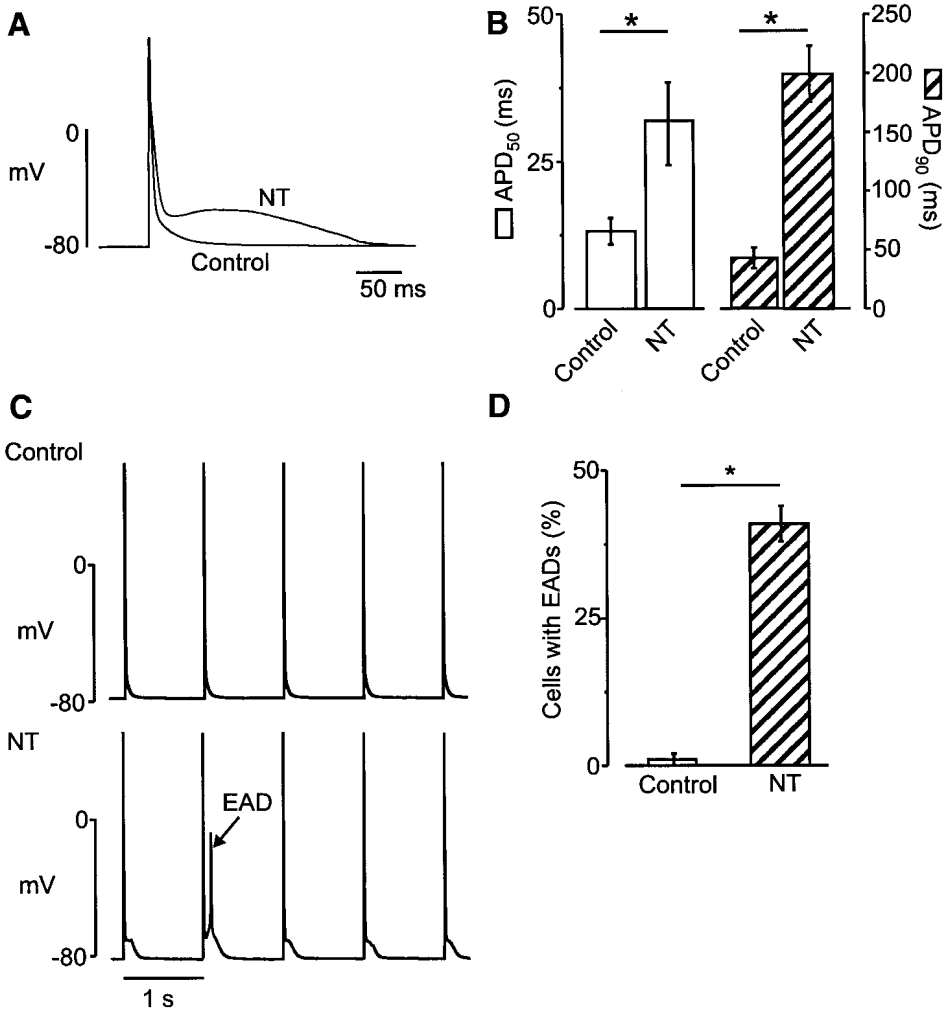

Fig. 7. Neuraminidase increases the duration of the action potential and the probability of occurrence of early afterdepolarizations (EADs) in ventricular myocytes. *A*: action potentials recorded from a representative control and NT cell. *B*: analysis of the action potential duration at 50% (APD_{50}) and 90% (APD_{90}) repolarization in control ($n = 24$) and NT cells ($n = 16$). *C*: trains of action potentials recorded from a representative control (*top*) and NT (*bottom*) cell. Arrow indicates an EAD in the NT cell. *D*: percentage of control ($n = 24$) and NT cells ($n = 16$) with EADs. *$P < 0.05$.

Differential contribution of sialic acids to the function of I_{to}, $I_{K,slow}$, and I_{ss} in the mouse ventricle. A series of recent papers have suggested that glycosylation is an important step in the processing of certain K^+ channels (25). However, these studies have been carried out with heterologously expressed K^+ channels only. These previous investigations have not directly examined the functional consequences (see below) of poor or deficient K^+ channel glycosylation on the native cell function. The experiments presented in this paper addressed these specific issues.

The observation that neuraminidase decreased the amplitude of I_{to}, but not I_{ss} and $I_{K,slow}$, in mouse ventricular myocytes suggests that the proteins forming the channels underlying these currents are differentially glycosylated during posttranslational processing. Thus the number of sugar residues linked to these channels differs, at least with respect to their sialic acid content. This situation is not unique for K^+ channels. In heart, it has been shown that neuraminidase modifies T-type Ca^{2+} current without affecting L-type Ca^{2+} current (36). Indeed, it would be interesting to investigate whether variations in the level of glycosylation between K^+ channels could account, at least in part, for differences in their voltage dependence. In addition, our results suggest that sialylation of K^+ channels is not an absolute requirement for the surface expression of functional K^+ channels. However, it was recently shown that in HERG channels, complete elimination of N-linked glycosylation either through pharmacological (i.e., tunicamicin) or molecular (mutation of potential N-linked glycosylation sites) strategies prevented the surface expression of this channel (25). Thus it may be that the degree of glycosylation of the channel determines surface membrane expression.

Sialic acids linked to Kv4 channels contribute to the voltage dependence of I_{to}. Although incorporation of sialic acid residues onto the Kv4 channels is not required for their surface expression, our results show that these negatively charged particles do contribute significantly to the voltage dependence of these channels. We found that sialic acid-deficient Kv4 channels produced currents that had depolarized voltage dependencies of activation and steady-state inactivation. However, we note that neuraminidase, in addition to shifting the G/G_{max}-voltage relationship of I_{to} toward depolarized potentials, reduces the conductance of I_{to} in mouse ventricular myocytes, which suggests a reduction in the total number of activatable channels over a physiological range of voltages or a reduction in

Kv4 single-channel conductance. These effects of neuraminidase on Kv4 channels would have the effect of reducing the amplitude of I_{to} under physiological conditions. The positive shift in the steady-state inactivation of I_{to} would not compensate the shift in activation and smaller conductance of this current because at the resting potential of these ventricular myocytes (approximately −80 mV), nearly 100% of the channels are available for activation.

Insight into how negatively charged particles on the external portion of Kv4.3 contribute to the voltage dependence of the channel could be gained by analyzing the surface potential model initially put forward by Frankenhaeuser and Hodgkin (10) and later expanded by Hille and colleagues (13). In this model, fixed, negatively charged particles (phospholipids and sugars) on the surface membrane of the cell produce a surface potential. The functional implication of such surface potential is that its magnitude determines the intramembrane voltage, which is the voltage "felt" by the voltage sensor of the K^+ channel. According to the surface potential model, reductions in the number of negative charges (i.e., ion channel deglycosylation or synthesis of sialic acid-deficient glycoproteins) has the effect of reducing the magnitude of the surface potential, which then leads to an increase in the intramembrane potential. Under these circumstances the surface potential model predicts that as the intramembrane potential is increased, a greater membrane depolarization will have to be applied for the channel to "feel" the same transbilayer field than under normal conditions. The surface potential model thus predicts that a reduction in the number of negatively charged particles should produce a positive shift in the threshold for activation of the channel.

Based on the surface potential model, our results suggest that negatively charged sialic residues in Kv4 channels contribute to the surface potential "felt" by the channel. This conclusion is supported by the observation that Kv4.3 currents expressed in the sialylation-deficient cell line lec2 had voltage dependencies of activation and inactivation shifted toward more positive potentials than those in control cells (pro5 and k1). Our experiments examining the effects of increasing external Ca^{2+} on the voltage dependence of activation of Kv4.3 channels also support the surface potential theory. We found that as external Ca^{2+} was increased and negatively charged particles are titrated, the $V_{½}$ of activation of Kv4.3 currents shifted toward more positive potentials. However, the activation of Kv4.3 currents in the glycosylation-deficient cell line was less sensitive to external Ca^{2+} than wild-type controls were.

In the surface potential model, both the negatively charged particles present on the channel itself and the surrounding lipids contribute to the voltage dependence of the channel. It is therefore possible that sialylated lipids could contribute to the voltage dependence of K^+ channels. We have examined the effect of desialylated lipids on the kinetic parameters of deglycosylated Kv4.3 channels. We found that most, perhaps as much as 90%, of the changes in the voltage dependence of the Kv4.3 current is produced by the removal of channel-linked sialic acids. These data suggest that sialic acid residues in Kv4.3 channels largely dominate the effects of these negatively charged particles in the gating of these channels.

We note that K^+ channel pore-forming subunits (α) are known to interact with accessory proteins to form a functional heteromultimeric channel. These accessory proteins include Kvβ (32), Kchip (2), MiRP (1), and minK (26) as well as γ-subunits (16). Thus we cannot rule out the possibility that deglycosylation of endogenous membrane-spanning accessory subunits could also contribute to the changes in the voltage dependence of I_{to} observed in lec2 cells. Similarly, in the mouse ventricle it is possible that different α-subunits of the Kv4 family (i.e., Kv4.2 and Kv4.3) could form the channels producing I_{to} in these cells (35), and hence deglycosylation of any one of these α-subunits and/or membrane-spanning accessory subunits could modify I_{to} function.

Sialic acid deficiency produces arrhythmogenic changes in native I_{to} in mouse ventricular myocytes. The positive shift in the voltage dependence of activation of I_{to} would have the consequence of reducing the magnitude of this current. At 60 mV, I_{to} in NT cells was ~35% smaller than in control myocytes. This reduction in amplitude of I_{to} induced by K^+ channel glycosylation contributed to a significant increase in the duration of the AP of ventricular myocytes. The increase in AP duration was evident at APD_{50} and APD_{90}. Although a reduction in I_{to} could account to a large extent for the observed increases in AP duration, it is important to note that neuraminidase treatment is known to modify the function of other channels, including T-type Ca^{2+} channels (18), Na^+ channels (7, 39), and several K^+ channels (19, 28). It also is possible that in these experiments neuraminidase could have deglycosylated a transporter such as the Na^+/Ca^{2+} exchanger (15), which could have altered its function in ways that prolong the AP. Thus the changes we detected in the AP in ventricular myocytes reflect changes in I_{to} and these other currents that are affected by neuraminidase. It is important to note that the increase we observed in AP duration was accompanied by an increase in the number of EADs observed. This suggests that proper glycosylation of K^+ channels is crucial for the normal electrical functioning of the heart.

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