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Glycosylation Influences Gating and pH Sensitivity of I_{sK}

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Abstract. The KvLQT1 and minK subunits that coassemble to form I_{sK} channels, contain potential N-glycosylation sites. To examine the role of glycosylation in channel function, a Chinese hamster ovary cell line deficient in glycosylation (Lec-1) and its parental cell line (Pro-5) were transiently transfected with human KvLQT1 (hKvLQT1) cDNA, alone and in combination with the rat (rminK) or human minK (hminK) cDNA. Functional KvLQT1 and I_{sK} currents were expressed in both cell lines, although amplitudes were larger in Pro-5 than Lec-1 cells transfected with hKvLQT1 and hKvLQT1/hminK. For I_{sK}, but not KvLQT1, the voltage-dependence of activation was shifted to more positive voltages and the activation kinetics were slower in the Lec-1 compared to the Pro-5 cells. The effect of extracellular acidification on recombinant KvLQT1 and I_{sK} currents was investigated in Pro-5 and Lec-1 cells. Changing external pH (pH_o) from 7.4 to 6.0 significantly decreased the amplitude and increased the half-activation voltage ($V_{1/2}$) of KvLQT1 currents in Pro-5 and Lec-1 cells. In Pro-5 cells, decreasing pH_o reduced I_{sK} amplitude without increasing $V_{1/2}$, whether rminK or hminK was coexpressed with hKvLQT. In contrast, changing pH_o from 7.4 to 6.0 did not significantly change I_{sK} amplitude in Lec-1 cells. Thus, oligosaccharides attached to the minK subunit affect not only the gating properties, but also the pH sensitivity of I_{sK}.

Key words: I_{sK} — minK — KvLQT1 — Lec mutant — Glycosylation — External pH

Introduction

Like other proteins, ion channels are charged, not only because of sequences which contain acidic and basic amino acids that are charged at physiological pH, but also because of post-translational attachment of acidic carbohydrate moieties. Mutagenesis studies and detailed analyses of cloned channel behavior have shown that charged amino acids can have important functional roles. For example, positively charged arginine and lysine residues in the S4 domain of Na^+ and K^+ channels function as a voltage sensor (Papazian et al., 1991), while the total net charge in extracellular loops of Shaker K^+ channels contributes to fixed surface charge that can be screened by divalent and trivalent cations (Elinder, Madeja & Arhem, 1996; Hice et al., 1994).

The ways in which oligosaccharide chains attached to asparagine residues in ion channel proteins affect channel function are variable and ill defined. Site-directed mutagenesis studies that rely on the *Xenopus* oocyte expression system have suggested that N-glycosylation exerts negligible effects on surface membrane expression and gating of Shaker K^+ channels (Deal, Lovinger, & Tamkun, 1994; Santacruz-Toloza et al., 1994). However, expression of Kv1.1 channels in mammalian cells deficient in glycosylation significantly altered voltage-dependence of activation, activation kinetics, and sensitivity to extracellular Ca^{2+} (Thornhill et al., 1996). This discrepancy may be a consequence of the different expression systems. Carbohydrate processing occurs in a host-dependent fashion; both qualitative and quantitative differences in glycosylation have been noted between *Xenopus* oocytes, yeast, and mammalian systems (Roitsch & Lehle, 1989*a,b*). N-linked glycosylation is required for surface membrane expression of HERG in HEK 293 cells (Petrecca et al., 1999). Moreover, N-glycosylation dramatically alters the open probability of the inward rectifier K^+ channel ROMK1 (Kir 1.1a) expressed in Sf9 cells (Schwalbe et al., 1995). Sialidase treatment of isolated muscle cells has produced inconsistent effects on native ion channel function. Enzymatic removal of the sialic acid associated with N-linked oligosaccharide affected functional properties of the sodium (Recio-Pinto et al., 1990; Zhang, Hartmann, & Satin, 1999) and L- and T-type calcium currents (Fer-

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mini & Nathan, 1991), but not the calcium-activated K^+ current (Wann & Richards, 1994), hyperpolarization activated pacemaker current (Fermini & Nathan, 1990), or composite cardiac delayed rectifier K^+ current (McDonagh & Nathan, 1990).

The slowly activating, noninactivating component of the cardiac delayed rectifier K^+ current (I_{Ks}) has been associated with two protein subunits: the six transmembrane domain K^+ channel protein known as KvLQT1 or KCNQ1, and the single transmembrane domain protein known as minK, I_{sK}, or KCNE1. Simultaneous expression of both these subunits is required to reproduce a current with the kinetic properties of native cardiac I_{Ks} (Barhanin et al., 1996; Sanguinetti et al., 1996). However, the stoichiometry of the underlying channels and the nature of the interaction between minK and KvLQT1 are incompletely understood (Romey et al., 1997; Tai & Goldstein, 1998; Tai, Wang & Goldstein, 1997; Wang et al., 1998).

Both KvLQT1 and minK possess charged amino acids in the extracellular portions of the channel proteins; furthermore, both channel subunits contain potential N-glycosylation sites (Fig. 1) (Takumi, Ohkubo & Nakanishi, 1988; Folander et al., 1990; Varnum et al., 1993; Zhang et al., 1994; Barhanin et al., 1996; Sanguinetti et al., 1996). There is virtually no information about the functional significance of these moieties in KvLQT1. Existing information about the functional significance of charged amino acids and N-linked oligosaccharide in the minK subunit has come exclusively from studies that rely on heterologous expression in *Xenopus* oocytes (Takumi et al., 1991; Hice et al., 1993, 1994; Salata et al., 1996). The nature of the oligosaccharide attached to minK has been studied in Sf9 cells transfected with minK alone without KvLQT1, but in the absence of any functional correlate. These experiments suggested that the attached sugar moiety was not rich in negatively charged sialic acid (Lesage et al., 1993).

Interspecies variation in the number of negatively charged amino acid residues proximal to the putative transmembrane domain of minK (see Fig. 1) is believed to underlie observed differences between the La^{+3} sensitivity of recombinant I_{sK} currents associated with coexpression of rat and human minK in *Xenopus* oocytes (Hice et al., 1993, 1994). It has also been suggested that protons, which block the recombinant current in a concentration-dependent, voltage-independent fashion, bind to these acidic residues (Yamane et al., 1993). In contrast, N-glycosylation of minK has not been shown to affect significant IsK channel function in *Xenopus* oocytes. minK mutants devoid of N-glycosylation sites elicit currents comparable in size, kinetics, and pharmacology to the wild-type (Salata et al., 1996).

The primary aim of this report was to determine if N-glycosylation of minK and KvLQT1 subunits influences the activation properties or pH sensitivity of recombinant I_{sK} currents in Chinese hamster ovary (CHO) cells with normal (CHO-K1, Pro-5) and deficient glycosylation (Lec-1). Human KvLQT1 (hKvLQT1) cDNA was coexpressed with either the rat or human species variants of minK to determine if the effects of N-glycosylation are influenced by the number of extracellular, acidic amino acids in the minK subunit.

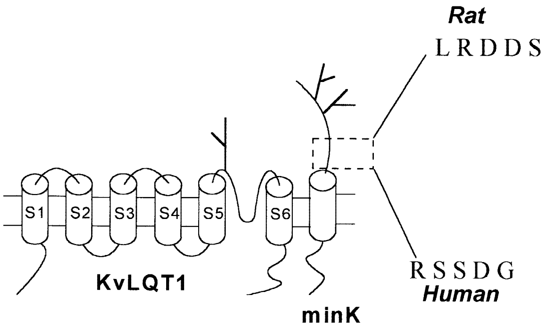

Fig. 1. Schematic diagram showing membrane topology of KvLQT1 and minK subunits that assemble to form I_{sK} channels. Potential N-linked glycosylation sites are shown in the S5-P segment of hKvLQT1, and at positions 5 and 26 in the extracellular N-terminus of minK. Interspecies variation in the number of negatively charged amino acid residues proximal to the hydrophobic domain of minK (positions 37–40 in the rat minK sequence) is also shown. The indicated residues contribute to interspecies differences in the La^{3+} sensitivity of I_{sK} (Hice et al., 1993, 1994).

Materials and Methods

Materials

The CHO-K1, Pro-5 and Lec-1 cells were obtained from American Type Culture Collection (Rockville, MD). As described previously, CHO-K1, parental of Pro-5, and Pro-5, parental of Lec-1, are control cell lines with normal glycosylation, while Lec-1 is mutant cell line that produces truncated glycoproteins (Stanley, Caillibot & Siminovitch, 1975; Stanley, 1984, 1989). The hKvLQT1 clone was a gift from Mark Keating, University of Utah (Sanguinetti et al., 1996). The human minK (hminK) clone was a gift from Robert Kass, Columbia University (Wang, Xia & Kass, 1998). The wild-type rat minK was provided by Steve Goldstein, Yale University (Hausdorff et al., 1991). The N5,26A rminK was a gift from Andreas Busch and Karen Wild (University of Tuebingen). The M2 FLAG antibody, pFLAG-CMV-2, and all other reagents were obtained from Sigma Chemical (St. Louis, MO) unless otherwise stated.

Culture and Transfection of CHO Cell Lines

Pro-5 and Lec-1 cells were maintained in α-modified minimum essential medium supplemented with fetal bovine serum (FBS, 10%) and penicillin (100 U/mL)-streptomycin (100 μg/mL) at 37°C under 5% CO_2. CHO-K1 cells were similarly maintained in F12 medium supplemented with L-glutamine (2 mM), FBS (10%), and penicillin (100 U/mL)-streptomycin (100 μg/mL). The hKvLQT1, rminK, and hminK cDNAs were subcloned into the eukaryotic expression vector pcDNA3.1, and transiently transfected into the CHO cell lines by lipofection using the conditions described by Sanguinetti et al. (1996). Forty-eight hours after transfection, CHO-K1, Pro-5, or Lec-1 cells were lifted off the 35 mm dishes using a nonenzymatic solution (Nozyme™, JRH Biosciences, Lenexa, KS), and assayed for expression of I_{sK} using standard whole-cell patch-clamp methods (described in detail below).

ELECTROPHYSIOLOGICAL RECORDING

Membrane currents were recorded using the whole-cell configuration of the patch clamp technique (Hamill et al., 1981). Recording pipettes were pulled to resistances of 2.5–6 Mohms when filled with intracellular solution containing (mM): 110 potassium aspartate; 1 $MgCl_2$; 11 EGTA; 1 $CaCl_2$; 10 HEPES; 10 K_2ATP (pH 7.3 was attained by addition of 1 N KOH to bring the final potassium concentration to 140). This concentration of HEPES has been shown previously to adequately buffer intracellular pH over the range of extracellular pH (pHe) used in these experiments (Krafte & Kass, 1988). Extracellular recording solutions consisted of (mM): 132 NaCl; 1.2 $MgCl_2$; 1 $CaCl_2$; 5 glucose; 4 KCl; and one of the following buffers, as indicated: 10 HEPES (pKa 7.5) for pHe 7.4; 10 MES (pKa 6.1) for pHe 6.0.

The reference electrode was an Ag/AgCl half-cell connected to the bath via a 3 M KCl-agar salt bridge. Tip potentials were zeroed prior to seal formation. Liquid junction potentials calculated for the pH 7.4 and pH 6.0 recording solutions were 18.4 and 18.5 mV, respectively, (pClamp 7.0). Measured liquid junction potentials ranged from 13.8 to 14.7 mV. Data were not corrected for either the calculated or the measured liquid junction potentials. Because the parameters reported are differences or changes in half-activation voltage, and all cells were studied under identical recording conditions, errors in voltage values due to liquid junction potential would have little effect on interpretation of the data.

As reported previously (Thornhill et al., 1996), Pro-5 and Lec mutant cells exhibited statistically indistinguishable membrane capacitances and series resistances. Membrane capacitance, calculated as the time integral of the capacitive response to a 5 mV hyperpolarizing step from a −40 mV holding potential was 29.2 ± 3.2 ($n = 13$); this value was similar to that reported previously for the parental CHO-K1 cell line (21 ± 7 pF, Peterson & Nerbonne, 1999). Series resistance, estimated by dividing the time constant of the decay phase of the uncompensated capacitive transient by the calculated membrane capacitance, was 12.7 ± 1.9 MΩ ($n = 13$), and was electrically compensated to minimize the duration of the capacity transient (75–95%). Peak tail currents used to derive activation curves did not exceed 1.6 nA, and the voltage errors associated with uncompensated series resistance (≤5 mV) were not corrected.

All recordings were performed at room temperature (22–24°C) from a plexiglass chamber mounted on an inverted microscope (Nikon Diaphot 300). Data acquisition and analysis was accomplished using an IBM compatible computer interfaced to an Axopatch 200-A amplifier driven by pClamp software (Axon Instruments, Burlingame, CA). Rundown of I_{sK} over time was rare; runup was observed occasionally after achieving whole-cell patch clamp. Only cells with stable IsK currents (no rundown or runup over 5–15 min) were included in the investigation.

The standard voltage clamp protocol for activation of I_{Ks} consisted of a series of 3–4 second depolarizing test pulses to test potentials (V_t) ranging from −40 to +60 mV at an interpulse interval of 14 sec; the standard holding potential was −40 mV. Deviations from this protocol are noted in figure legends. Isochronal activation curves for I_{Ks} were determined from the amplitudes of tail currents recorded on return to the holding potential. Tail currents (I_{Ktail}) were normalized to the maximum measured amplitude (I_{Kmax}), and the voltage dependence of activation was fit to a Boltzmann distribution of the form $I_{Ktail}/I_{Kmax} = 1/[1+ \exp\{V_{1/2} - V_t)/k\}]$ with a nonlinear least squares fitting routine (Origin, Microcal Software, Northampton, MA) to estimate the half-activation voltage ($V_{1/2}$) and slope factor (k) for this relationship. The time courses of I_{Ks} activation and deactivation were fit with a double-exponential relationship of the form $I_t = A_0 + A_1 e^{-t/\tau 1} + A_2 e^{-t/\tau 2}$ using a Chebyshev noniterative fitting technique (pClamp, Axon Instruments). The time course of I_{Ks} activation was also described using a model-independent parameter; the isochronal 90% rise time was defined as the time required for the whole cell current to reach 90% of its final value following a 4 sec depolarization from −40 to +60 mV.

Currents associated with expression of KvLQT1 alone were elicited from a holding potential of −50 mV using a protocol similar to the standard I_{Ks} activation protocol with shorter duration (2 sec) test pulses. The voltage dependence of KvLQT1 channel activation was determined by fitting normalized extrapolated tail current *vs.* test potential to a Boltzmann function, as described by Tristani-Firouzi & Sanguinetti (1998). Time constants for activation of KvLQT1 channel current were determined by fitting currents activated during the test-pulse with a double exponential function as described previously. Time constants for recovery from inactivation (initial increase) and deactivation (slow decline) were derived by fitting the relevant portions of the tail currents with a single exponential function (Tristani-Firouzi & Sanguinetti, 1998).

STATISTICAL ANALYSIS

The data are presented as mean ± SEM unless otherwise indicated (n = no. of cells). The statistical significance of species (rat or human) and cell line (Pro-5 or Lec-1) was determined using analysis of variance to test for these two main effects and their interaction (GLM ANOVA, Statististix Analytical Software, Tallahassee, FL). Where independent main effects of species and cell line were detected in the absence of any interaction, data obtained using rminK and hminK were analyzed separately. Pairwise comparisons were performed using Least Significant Differences or *t*-test for independent samples, as appropriate. Effects of changing extracellular pH were analyzed using the paired *t*-test. A $P < 0.05$ was considered significant.

IMMUNOBLOT ANALYSIS

To confirm the expected difference in glycosylation between Lec-1 and Pro-5 cells (Stanley et al., 1975; Stanley, 1984, 1989), Pro-5 and Lec-1 cells were transiently transfected with cDNAs encoding hminK and FLAGM2-tagged hKvLQT1. Whole cell lysates were made from confluent monolayers 48 hr after transfection by standard techniques using a lysis buffer consisting of PBS with 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS and protease inhibitor cocktail (Sigma). Lysis buffer was added to the culture dish after washing with cold PBS 3 times. The culture dishes were scraped and the lysate was aspirated into a syringe with a 21 gauge needle to shear DNA. The lysates were rocked in the cold for 1 hr and centrifuged for 10 min at 10,000 × *g*. Protein was determined by the Micro BCA method (Pierce). For immunoprecipitations, 100 μL aliquots of lysate containing 1 mg/mL protein were incubated overnight at 4°C with 4 μg FLAGM2 antibody. Immune complexes were then precipitated by incubation with 20 μL protein G-agarose (Pierce). Immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose. The membrane was blocked by overnight incubation in 5% nonfat dry milk in TBS at 4°C, probed with the FLAGM2 antibody (10 μg/mL) in TBS for 1 hr at room temperature. After extensive washing, the membrane was incubated with the horseradish peroxidase-linked secondary antibody (sheep anti-mouse IgG) in TBS for 1 hr at room temperature. After additional washing, bound antibodies were detected using enhanced chemiluminescence (ECL, Amersham).

The total carbohydrates of protein in the cell lysates were biotinylated using an Immuno-Blot Kit for Glycoprotein Detection (Bio-Rad). The resulting proteins were analyzed by SDS-PAGE, transferred to nitrocellulose, probed with a streptavidin-alkaline phosphatase conjugate and detected by a colorimetric alkaline phosphatase reaction.

Results

Confirmation of Deficient Glycosylation in Lec-1 Mutant

The cDNAs encoding hKvLQT1 and either hminK or rminK were cotransfected into a control cell line, Pro-5 (the parental cell line of Lec-1), and one glycosylation mutant line, Lec-1 (Stanley et al., 1975; Stanley, 1984, 1989). Lec-1 cells produce truncated cell surface glycoproteins, because decreased activity of N-actylglucosamine transferase (GlcNAc) results in mature carbohydrate blocked at the Asparagine-GlcNAc$_2$-Mannose$_5$ intermediate (Fig. 2*A*). Immunoblot analysis of FLAGM2-tagged hKvLQT1 protein expressed in CHOK1, Pro-5 and Lec-1 cell lines showed a more discrete band in Lec-1 compared to the broader bands seen in the CHOK1 and Pro-5 lanes (Fig. 2*B*). These results are consistent with the expected glycosylation deficiency (Thornhill et al., 1996) for Lec-1. To demonstrate this glycosylation deficiency further, the carbohydrate moieties of the proteins expressed in Lec-1 and Pro-5 cell lines cotransfected with hKvLQT1/hminK were biotinylated, then subsequently analyzed by SDS-PAGE, transfer to nitrocellulose and detection of biotinylated proteins by streptavidin-linked alkaline phosphatase. The differences in glycosylation between Lec-1 and Pro-5 are shown in Fig. 2*C*. At the molecular weights expected for both KvLQT1 and minK, broad dark bands are apparent in Pro5 compared to faint, more discrete bands in Lec-1; this result is consistent with relatively deficient glycosylation of the heterologously expressed channel proteins in the Lec-1 mutant.

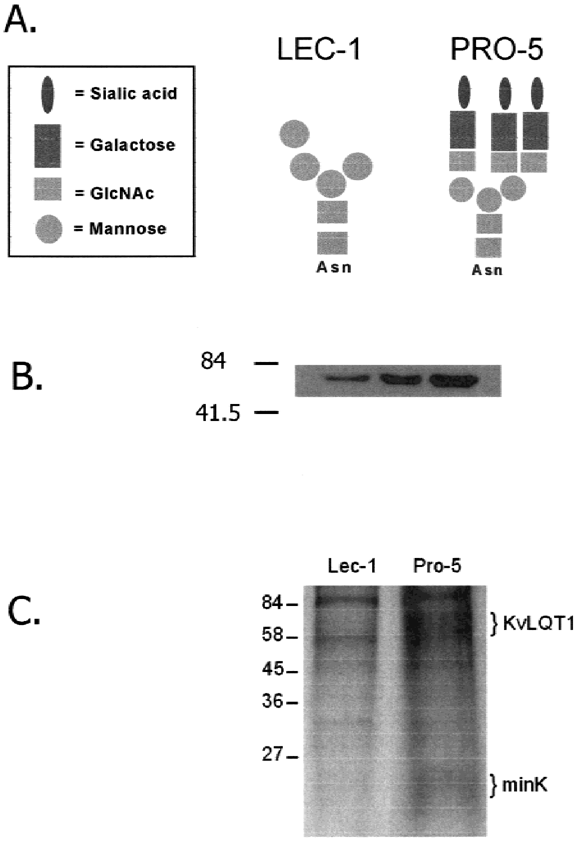

Fig. 2. (*A*) Theoretical comparison of N-linked glycoprotein oligosaccharides in Pro-5 and Lec-1 cells. Lec-1 was derived from the parental CHO clone Pro-5. Lec-1 cells lack GLcNAc glycosyl transferase so that N-linked carbohydrates are truncated at the Man5-GlcNAC2-Asn intermediate. (Man = mannose; GLcNAc = N-acetylglucosamine). (*B*) FLAGM2-tagged KvLQT1 protein was immunoprecipitated, and subsequently detected using anti-FLAGM2 antibody in lysates from (left to right) Lec-1, CHO-K1, and Pro-5 cells transiently cotransfected with cDNAs encoding hminK and FLAGM2-tagged hKvLQT1. (*C*) Biotinylation of glycoproteins in Lec-1 and Pro-5 cells lysates. Glycoproteins in cell lysates from Lec-1 and Pro-5 cells cotransfected with hminK/hKvLQT1 were labeled by biotinylation of carbohydrate moieties. Equal amounts of protein (30 μg) for Lec-1 and Pro-5 were treated and loaded on an SDS-PAGE gel. After transfer to nitrocellulose, biotinylated proteins were detected by probing with an streptavidin-linked alkaline phosphatase conjugate followed by a standard colorimetric alkaline phosphatase assay. The brackets indicate reported molecular weight ranges for KvLQT1 and minK.

Expression of minK and KvLQT1 Subunits in CHO Cell Lines

Pro-5 and Lec-1 cells transiently cotransfected with rminK/KvLQT1 or hminK/KvLQT1 exhibited robust outward K$^+$ currents with the slow activation and lack of inactivation characteristic of native I_{Ks} and recombinant I_{sK} currents (*see* Figs. 3 and 7). As expected from previous reports describing a lack of overlapping voltage-gated currents in CHO cells (Lesage et al., 1993; Sanguinetti et al., 1996), identical depolarizations of either untransfected or sham-transfected Pro-5 or Lec-1 cells did not produce similar currents (n = 3, 12 cells total, *data not shown*). There was no significant difference in the amplitudes of the I_{sK} currents expressed in Pro-5 (n = 12) and Lec-1 (n = 16) cells after transfection of rminK/KvLQT1; time-dependent currents measured at the end of 4 sec depolarizing test pulse to +60 mV averaged 2.96 ± 0.51 and 2.96 ± 0.38 pA, respectively. In contrast, the mean amplitude of similarly measured time-dependent current was significantly greater for hminK/KvLQT1 expressed in Pro-5 (6.91 ± 0.8 pA, n =

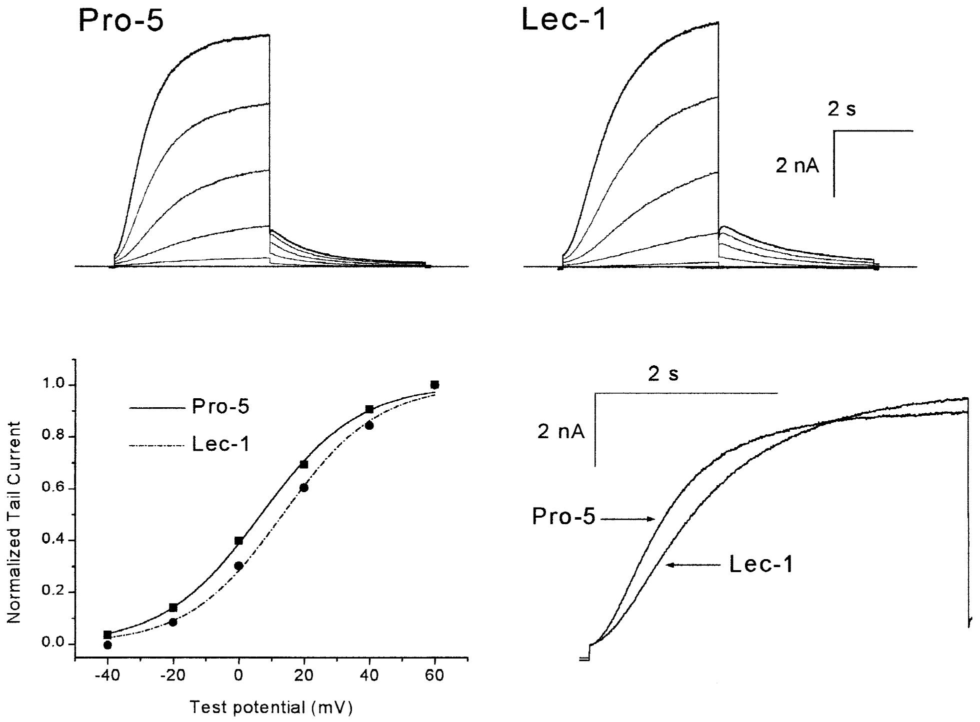

Fig. 3. I_{sK} associated with coexpression of hminK/hKvLQT1 in Pro-5 and Lec-1 cells. Upper panels: Representative currents eliminated by a series of depolarizing test pulses (−40 to +60, step 20) from the −40 mV holding potential. Lower left: Isochronal (4 sec) activation curves for families of current traces shown in upper panels. Normalized tail currents are plotted as a function of test potential. Continuous lines are best fit Boltzmann functions of the form $I/I_{max} = 1/\{1 + \exp[(V_{1/2} - V_t)/k]\}$. For Pro-5, $V_{1/2} = 6.8$ mV and $k = 14.9$ mV. For Lec-1, $V_{1/2} = 13.5$ mV and $k = 14.5$ mV. Lower right: Superposition of time-dependent currents recorded during a 4 sec pulse to +60 mV. For Pro- and Lec-1, the times required for elicited currents to reach 90% of their final amplitudes were 1787 and 2448 msec, respectively.

9) than Lec-1 (3.70 ± 0.57 pA, $n = 11$). Overall, current amplitudes associated with coexpression of hminK/KvLQT1 were significantly greater than those associated with coexpression of rminK/KvLQT1. Moreover, hKvLQT1 current amplitude measured at the end of a +40 mV test pulse was significantly larger in Pro-5 (2.00 ± 0.36 nA, $n = 11$) than in Lec-1 cells (0.96 ± 0.33 nA, $n = 11$), when hKVLQT1 was expressed without minK.

FUNCTIONAL ANALYSIS OF I_{sK} AND KvLQT1 CURRENTS EXPRESSED IN CHO CELL LINES

To determine if glycosylation influences KvLQT1 or I_{sK} channel function, the kinetics and gating of recombinant currents expressed in Pro-5 and Lec-1 were compared. Data obtained using hminK and rminK were analyzed separately, because the slow activation time constants (τ_{slow}) and the isochronal 90% rise time (time required for whole cell current to reach 90% of its final value following a 4 sec depolarization from −40 to +60 mV) were influenced independently by cell line and minK species variant. As reported previously (Hice et al., 1994; Yang & Sigworth, 1998), I_{sK} associated with expression of hminK activated faster than I_{sK} associated with expression of rminK, as evidenced by the shorter isochronal 90% rise time and slow time constant of activation. Gating parameters for I_{sK} currents associated with hminK and rminK are summarized in Tables 1 and 2, respectively.

Irrespective of the expressed minK species variant, I_{sK} activation was slower and exhibited more positive $V_{1/2}$ in the glycosylation mutant Lec-1 cells than in the control Pro-5 cells. The isochronal 90% rise time and slow time constant of activation were significantly longer for recombinant I_{sK} currents expressed in Lec-1 cells compared to Pro-5 cells, although the fast time constant of activation did not differ significantly between cell lines (Tables 1 and 2).

In contrast, there was no significant difference ($P = 0.32$) in the $V_{1/2}$ for currents associated with expression of hKvLQT1 alone in CHO-K1 (−14.7 ± 2.2 mV, $n = 5$), Pro-5 (−14.2 ± 2.8 mV, $n = 12$) and Lec-1 (−17.9 ± 2.4 mV, $n = 10$) cells. Slope factors were also similar ($P = 0.89$) for hKvLQT1 currents expressed in CHO-K1 (11.6 ± 0.8), Pro-5 (11.2 ± 0.7 mV) and Lec-1 (11.4 ± 1.2 mV). The effects of glycosylation on the activation and inactivation kinetics of KvLQT1 channel current are shown in Fig. 4. The fast time constant of activation was significantly faster for KvLQT1 currents in Lec-1 cells than Pro-5 cells, at 0, +20, +40 and +60 mV, but there

Table 1. I_{sK} Characteristics: coexpression of hKvLQT1 and hminK cDNAs

Cell line (n)	Isochronal 90% rise-time (msec)	τ Activation (msec)		τ Deactivation (msec)		$V_{1/2}$ (mV)	k (mv)
		τ_{fast}	τ_{slow}	τ_{fast}	τ_{slow}		
Pro-5 (10)	2042.4 ± 130.9	367.9 ± 63.8	743.0 ± 46.1	246.1 ± 81.8	1885.1 ± 289.4	−0.3 ± 3.8	16.3 ± 0.9
Lec-1 (11)	2448.8 ± 93.9*	236.2 ± 24.2	1165.9 ± 24.2*	340.0 ± 89.1	1889.1 ± 460.9	11.1 ± 1.7*	15.9 ± 0.4

Each value is mean ± SEM (*, significantly different from Pro-5 at $P < 0.05$).

Table 2. I_{sK} Characteristics: coexpression of hKvLQT1 with wild-type and N5,26A mutant rminK cDNAs

Cell line (n) minK cDNA	90% Rise-time (msec)	τ Activation (msec)		τ Deactivation (msec)		$V_{1/2}$ (mV)	k (mv)
		τ_{fast}	τ_{slow}	τ_{fast}	τ_{slow}		
Pro-5 (12) wt rminK	2466.8 ± 154.5^{a}	262.5 ± 43.9	1255.6 ± 142.4^{a}	206.5 ± 51.5	1980.4 ± 238.8	4.2 ± 3.9^{a}	18.0 ± 0.6
Lec-1 (16) wt rminK	2931.2 ± 112.1^{b}	383.3 ± 53.2	2280.6 ± 345.1^{b}	289.9 ± 75.9	1755.3 ± 280.4	14.0 ± 2.6^{b}	17.2 ± 0.6
Pro-5 (12) N5,26A rminK	2739.8 ± 104.1^{ab}	274.3 ± 38.8	1564.6 ± 166.1^{ab}	335.4 ± 110.8	2014.1 ± 332.7	7.3 ± 2.5^{c}	16.2 ± 0.5

Each value is mean ± SEM. Different superscripts indicate a statistically significant difference between a given parameter among the different cell lines (rows) at $P < 0.05$. wt = wild type.

were no significant differences in the slow time constant of activation (Fig. 4, upper right). The relative amplitude of the fast component of activation was similar at all test potentials for KvLQT1 expressed in Pro and Lec cells (Fig. 4, lower left). The time constant for deactivation was slower in Lec-1 than Pro-5 cells at all voltages; however, the difference was statistically significant only at +20 mV (Fig. 4, lower right). Time constants for recovery from inactivation were similar for recombinant KvLQT1 currents expressed in Pro-5 and Lec-1 cells (Fig. 4, lower right).

To provide additional evidence that differences in glycosylation contribute to the differences observed in gating between recombinant I_{sK} currents expressed in Pro-5 and Lec-1, the kinetics and voltage-dependence of I_{sK} associated with expression of rminK/hKvLQT1 in these cells were compared to those associated with coexpression of a mutant rminK (N5,26A) and hKvLQT1 in Pro-5 cells. The N5,26A mutant lacks the two N-glycosylation sites found in rat and human species variants of minK. We hypothesized that if glycosylation of the minK subunit were a significant determinant of activation rate and $V_{1/2}$, then the I_{sK} current associated with expression of the N5,26A rminK mutant in Pro-5 would activate more slowly and exhibit a more positive $V_{1/2}$ than current associated with similar expression of wild-type rminK. The data obtained are consistent with this prediction. The N5,26A mutant expressed as well as the wild-type rminK in Pro-5 cells; the mean amplitude of time-dependent current recorded at the end of 4 sec depolarizing test pulse to +60 mV was 3.67 ± 0.51 pA (n = 12). The isochronal 90% rise time, activation time constants, and $V_{1/2}$ for N5,26A were intermediate between those determined for Pro-5 and Lec-1 (Table 2).

pH SENSITIVITY OF I_{sK} AND KvLQT1 CURRENTS EXPRESSED IN CHO CELL LINES

The pH sensitivities of recombinant KvLQT1 currents expressed in Pro-5 and Lec-1 were determined by the measuring the changes in $V_{1/2}$, k, and current amplitude associated with changing external pH from 7.4 to 6.0. Decreasing extracellular pH significantly decreased the amplitude and induced a positive shift in the half-activation voltage of KvLQT1 currents expressed in Pro-5 and Lec-1 cells (Fig. 5). In Pro-5 cells (n = 5), tail current amplitudes were decreased by 69 ± 1, 63 ± 1, 61 ± 1, and 63 ± 1% following test depolarizations to 0, +20, +40 and +60 mV, and $V_{1/2}$ was increased significantly by +6.9 ± 2.3 mV. In Lec-1 cells (n = 5), tail current amplitudes were decreased by 66 ± 1, 61 ± 1, 61 ± 1, and 58 ± 1% following test depolarizations to 0, +20, +40 and +60 mV, and $V_{1/2}$ was increased significantly by +8.3 ± 2.5 mV. The magnitudes of the pH 6.0-induced block and shift were not significantly different in Pro-5 and Lec-1 cells. The slope of the Boltzmann relationship was significantly decreased by changing external pH from 7.4 to 6.0 in Pro-5 ($\Delta k = -2.5 \pm 0.2$ mV), but not Lec-1 cells ($\Delta k = 0.3 \pm 0.7$ mV).

The pH sensitivities of recombinant I_{sK} currents expressed in Pro-5 and Lec-1 were determined by the measuring the changes in $V_{1/2}$, k, and current amplitude associated with changing external pH from 7.4 to 6.0. In

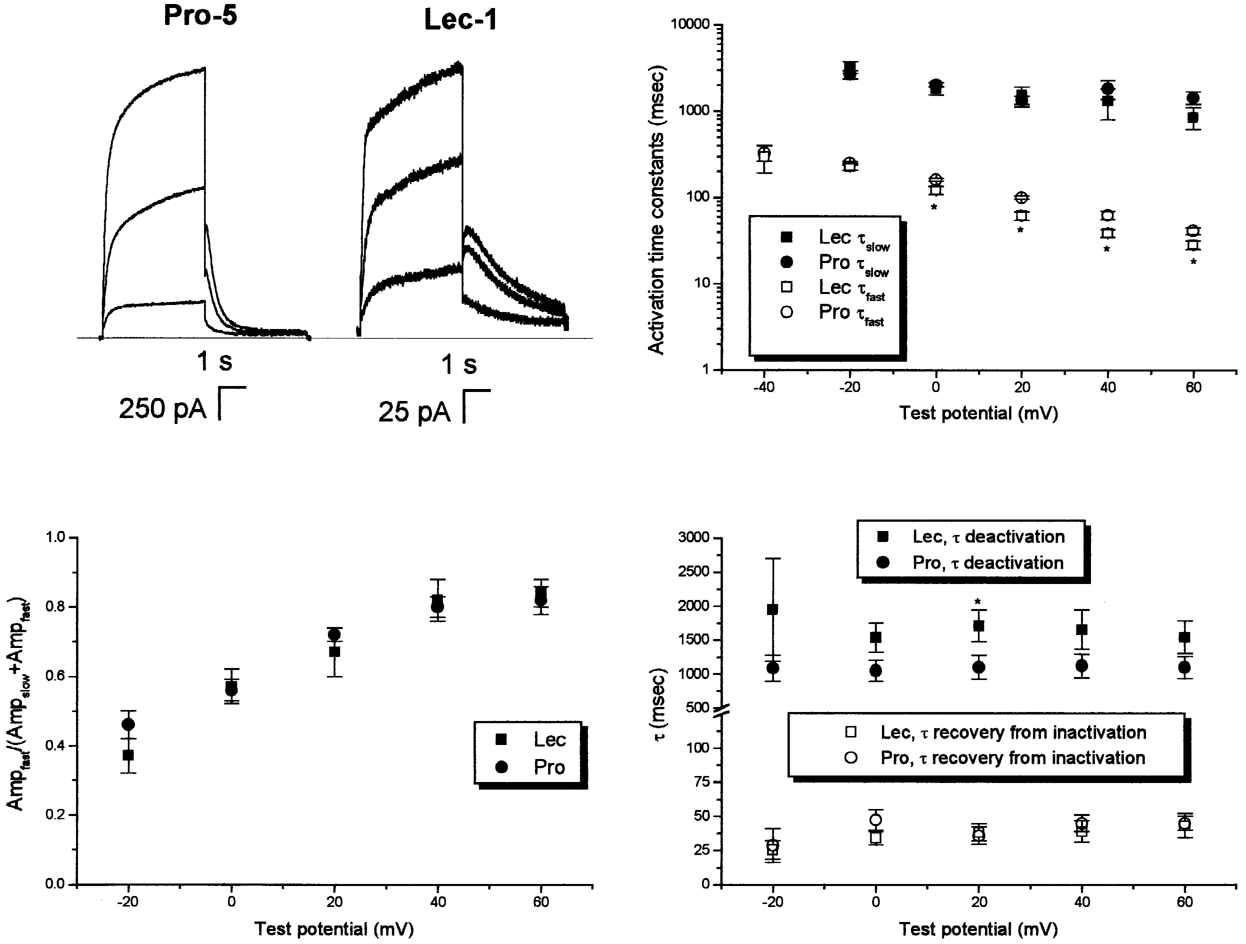

Fig. 4. Glycosylation influences KvLQT1 current amplitude, fast component of KvLQT1 channel activation, and KvLQT1 channel deactivation. Upper left: Currents recorded from Pro-5 and Lec-1 cells transfected with hKvLQT1 cDNA during 4 sec pulses from the −50 mV holding potential to −20, 0 and +20 mV. Deactivating tail currents were elicited by repolarization to −40 mV. Upper right: Time constants for activation of KvLQT1 channel current in Pro-5 (Pro, n = 12) and Lec-1 cells (Lec, n = 12), determined from two-exponential fits to the activating phase. Lower left: Relative amplitude of the fast component of KvLQT1 channel activation in Pro5 (Pro, *n* = 9) and Lec-1 (Lec, *n* = 8) cells. Lower right: Time constants of deactivation (slow decline) and recovery from inactivation (initial increase in current). KvLQT1 tail currents were measured in Pro-5 (*n* = 12) and Lec-1 cells (*n* = 11) at −40 mV after 2 sec depolarizing pulses to −40 to +60 mV (step 20).

Pro-5 cells, this decrease in external pH was associated with a significant decrease in IsK amplitude whether rminK or hminK was coexpressed with hKvLQT (Fig. 6). Changing external pH from 7.4 to 6.0 decreased Isk tail current amplitude in Pro-5 cells, measured at −40 mV after 4 sec pulses to +20, +40 and +60 mV by 19 ± 6%, 21 ± 5% and 23 ± 7%, respectively ($n = 12$). The H^+-induced block of I_{sk} in Pro-5 cells was neither strongly voltage-dependent, nor associated with a positive shift in the voltage-dependence of activation (Table 3). In fact, the $V_{1/2}$ of I_{sK} activation significantly decreased when external pH was decreased from 7.4 to 6.0 (Table 3).

In contrast to Pro-5, changing extracellular pH from 7.4 to 6.0 did not decrease I_{sK} amplitude in Lec-1 cells. Tail current amplitudes measured at −40 mV after 4 sec pulses to +20, +40 and +60 mV were increased by 3 ± 4%, 4 ± 3% and 3 ± 3%, respectively; these changes in current amplitude were not statistically significant. In Lec-1 cells, the decrease in the $V_{1/2}$ of I_{sK} activation associated with changing external pH from 7.4 to 6.0 was not statistically significant (Table 3). The slopes of the Boltzmann relationships describing I_{sK} activation were unaltered by changing extracellular pH in Lec-1 and Pro-5 cells (Table 3).

To provide additional evidence that differences in glycosylation contributed to the significant differences in pH sensitivity between recombinant I_{sK} currents expressed in Pro-5 and Lec-1 cells, the currents associated with expression of rminK/hKvLQT1 in these cells were compared to the currents associated with coexpression of rminK and hKvLQT1 in CHO-K1 cells (parental cell line of Pro-5), and coexpression of rminK N5,26A (the

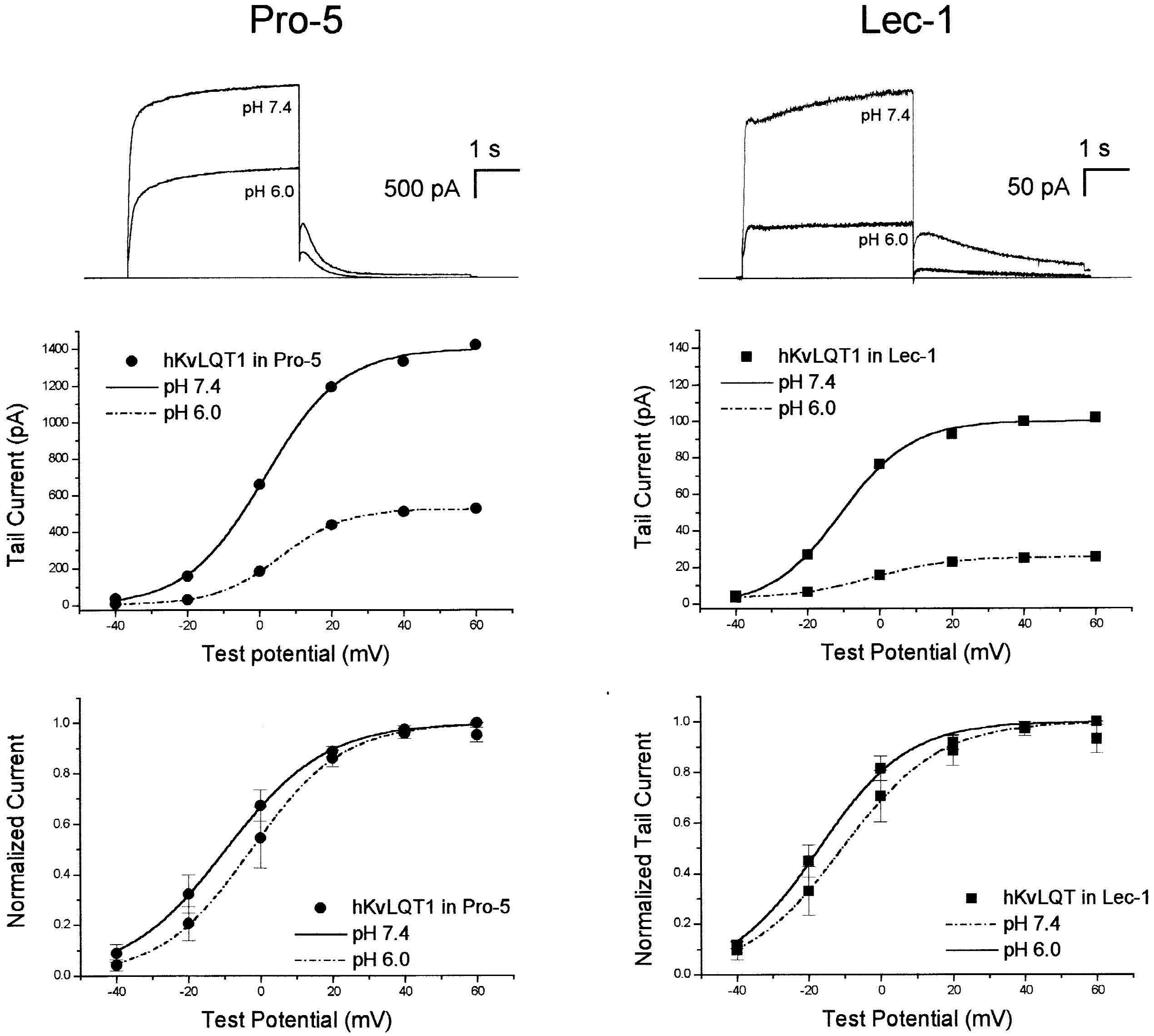

Fig. 5. Glycosylation does not affect pH sensitivity of KvLQT1 currents. Pro-5 (left, $n = 5$) and Lec-1 ($n = 5$) cells were transfected with hKvLQT1. Top panels: Representative currents recorded from Pro-5 (left) and Lec-1 (right) cells in response to a 4 sec depolarizing pulse to +60 mV from the −50 mV holding potential at extracellular pH of 7.4 and 6.0. Tail currents were measured at a −40 mV return potential. Middle panels: Extrapolated tail current amplitude as a function of test potential for the Pro-5 (left) and Lec-1 (right) cells shown in top panels. The curves are Boltzmann fits to the data at each pH. Data in pH 6.0 were described by curves which incorporated both depolarized shifts in gating, and scaled-down current amplitudes. Bottom panels: Normalized tail current amplitude as a function of test potential at pH 7.4 and pH 6.0 for Pro-5 (left, $n = 5$) and Lec-1 ($n = 5$, right) cells. Continuous lines are best fit Boltzmann functions of the form $I/I_{max} = 1/\{1 + \exp[(V_{1/2} - V_t)/k]\}$. Decreasing the extracellular pH from 7.4 to 6.0 significantly induced a significant positive shift in the $V_{1/2}$ of KvLQT1 currents expressed in Pro-5 ($\Delta V_{1/2} = +7.0$ mV) and Lec-1 ($\Delta V_{1/2} = +7.2$ mV) cells.

rminK mutant devoid of N-glycosylation sites) and hKvLQT1 in Pro-5 cells. In CHO-K1 cells transfected with rminK/KvLQT1, changing external pH from 7.4 to 6.0 decreased tail current amplitude measured at −40 mV after 4 sec pulses to +20 mV, +40 mV and +60 mV by 53 ± 18%, 53 ± 18% and 53 ± 17%, respectively ($n = 4$). Thus, I_{sK} currents expressed in CHO-K1 cells were pH sensitive, like I_{sK} currents expressed in Pro-5 cells. In contrast, I_{sK} currents associated with co-expression of KvLQT1 and the N5,26A rminK mutant in Pro-5 cells were relatively pH insensitive, like the IsK currents expressed in the glycosylation mutant Lec-1 cells (Figs. 4 and 5).

Discussion

Although the role of glycosylation in K^+ channel function is not completely understood, previous investiga-

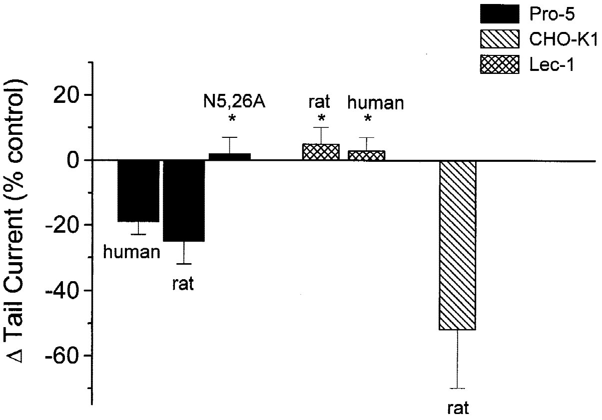

Fig. 6. Glycosylation of minK subunit affects pH sensitivity of I_{sk}. Pro-5, Lec-1, and CHO-K1 cells were transfected with hKvLQT1 in combination with one of the following minK constructs: wild-type rat minK (rminK: Pro-5, $n = 9$; Lec-1 $n = 9$; CHO-K1, $n = 4$); wild-type human minK (hminK: Pro-5, $n = 7$; Lec-1, $n = 9$) or rminK mutant lacking N-glycosylation sites (N5, 26A: Pro-5, $n = 5$). Tail current amplitudes were measured at a −40 mV return potential following a depolarizing test pulse to +40 mV. Changes in tail current amplitude induced by decreasing extracellular pH from 7.4 to 6.0 are shown. Asterisks indicate significant difference from Pro-5 transfected with hKvLQT1/hminK ($P < 0.05$).

tions of Kv1 and HERG channels have shown that the nature and extent of N-glycosylation may dramatically affect either the voltage-dependence of channel activation (Thornhill et al., 1996; Bennett et al., 1997), or the cell surface expression and subunit assembly (Petrecca et al., 1999). Data presented here demonstrate that N-glycosylation of KvLQT1 and minK can not only influence the size of expressed KvLQT1 and I_{sK} currents, but also profoundly impact I_{sK} channel function and pH sensitivity.

GLYCOSYLATION AFFECTS THE AMPLITUDE OF hKvLQT1 AND hKvLQT1/minK CURRENTS

Whole-cell currents associated with expression of KvLQT1 have been shown to be substantially smaller in amplitude than whole-cell currents associated with coexpression of KvLQT1 and minK in variety of heterologous expression systems (Barhanin et al., 1996; Sanguinetti et al., 1996; Romey et al., 1997; Yang & Sigworth, 1998; Sesti & Goldstein, 1998). Our data are consistent with these previous reports. Coexpression with minK is believed to increase not only the number of functional channels expressed on the cell surface (Romey et al., 1997), but also their unitary conductance (Yang & Sigworth, 1998; Sesti & Goldstein, 1998; Pusch, 1998). Although indirect evidence suggests that some homomeric KvLQT1 channels may contribute to I_{Ks} in cardiac myocytes (Salata et al., 1998), in most native cells I_{Ks} has been linked to functional channels formed by coassembly of KvLQT1 with minK or a related β-subunit from the KCNE family (Barhanin et al., 1996; Sanguinetti et al., 1996; Marcus & Shen, 1994; Kim & Greger, 1999; Schroeder et al., 2000). Together, these findings suggest that synthesis, trafficking, assembly and/or turnover of homomeric KvLQT1 channels may be relatively inefficient and more vulnerable to disruption in the absence of KCNE subunits.

In the present study, we observed a significant reduction in the amplitude of whole-cell KvLQT1 currents expressed in Lec-1 cells, as compared to Pro-5 cells. This difference occurred in the absence of significant differences in either cell membrane capacitance or time course of transfection. These results suggest that the density and/or the activity of KvLQT1 channels may be altered by incomplete glycosylation. Fluctuation analysis of KvLQT1 channels in Pro-5 and Lec-1 cells could be used to determine if deficient glycosylation diminishes the number of functional channels expressed on the cell surface, their open probability, or their unitary conductance. Any of these is plausible. N-glycosylation is believed to play a role in trafficking and assembly of other six transmembrane domain voltage-gated potassium channel α-subunits (Shi & Trimmer, 1999), and is known to be essential for cell surface expression of HERG (Petrecca et al., 1999), another α-subunit that interacts with minK and related peptide subunits of the KCNE family (Abbott et al., 1999). Furthermore, diminished glycosylation has been shown to decrease the open probability of ROMK1 (Kir 1.1) channels (Schwalbe et al., 1995).

A previous study showed no significant effect of glycosylation on I_{sK} current density in *Xenopus* oocytes injected with mRNA encoding either wild-type or N5Q (glycosylation mutant) rabbit minK (Salata et al., 1996). However, the general applicability of this result is questionable. The rabbit species variant of minK is unique among cloned KCNE subunits in having only one rather than two NH_2-terminal N-glycosylation sites (Salata et al., 1996). Thus, I_{sK} channels formed with this species variant of minK may be less influenced by N-glycosylation than other recombinant I_{sK} channels. Alternatively, the lack of observed effect of N-glycosylation on current density could be specific to the *Xenopus* oocyte expression system and the interaction between the endogenous *Xenopus* KvLQT1 (xKvLQT1) and rabbit minK. Expression environment can profoundly impact K^+ current properties (Petersen & Nerbonne, 1999).

In the present study, there was no significant difference between the amplitudes of the I_{sK} currents expressed in Pro-5 and Lec-1 cells cotransfected with hKvLQT1 and rminK. However, I_{sK} amplitude was significantly larger for hKvLQT1 and hminK coexpressed in Pro-5 than Lec-1. Furthermore, the amplitudes of IsK associated with coexpression of hKvLQT1 and hminK were significantly greater than those associated with coexpression of hKvLQT1 and rminK.

Table 3. Effect of decreasing external pH from 7.4 to 6.0 on Boltzmann parameters associated with coexpression of hKvLT1 and either hminK or rmin*K* in Pro-5, and Lec-1

Cell Line (n) cDNAs	pH 7.4		pH 6.0	
	$V_{1/2}$ (mV)	*k* (mv)	$V_{1/2}$ (mV)	*k* (mv)
Pro-5 (7) hKvLQT1/hminK	−0.3 ± 3.3	16.9 ± 0.8	−8.6 ± 4.9*	17.8 ± 1.0
Lec-1 (9) hKvLQT1/hminK	12.3 ± 1.8	15.9 ± 0.5	7.3 ± 2.3	16.7 ± 0.8
Pro-5 (5) hKvLQT1/rminK	1.2 ± 4.6	17.7 ± 0.9	−4.6 ± 5.1*	18.6 ± 2.0
Lec-1 (9) hKvLQT1/rminK	9.5 ± 2.1	16.9 ± 0.6	6.2 ± 2.5	17.6 ± 0.7

Each value is mean ± SEM (*, significantly different from pH 7.4 at $P < 0.05$).

It is unlikely that the difference in current amplitude associated with transfection of rat *vs.* human minK reflects a difference in the unitary conductance of the formed I_{sK} channels, because the single channel conductances of I_{sK} channels formed by hKvLQT1 in combination with hmink and rminK have been reported to be similar (Yang & Sigworth, 1998). The larger current amplitudes associated with coexpression of conspecific hminK and hKvLQT1 cDNAs, and N-glycosylation of these channels, could result from increased cell surface expression and/or greater open probability of these channels. Additional experiments are necessary to determine if differences in the synthesis, assembly, trafficking, cell surface expression, or turnover of I_{sK} channels occur as the result of varying either the minK species variant expressed with hKvLQT1, or the degree of glycosylation of hminK/hKvLQT1 channel proteins. Differences in the gating of hKvLQt1/hminK and rminK/LvLQT1 channels expressed in Pro-5 and Lec-1 cells are discussed below.

GLYCOSYLATION AFFECTS hKvLQT1 CHANNEL FUNCTION

A previous investigation of recombinant Kv1.1 channels expressed in CHOK1, Pro-5 and Lec mutant cell lines found that the voltage dependence of activation ($V_{1/2}$) was shifted to more positive potentials and the activation kinetics were slower in the Lec mutant cell lines, compared to the controls (Thornhill et al., 1996). These findings suggest that reducing the amount of N-linked carbohydrate on Kv1.1 channels lessens the negative potential detectable by the voltage sensor. Deficient glycosylation may either reduce the negative surface potential, or induce conformational changes that shift negatively charged amino acids to positions that no longer influence the voltage sensor.

In the present study, there were no significant differences in the $V_{1/2}$ or slope of the Boltzmann distribution describing activation of hKvLQT1 channels in CHO-K1, Pro-5, and Lec-1 cells. However, the fast component of KvLQT1 channel activation was accelerated, and KvLQT1 channel deactivation was slower in Lec-1 compared to Pro-5 cells. The unchanged activation midpoint and altered kinetics of KvLQT1 currents in Lec-1 compared to Pro-5 cells cannot be explained easily by surface charge considerations. The different responses of Kv1.1 and KvLQT1 channels to deficient glycosylation are likely to reflect the distant phylogenetic relationship (Barhanin et al., 1996) and distinct gating behavior (Pusch et al., 1998; Tristani-Firouzi & Sanguinetti, 1998) of these K^+ channels.

Both the Kv1.1 and the KvLQT1 α-subunits have one extracellular N-glycosylation site (Barhanin et al., 1996; Sanguinetti et al., 1996). The site in Kv1 channels is on the extracellular loop between S1 and S2 (Shi & Trimmer, 1993), while the site in KvLQT1 is on the extracellular region between S5 and the pore (P loop) (Barhanin et al., 1996; Sanguinetti et al., 1996). In *Shaker* channels, fixed charges associated with amino acids on the S5-P loop have been identified as the functional surface charges that influence $V_{1/2}$ (Elinder et al., 1996). We were unable to demonstrate a similar role for negative charge associated with N-linked oligosaccharide attached to the S5-P region of KvLQT1. Mutations that cause accelerated activation of KvLQT1 involve residues in the cytoplasmic S4-S5 linker (Wang et al., 1999) rather than any extracellular region. Thus, the basis for the similar influence of deficient glycosylation is not obvious. The mechanism underlying the observed effect of deficient glycosylation on KvLQT1 channel deactivation is also unclear.

GLYCOSYLATION AFFECTS I_{sK} CHANNEL FUNCTION

In *Xenopus* oocytes, expression of hminK (in combination with the endogenous xKvLQT1) produces I_{sK} currents that not only activate faster, but also exhibit a more positive half-activation voltage than I_{sK} currents induced by similar expression of rminK (Hice et al., 1994). When rminK and hminK are coexpressed with hminK in *Xenopus* oocytes, the difference in activation kinetics is still apparent, but the difference in half-activation voltage is not (Yang & Sigworth, 1998). The reported difference in half-activation voltage is consistent with a surface charge effect; rat minK has a higher density of acidic residues in the NH2-terminal domain (Fig. 1). However, the consistently described difference in activation kinetics cannot be explained easily by a difference in effective surface potential.

In the present study, we coexpressed hKvLQT1 with either rminK or hminK in CHO cell lines. I_{sK} associated with expression of hminK activated more quickly than I_{sk} associated with expression of rminK, as reported previously (Hice et al., 1994; Yang & Sigworth, 1998). There was no significant effect of minK species variant on the $V_{1/2}$ of I_{sK} currents expressed in the control cell line, Pro-5 (Tables 1–3).

Coexpression of minK and KvLQT1 in Lec-1 cells was associated with a positive shift in the voltage-dependence of I_{sK} activation and significantly slowed activation kinetics. Significant changes in these parameters were seen whether rminK or hminK was coexpressed with hKvLQT1. Furthermore, the differences in I_{sK} gating associated with altered N-glycosylation were independent of and comparable in magnitude to those associated with interspecies variation in the minK amino-acid sequence (*see* Tables 1 and 2). Negative fixed charges that influence the gating of I_{sK} channels could reside on acidic phospholipids such as phosphatidyl serine, carbohydrates such as sialic acid, or charged amino acids such as aspartate in the channel protein. Our data suggest that the acidic carbohydrate modifications to the I_{sK} channel subunits play a functional role equal to or greater than that of the charged amino acids proximal to the hydrophobic domain in the minK protein.

As discussed above, neither a shift in $V_{1/2}$ nor a slowing of activation kinetics was apparent for homomeric KvLQT1 channels in Lec-1 compared to Pro-5 and CHOK1 cells. Thus, N-linked oligosaccharides appear to contribute more to the functional surface charge of the I_{sK} channels than KvLQT1 channels. This may reflect not only the presence of two N-glycosylation sites on rminK and hminK compared with one N-glycosylation site on hKvLQT1, but also the different stoichiometries of the assembled channels. Experimental evidence suggests that KvLQT1 channels are homotetramers, while I_{sK} channels may contain two or more minK in combination with four KvLQT1 α subunits (Wang & Goldstein, 1995; Tai & Goldstein, 1998; Wang et al., 1998).

The gating properties of channels formed in Pro-5 cells with the glycosylation mutant (N5,26A) rminK suggest that deficient glycosylation of both the KvLQT1 and the minK subunits can influence I_{sK} gating. I_{sK} currents associated with coexpression of hKvLQT1 and N5,26A rminK in Pro-5 exhibited $V_{1/2}$, isochronal 90% rise time, and activation time constants intermediate between those associated with Pro-5 and Lec-1 expression of hKvLQT1 and wild-type rminK (Table 2). The differences between the I_{sK} channels formed by combination of hKvLQT1 with N5,26A and wild-type rminK in Pro-5 cells can be attributed to lack of glycosylation of the minK subunit. Comparison of the I_{sK} channels formed by combination of hKvLQT1 with N5,26A in Pro-5 cells and wild-type rminK in Lec-1 cells provides a means to assess the functional importance of KvLQT1 glycosylation. The data indicate that lack of minK glycosylation affects IsK less than deficient glycosylation of both minK and KvLQT1.

The changes in both activation rate and half-activation voltage for I_{sK} channels expressed in Lec-1 cells are consistent with the hypothesis that negatively charged sialic acid groups on N-Linked oligosaccharides contribute significantly to cell surface negative charges, and that as a result, truncation of N-linked carbohydrates effectively alters the electric field sensed by the channel gating mechanism. However, surface charge theory would predict that channel activation and deactivation were equally affected by loss of negative cell surface charge. Results shown in Tables 1 and 2 indicate that deficient glycosylation affects activation without significantly affecting deactivation. Wang et al. (1998), reported that cotransfection with additional hminK slowed the activation but had no effect on the deactivation of I_{sK} currents associated with transfection of a tandem construct consisting of hminK and hKvLQT1 linked 1:1 in frame. These data suggest not only that modulation of I_{sK} gating kinetics may involve minK interaction sites peripheral to the pore, but also that the ratio of minK to KvLQT1 may not be fixed in assembled I_{sK} channels. Thus, the changes in I_{sK} gating associated with deficient glycosylation could be the result of altered subunit stoichiometry as well as altered surface potential.

GLYCOSYLATION AFFECTS pH SENSITIVITY OF I_{sK} BUT NOT KvLQT1

Ion channel modulation by extracellular pH is both biophysically interesting and physiologically important. Investigation of the effects of H^+ ions on channel behavior has provided not only information about interactions between native channels and their environment in the membrane phospholipid, but also a basis for understanding the marked effects of myocardial ischemia and acidosis on cardiac rate and rhythm (Gende, Camilion & Cingolani, 1978; Orchard & Cingolani, 1994). Many ion channels that contribute to the cardiac action potential are sensitive to extracellular hydrogen ions, including: sodium channels (Yatani et al., 1984; Zhang & Siegelbaum, 1991; Benitah et al., 1997), L-type calcium channels (Krafte & Kass, 1988), HERG (Anumonwo et al., 1999; Berube, Chanhine & Daleau, 1999; Jo et al., 1999) and Kv1.5 (Furukawa et al., 1995; Steidl & Yool, 1999).

The effects of extracellular pH on recombinant I_{sK}

currents in *Xenopus* oocytes (Yamane et al., 1993), and native I_{Ks} in guinea pig ventricular myocytes (Davies et al., 1995; Kass, Davies & Freeman, 1996) have been described previously, and found to differ substantially. External acidification reduces the maximal conductance of I_{sK} associated with *Xenopus* expression of rminK in a voltage-independent fashion without affecting the voltage-dependence of activation (Yamane et al., 1993). In contrast, the effects of extracellular acidification on native cardiac I_{Ks} are mediated by more than one mechanism. Decreasing extracellular pH not only blocks cardiac I_{Ks}, but also causes a profound depolarizing shift in the voltage-dependence of activation (Davies et al., 1995; Kass et al., 1996).

We hypothesized that differences in post-translational processing could contribute to the discrepant effects of external acidification on recombinant I_{sK} in the *Xenopus* expression system and native I_{Ks} in guinea pig cardiac myocytes. This investigation was designed to determine if N-glycosylation can influence the pH sensitivity of I_{sK} currents expressed in mammalian cells. H^+ ion effects on homomeric hKvLQT1 channels were determined for comparison.

Recombinant KvLQT1 currents were extremely sensitive to extracellular acidification (Fig. 5). Decreasing external pH from 7.4 to 6.0 significantly decreased the amplitude and induced a positive shift in the half-activation voltage of recombinant KVLQT1 currents expressed in Pro-5 and Lec-1 cells. The magnitudes of the shift in the midpoint of the activation curve (~7 mV) and the H^+-induced block (~60% reduction in current amplitude) were similar in the control (Pro-5) and the glycosylation-deficient (Lec-1) cell lines. However, the slope of the activation curve was significantly decreased at an external pH of 6.0 in Pro-5 ($\Delta k = -2.5 \pm 0.2$ mV), but not Lec-1 cells.

The depolarizing shift in the $V_{1/2}$ of KvLQT1 induced by extracellular acidosis is consistent with binding and/or screening of negative surface charges by H^+ ions. Protons have been reported to have similar effects on voltage-gated Na^+ (Yatani et al., 1984; Zhang & Siegelbaum, 1991; Benitah et al., 1997), Ca^{2+} (Krafte & Kass, 1988) and K^+ channels (Furukawa et al., 1995; Ahn & Hume, 1997; Anumonwo et al., 1999; Berube, Chanhine & Daleau, 1999; Jo et al., 1999; Perez-Cornejo, 1999). Our observation of equivalent gating shifts in Pro-5 and Lec-1 cells indicates that the titratable H^+-sensitive negative charges on the surface of these CHO cell lines are not associated with the terminal regions of N-linked oligosaccharides. In addition, the data suggest that H^+ ions modulate KvLQT1 current amplitude by more than one mechanism, because the relatively modest (~7 mV) shift in half-activation voltage does not adequately explain the profound (~60%) block of KvLQT1 currents observed in Pro-5 and Lec-1 cells. The decreased slope of the voltage-dependence of activation seen at pH 6.0 in Pro-5 cells provides additional support for the hypothesis that H^+ ions can affect the gating of normally glycosylated KvLQT1 channels by mechanisms other than neutralization of surface charge. More detailed analysis of proton effects on KvLQT1 channel gating and conductance is warranted, but beyond the scope of this investigation.

In the present study, where mammalian cell lines were cotransfected with hKvLQT1 and either rminK or hminK, decreasing the extracellular pH reduced the amplitude of I_{sK}, without inducing a depolarizing shift in the voltage-dependence of activation (Table 3, Figs. 4 and 5). These observed effects of external acidification on recombinant IsK in CHO-K1 and Pro-5 cells are similar to those obtained previously using the heterologous *Xenopus* expression system (Yamane et al., 1993). Thus, the previously documented differences between native and recombinant I_{sK} currents cannot be characterized as artifacts of the *Xenopus* expression system, or attributed to the lack of an N-glycosylation site in the S5-P loop of *Xenopus* KvLQT1 (Sanguinetti et al., 1996).

Furthermore, it is unclear if differences between the surface potentials of cardiac myocytes and epithelial cells can be responsible for the presence of pH-induced gating shifts in only the former. Although Pro-5 and Lec-1 cells may not process minK and KvLQT1 proteins in a manner identical to cardiac tissue, the data obtained from heterologous expression of minK and KvLQT1 subunits in these CHO cell lines suggests that profound differences in the extent of N-glycosylation can be associated with an absence of pH-induced gating shifts. Another possibility is that the H^+-induced shift in the voltage dependence of I_{Ks} activation in cardiac myocytes reflects contributions to native cardiac I_{Ks} of both homotetrameric KvLQT1 channels and heteromultimeric channels formed by coassembly of KvLQT1 with minK subunits (Salata et al., 1998). Further investigation will be required to resolve the basis of the differences between H^+ ion effects on the gating of recombinant I_{sK} and native cardiac I_{Ks}.

Our experimental results also refute the hypothesis that the negatively charged residues just proximal to the hydrophobic domain of rminK represent the sites for proton binding and IsK block (Yamane et al., 1993). The human species variant of minK differs from rminK with respect to the number of charged residues in this region (Fig. 1), and, as a result, the associated IsK exhibits ~8-fold reduced La^{+3} sensitivity (Hice et al., 1993, 1994). In contrast, the H^+ sensitivity of I_{sK} associated with expression of rminK and hminK in Pro-5 cells cotransfected with hKvLQT1 did not differ significantly in magnitude (Fig. 4).

Most importantly, our data suggest strongly that N-glycosylation of the minK subunit modulates the voltage-independent block of I_{sK} by H^+ ions. Expression of

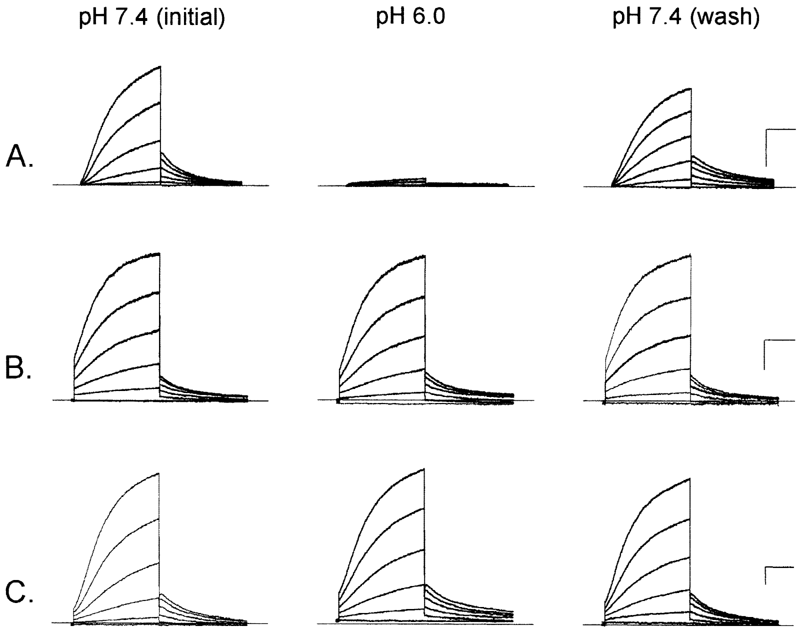

Fig. 7. I_{sk} currents recorded at pH 7.4 and 6.0. Currents were elicited by depolarizing test pulses (−40 to +60, step 20) from a −40 mV holding potential, at an initial extracellular pH (pH_{out}) of 7.4 (left), after changing pH_{out} to 6.0 (middle), and then returning to the original pH_{out} of 7.4 (left). (*A*) Currents recorded from a CHO-K1 cell cotransfected with wild-type rminK and hKvLQT1. (*B*) Currents recorded from a Lec-1 cell cotransfected with hminK and hKvLQT1. (*C*) Currents recorded from a Pro-5 cell cotransfected with mutant N5,26A rminK and hKvLQT1. Calibration bars: 500pA (vertical); 2 sec (horizontal).

KvLQT1/minK in the glycosylation deficient cell line, Lec-1 eliminated the reduction of I_{sK} amplitude induced by acid pH in CHO-K1 and Pro-5 cells (Figs. 6 and 7). Furthermore, coexpression of hKvLQT1 with a rat mutant minK lacking N-glycosylation sites (N5,26A) had a similar effect.

Comparisons between the effects of extracellular acidification on I_{sK} and KvLQT1 channels suggests that coassembly with the minK subunit alters the pH sensitivity of the channels formed in at least three ways. First, incorporation of minK limits proton access to the negative surface charges that are titratable in homomeric KvLQT1 channels. In contrast to KvLQT1, changing pH from 7.4 to 6.0 had no effect on the midpoint of IsK activation. Coassembly with minK also diminishes the reduction in I_{sK} current amplitude induced by acid pH. Whereas hKvLQT1 currents in Pro-5 cells were blocked on average by ~60% at pH 6.0, I_{sK} currents associated with similar coexpression of hKvLQT1 and hminK were blocked on average by ~20%. Finally, N-linked oligosaccharide on minK contributes to the I_{sK} channel's pH sensor.

The correlation between the H^+ sensitivity of I_{sK} and the structure of the oligosaccharide chain attached to the minK subunit is most intriguing. If the nature and extent of N-glycosylation can modulate H^+ ion effects on K^+ current amplitude, then differential expression of glycotransferases may represent a means of fine-tuning tissue response to acidosis and ischemia. Two N-terminal consensus N-glycosylation sites are present not only on the minK subunit (KCNE1), but also on the related β-subunits, KCNE2-4 (Schroeder et al., 2000). Thus, this modulatory mechanism could affect K^+ channel response to altered pH not only in the heart, but also in the CNS and intestines where other KCNQ α-subunits and KCNE β-subunits interact to form physiologically important K^+ currents (Schroeder et al., 1998, 2000).

In summary, data presented here suggest that N-glycosylation of the KvLQT1 and/or minK ion channel subunits can influence the gating and pH sensitivity of formed I_{sK} channels. The results contribute to our understanding of mechanisms underlying pH regulation of protein function, and suggest a novel regulatory role for minK in modulating the cardiac electrophysiological response to the extracellular acidosis that can accompany disease states such as respiratory or renal failure, diabetic ketoacidosis and myocardial ischemia.

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