Biochemical engineering of the N-acyl side chain of sialic acids alters the kinetics of a glycosylated potassium channel Kv3.1

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The sialic acid of complex N-glycans can be biochemically engineered by substituting the physiological precursor N-acetylmannosamine with non-natural N-acylmannosamines. The Kv3.1 glycoprotein, a neuronal voltage-gated potassium channel, contains sialic acid. Western blots of the Kv3.1 glycoprotein isolated from transfected B35 neuroblastoma cells incubated with N-acylmannosamines verified sialylated N-glycans attached to the Kv3.1 glycoprotein. Outward ionic currents of Kv3.1 transfected B35 cells treated with N-pentanoylmannosamine or N-propanoylmannosamine had slower activation and inactivation rates than those of untreated cells. Therefore, the N-acyl side chain of sialic acid is intimately connected with the activation and inactivation rates of this glycosylated potassium channel.

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1. Introduction

Voltage-gated potassium channels (Kv) are critical determinants in control and modulation of the action potential repolarization in neurons, cardiac myocytes and skeletal muscle. Kv3.1 is a member of the Kv3 subfamily and all members have two absolutely conserved N-glycosylation sites within the S1–S2 extracytoplasmic linker [1,2]. It was demonstrated that the Kv3.1 pore-forming α-subunit heterologously expressed in a cultured neuronal-derived cell model, B35 neuroblastoma cell, contains sialylated N-glycans [3,4], similar to that throughout the central nervous system [5]. The lack of sialylated N-glycans attached to the Kv3.1 protein led to decreases in activation, inactivation and deactivation kinetics of the ionic currents expressed from Kv3.1 transfected B35 cells [4]. Other potassium channels which lack glycan attachment, as well as sialic acid residues, have also been shown to alter channel gating [6–13]. Since modifications of Kv channel regulation and expression can lead to changes in action potential repolarization [14], the expression of different Kv glycoforms and aglycoforms are important in modulation of electrical signaling, and thus have substantial impact on the development and maintenance of an organism.

Sialic acids represent a family of about 50 amino sugars. They are found at terminal positions of carbohydrate chains and the carboxylate group at the 1-carbon position is typically ionized at physiological pH [15]. This structural feature of sialic acid has been ascribed to modifying Kv channel gating [8,11–13]. Another main structural and functional feature of sialic acids is the N-acyl side chain at the 5-carbon position. The size of the N-acyl side chain can be altered in cell culture and in vivo by substituting the first direct precursor of sialic acid, N-acetylmannosamine (ManNAc), with non-natural N-acylmannosamines [16,17]. Moreover, the enzymes which convert ManNAc to N-acetylmannosaminic acid (Neu5Ac) are promiscuous and can therefore change non-natural N-acylmannosamines, N-pentanoylmannosamine (ManNPent) and N-propanoylmannosamine (ManNPprop), to the respective non-natural sialic acids, N-pentanoylmannosaminic acid (Neu5Pent) and N-propanoylmannosaminic acid (Neu5Prop), with the result that these non-natural sialic acids partially replace the natural sialic acids of the glycoconjugates [18,19].

The primary aim of this paper was to determine whether the N-acyl side chain at the 5-carbon position of sialic acid could impact the conducting properties of the Kv3.1 channel heterologously expressed in neuronal-derived cells. Treatment of Kv3.1 transfected B35 cells with ManNPent or ManNPprop led to decreases in the activation and inactivation rates of the outward ionic currents for the inactivating current type while the deactivation rates were unchanged. Additionally, the whole cell current patterns of the...
non-inactivating currents were similar to those of Kv3.1 transfected B35 cells with natural sialic acids.

2. Materials and methods

2.1. B35 neuroblastoma cells and N-acylmannosamines

Wild type Kv3.1 (pore-forming α-subunit) transfected B35 neuroblastoma cells were established and maintained as described elsewhere [4]. Stable transfected cells were utilized for Western blotting and patch clamp studies. To change the natural sialic acid residues to non-natural sialic acid residues, Kv3.1 transfected cells were treated with ManNPent (5 mM) or ManNProp (5 mM) for 48–144 h. Preparation and analysis of N-acyl-n-mannosamines were performed as previously described [17].

2.2. Western blots of M2 immunoaffinity purified Kv3.1 glycoprotein

Kv3.1 glycoproteins were immunoaffinity purified from transfected B35 cells [4]. Neuraminidase (0.83 U/μl) (New England Biolabs) was used to remove sialic acid residues from glycoconjugates. The control was buffer substituted for the enzyme. Untreated and treated M2 immunoaffinity purified samples of Kv3.1 glycoproteins were immunoblotted as described in [4]. The rabbit anti-Kv3.1 antibody (Alamone Labs) was used to identify the Kv3.1 glycoprotein [3,4].

2.3. Whole cell recordings

Electrophysiological measurements were obtained from transfected B35 cells using the whole cell configuration of the patch clamp technique as described elsewhere [1,4]. Briefly, the external bath solution had the following composition (in mM): 5 potassium aspartate, 135 sodium aspartate, 1 MgCl₂ hexahydrate, 10 Mes, 60 mannitol (pH 6.3) and 295–312 mOsm. The intracellular solution consisted of (in mM): 140 potassium aspartate, 10 EGTA, 5 MgCl₂ hexahydrate, 10 HEPEs, 50 mannitol (pH 7.2) and 320–340 mOsm. Whole cell recordings were conducted with untreated and treated cells on similar days. Cell capacitance and series resistance were compensated throughout the course of experiments. Kv3.1 currents were sampled at 10 kHz and subsequently filtered at 1 kHz. Whole cell current recordings were accepted when membrane seal resistance was ≥ 1 GΩ, maximum current amplitudes were ≥ 400 pA, and minimal cell capacitance was displayed after compensation [4].

2.4. Data analysis

Digitized whole cell current recordings from B35 cells were analyzed using the CLAMPFIT 9.0 analysis program (Axon Instruments) [4]. Normalized conductance–voltage relationships were fitted with a Boltzmann equation of the form: 

$$ g = g_{max}[1 + \exp(V_m - V_{0.5})/q] $$

where q denotes the slope factor, Vₘ stands for the test potential, Vₐₜₜ is the potential at which the conductance was half maximal, gₚₜ is the conductance and gₚₜₜ is the maximal conductance. Whole cell current recordings were analyzed for rise times, activation time constants, inactivating current rates, and deactivation time constants as previously described [4]. Kodak 1D image analysis software was used to acquire Western blot images and Adobe Photoshop was used for preparation of figure. Whole cell current figures were generated using Origin 7.5 (OriginLab). Data are presented as the mean ± S.E. and n represents the number of cells tested. Student’s t-test was utilized to evaluate the statistical comparisons. Statistical significance was considered at P < 0.05.

3. Results and discussion

3.1. Sialylated N-glycans of the Kv3.1 glycoprotein

Treatment of several cultured cell lines with ManNPent and ManNProp results in production of Neu5Pent and Neu5Prop on the cell surface [18]. Transfected B35 cells expressing Kv3.1 were treated with ManNPent and ManNProp and then the Kv3.1 glycoproteins were analyzed by Western blots (Fig. 1). A predominant slower migrating band and a faint faster migrating band were observed for cells untreated and treated with N-acylmannosamines. As previously shown, the upper band represents two sialylated complex N-glycans of the Kv3.1 glycoprotein while the lower band consists of two oligomannose N-glycans [4]. In all cases, the upper bands were sensitive to neuraminidase treatment. A difference was that when cells were treated with ManNPent or ManNProp the upper band migrated slightly faster than that from untreated cells. Based on the most plausible explanations of the Western blots and previous studies [3,4,18], the oligosaccharides of the Kv3.1 glycoprotein isolated from untreated cells are referred to as natural sialylated N-glycans and those from treated cells as non-natural sialylated N-glycans.

3.2. Treatment of Kv3.1 transfected B35 cells with non-natural N-acylmannosamines leads to decreased activation kinetics of ionic currents

It was demonstrated that B35 cells expressing the Kv3.1 protein with zero or one occupied N-glycosylation sites had outward ionic currents with slower activation rates compared to those of the Kv3.1 glycoprotein with both sites occupied [4]. A similar activation protocol was employed to analyze the ionic currents of Kv3.1 transfected B35 cells which generated Kv3.1 glycoprotein with natural and non-natural sialylated N-glycans (Fig. 2). Both
inactivating (Fig. 2A) and non-inactivating (Fig. 2B) current types were observed for Kv3.1 transfected B35 cells untreated (control, top panels) and treated with ManNPent (middle panels) and ManNProp (bottom panels) from the indicated voltage protocol. Time scales were expanded for inactivating current types to show differences in activation kinetics (A, right panel). Gray lines denote currents at +20 and +40 mV. Conductance–voltage ($g/g_{\text{max}}$) curves of both inactivating (left panel) and non-inactivating (right panel) current types (C). Rise times of inactivating (right panel) and non-inactivating (left panel) currents types (D). n represents number of cells. Asterisks denote significant difference from control.

Conductance–voltage ($G$–$V$) relationships were fitted with the Boltzmann isotherm for inactivating (left panel) and non-inactivating (right panel) current types (Fig. 2C). Currents were observed when the test potential was greater than $-30$ mV. However, current amplitudes reached saturation much more readily for inactivating current type than non-inactivating current type. Boltzmann parameters of inactivating current and non-inactivating current types were similar for Kv3.1 transfected cells untreated (test potential at which $g/g_{\text{max}} = 0.5(V_{0.5})$ was $10.3 \pm 1.3$ mV and $24.0 \pm 0.8$ mV and the slope of the current voltage plot ($dV$) was $9.7 \pm 0.7$ mV and $21.9 \pm 0.8$ mV) and treated with ManNPent ($V_{0.5}$ was $11.6 \pm 1.3$ mV and $24.8 \pm 0.5$ mV and $dV$ was $8.3 \pm 0.6$ mV and $23.0 \pm 0.5$ mV) and
ManNProp ($V_{0.5}$ was 13.2 ± 1.2 mV and 26.4 ± 0.8 mV and $dV$ was 9.1 ± 0.7 mV and 21.4 ± 0.8 mV), respectively. These values were similar to [4]. To illustrate differences in activation kinetics of the ionic currents, time scales were expanded for the inactivating current type (Fig. 2A, right panel). The rise times (time needed for the current to rise from 10% to 90% of its peak current at a test potential) were slowest at the lowest test potential and fastest at the highest potential for all Kv3.1 glycoforms which indicates the voltage dependence of the ionic currents. Rise times were faster for the glycosylated Kv3.1 channel with natural sialylated $N$-glycans than those with non-natural sialylated $N$-glycans for the inactivating current types (Fig. 2D, left panel). On the other hand, differences were not observed for non-inactivating current types (Fig. 2D, right panel). These results are further supported by the activation time constants (single exponential fits of current at a test potential) plotted against voltage which revealed that the activation rates of the inactivating current types of the Kv3.1 channel were faster for the natural glycoform than both of the non-natural glycoforms while those of the non-inactivating current types were unchanged (not shown).

3.3. Inactivation kinetics of ionic currents were slowed for Kv3.1 transfected B35 cells treated with N-acylmannosamines

Inactivation kinetics of ionic currents of B35 cells expressing the glycosylated Kv3.1 channels were faster than those from unglycosylated or partially glycosylated Kv3.1 channels [4]. Whole cell recordings showed that initially the currents rapidly increased with time, and then steadily reduced as the duration of the pulse (2 s) continued for Kv3.1 transfected B35 cells untreated (left panel) and those treated with ManNPent (middle panel) and ManNProp (right panel) (Fig. 3A). Further the decrease in current amplitudes at the end of pulses relative to the maximum current amplitudes was greater for untreated cells versus treated cells. The percent of current remaining for the Kv3.1 glycoform with natural sialic acids was 50 ± 4% ($n = 9$) when the test potential was +40 mV while those with Neu5Pent and Neu5Prop were 63 ± 4% ($n = 5$) and 67 ± 2% ($n = 8$), respectively (Fig. 3B). Therefore, the non-natural sialic acids influence inactivation rates of the outward ionic currents produced by the Kv3.1 channel.

3.4. Deactivation kinetics of ionic currents were similar for Kv3.1 transfected B35 cells untreated or treated with N-acylmannosamines

$N$-Glycans attached to the Kv3.1 protein enhance deactivation kinetics of the outward ionic currents [4]. To examine the deactivation rates (fitting the current at a test potential with a single exponential) of transfected B35 cells untreated and treated with N-acylmannosamines, cells were clamped at +40 mV for 25 ms to activate Kv3.1 channels, and then channels were deactivated by clamping cells to less depolarized potentials (Fig. 4A). All Kv3.1 glycoforms displayed rapid deactivation rates for inactivating and non-inactivating current types (Fig. 4B), unlike the aglycoform [4]. Here we have shown for the first time that outward ionic current kinetics of the Kv3.1 channel depended on the size of the $N$-acyl chain at the 5-carbon position of sialic acid residues of the Kv3.1 glycoprotein. The activation and inactivation rates of the inactivating current type were slowed when natural sialic acid residues of the Kv3.1 glycoprotein were substituted with non-natural sialic acid residues. This reduction in the activation rates was more similar to the partially glycosylated Kv3.1 channel than the aglycoform [4]. Further shifts in the activation kinetics were not due to shifts in the voltage midpoints of channel activation, and therefore support the gating stabilizing theory in describing the gating mechanism of the Kv3.1 channel [4,8,13,14]. Although size differences of the
N-acyl chain exist between the two non-natural sialic acid residues, channel kinetics of the two glycoforms were similar. This lack of difference may be due to a combination of the length of the N-acyl chain and the amount of non-natural sialic acid incorporated. For instance, the amount of Neu5Pent incorporated may be less than that of Neu5-Prop [18,19]. In earlier studies, the functional importance of the N-acyl side chains was demonstrated when cells (e.g. lymphoma or Vero cells) became less sensitive to the uptake of polyoma or influenza A viruses upon treatment with ManNProp or ManNPent [17,19,21]. Taken together, these results have shown a new important biological characteristic in that the N-acyl side chain of neuraminic acid modulates the activation and inactivation rates of the ionic currents of Kv3.1 expressed in transfected B35 cells. We speculate that physiological changes may result from modification of the electrical signaling in neurons, cardiac myocytes and skeletal muscle when changing the size of the N-acyl chain of sialic acid residues of the Kv3.1 glycoprotein.

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