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Modulation of human Kv1.5 channel kinetics by N-cadherin

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Abstract

Kv1.5 is expressed in multiple tissues including heart, brain, macrophages, as well as vascular, airway, and intestinal smooth muscle cells. Kv1.5 currents contribute to cardiac repolarization. In cardiac myocytes Kv1.5 colocalizes with N-cadherin. As Kv1.5 expression increases following establishment of cell–cell contacts and N-cadherin influences the activity of other ion channels, we explored whether N-cadherin participates in the regulation of Kv1.5 activity. To this end, we expressed Kv1.5 in *Xenopus* oocytes with or without additional expression of N-cadherin. Coexpression of N-cadherin was followed by a ~2- to 3-fold increase of Kv1.5 induced current. The effect of N-cadherin was not paralleled by significant alterations of Kv1.5 channel abundance within the oocyte cell membrane but resulted primarily from accelerated recovery from inactivation. In conclusion, N-cadherin modifies Kv1.5 channel activity and is thus a novel candidate signaling molecule participating in the regulation of a variety of functions including cardiac action potential and vascular tone.

Keywords: K^+ channels; Atrial fibrillation; Adhesion; Cell membrane potential; Heart

The voltage-gated K^+ channel Kv1.5 is expressed in the human heart, including the atria [1,2] and ventricles [1], brain [1], macrophages [3], as well as vascular [4,5], intestinal, and airway [6] smooth muscle. In the heart, it contributes to the repolarization of the cardiac action potential [7,8] and is responsible for the ultrarapid delayed rectifier current (I_{Kur}) [9]. Mice with a long QT phenotype showed improved action potential duration upon adenoviral expression of Kv1.5 [10]. Kv1.5 is preferably expressed in atrial myocytes [11] and loss-of-function Kv1.5 mutations were shown to contribute to idiopathic atrial fibrillation [12]. Kv1.5 is thus considered an attractive target for the treatment of atrial arrhythmias [13,14].

Expression of Kv1.5 is upregulated by establishment of cell–cell contacts [15]. Kv1.5 is localised at intercalated disks in proximity to N-cadherin [16], which may thus participate in the regulation of Kv1.5 activity. Classical cadherins are a family of single-pass transmembrane glycoproteins, responsible for Ca^{2+}-dependent cell–cell adhesion. Neuronal cadherin (N-cadherin) is a classical cadherin expressed in various cell types, including the cardiac muscle [17,18]. In the heart N-cadherin is an integral part of the intercalated disk junction and is essential for the assembly of the adherens junctions in myocytes and thus the establishment of cell–cell contacts [19,20]. It plays a critical role in early cardiac development and morphogenesis as well as in cardiac function [21–23]. In fact, mice with cardiac overexpression of N-cadherin suffer from cardiac hypertrophy and further structural abnormalities [24]. Conversely, expression of a dominant negative form of N-cadherin (with deleted extracellular domain) in adult rat cardiomyocytes resulted in impaired cell–cell adhesion, destruction of intercalated disc-like structures and impairment of myofibrillar structures [19]. N-cadherin has previously been shown to regulate voltage-activated Ca^{2+} channels [25], an effect involving the juxtamembrane

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domain of N-cadherin [25]. Thus, at least in theory, N-cadherin could similarly affect K^+ channels.

The aim of the current study was to determine whether N-cadherin affects the function of Kv1.5. We show that N-cadherin indeed enhances Kv1.5 activity. The effect results from alterations of channel activity and not from effects on channel cell surface expression. N-cadherin dependent regulation of Kv1.5 channel activity suggests a link between the expression of adhesion molecules and the electrophysiological properties of cardiac myocytes.

Materials and methods

Constructs and cRNA synthesis. The human N-cadherin DNA clone (RZPD clone ID: IRATp970D0560D) was purchased from RZPD German Resource Center for Genome Research. The human Kv1.5 clone was a kind gift from Dr. Michael Sanguinetti (University of Utah). The T7-tagged Kv1.5 clone was a kind gift from Dr. David Fedida (University of British Columbia). Template cDNA was linearized with NheI (for Kv1.5) SmaI (for T7-Kv1.5) with BamHI (for N-cadherin). cRNA was synthesized from 1 μg of linearized DNA using an *in vitro* transcription kit (mMessage mMachine T7, Ambion). cRNA concentrations were evaluated using photospectrometry and transcript quality was checked by agarose gel electrophoresis.

Isolation, injection, and two-microelectrode voltage clamp recordings of Xenopus oocytes. Adult *Xenopus laevis* were anesthetized by immersion in 0.1% tricaine methanesulfonate (Sigma) and oocytes were surgically removed. In order to remove follicular cells, oocytes were transferred into 20 ml collagenase-OR-2 solution (containing (in mM): collagenase type 2 (1 mg/ml), 82.5 NaCl, 2 KCl, 1 $MgCl_2$, 5 Hepes, pH 7.4) and shaken gently at room temperature for 90 min (replacing the old solution for 20 ml fresh solution at 45 min). After collagenase treatment, the oocytes were washed 10× in ND96 solution (containing (in mM): 96 NaCl, 4 KCl, 1.8 $MgCl_2$, 1 $CaCl_2$, 5 Hepes, pH 7.4), and then stored at 18 °C in ND96 supplemented with 2.5 mM sodium pyruvate, 50 mg/l gentamycin, and 90 mg/l theophylline. Oocytes were injected with 40 nl of RNA (3 ng for Kv1.5 and 3 ng for N-cadherin) and incubated at 18 °C for 72 h in ND96 solution plus sodium pyruvate, gentamycin, and theophylline. For recordings, the oocytes were placed in a chamber at room temperature and continuously perfused with ND96 solution. Recordings were obtained using a two-microelectrode voltage clamp system (NPI electronics). Standard recording pipettes were filled with 3 M KCl and had resistances between 0.5 and 2 MΩ. For fast macroscopic current recordings, pipettes were coated with Sylgard elastomer (Dow Corning) to reduce capacitative currents and agar cushions (tips filled with 1.5% agarose in 3 M KCl) in low resistance pipettes (0.1–0.3 MΩ) were produced to allow high speed recordings of the amplifier with virtually no leakage of KCl solution into oocytes.

To obtain *I–V* curves, oocytes were held at −80 mV and current was measured following voltage steps between −80 and +80 mV. For activation and inactivation analysis, single exponential functions were fitted to the rising or decaying portions of the curves, respectively, and time constants of activation and inactivation were determined. For analyzing recovery after inactivation, peak values were determined from a series of 20 ms pulses from −100 to +60 mV, preceded by a 2000 ms pulse to +60 mV from an initial potential of −100 mV (see Fig. 4A). Deactivation time constants were analyzed by fitting single exponential functions to tail currents at each 10 mV step between −60 and 0 mV after a 50 ms depolarization to +50 mV. For conductance analysis, the decaying phase of tail currents were fitted with a single exponential function and the fit was extrapolated to the beginning of the repolarization pulse. Extrapolated tail current amplitudes were fitted with single Boltzmann functions: $y = 1/(1 + \exp[-(V - V_{1/2})/k])$, where $V_{1/2}$ represents the voltage at which 50% of all available channels were activated, V is the membrane potential, and k is the slope factor of the conductance $(G)/V$ relationship.

Detection of cell surface proteins by biotin labelling. To identify the amount of channel protein present in the plasma membrane, surface proteins were tagged with biotin and isolated by neutravidin-mediated precipitation of the biotin–protein complexes. Briefly, 20 intact oocytes were incubated in 10 μM EZ Link Sulpho-NHS-LC Biotin (Pierce) at room temperature for 30 min to label the extracellular proteins followed by five 10-min washes in ND96 solution. The oocytes were then homogenised with a Teflon pestle in 400 μl H-buffer (100 mM NaCl, 20 mM Tris–HCl, pH 7.4, 1% Triton X-100, plus a mixture of proteinase inhibitors (Complete™ tablets, Roche Applied Science) and rotated for 1 h at 4 °C. Lysates were spun twice for 60 s at 16,000*g*, to remove the yolk and 20 μl of the supernatant were removed (total protein fraction) and supplemented with 5× SDS sample buffer. The remaining supernatant was supplemented with 25 μl of previously washed immobilised neutravidin beads (Pierce) and incubated at 4 °C for 2.5 h on a rotator. The beads were then pelleted by a 120-s spin at 16,000*g* and washed three times in H-buffer. The final pellets containing the membrane fraction were boiled in SDS–PAGE loading buffer (0.8 M β-mercaptoethanol, 6% SDS, 20% glycerol, 25 mM Tris–HCl, pH 6.8, 0.1% bromophenol blue).

Gel electrophoresis and Western blotting. Proteins from homogenised oocytes were separated by SDS electrophoresis and transferred to Polyscreen® PVDF membranes (Perkin-Elmer™ Life Sciences) following an established protocol (Mini-PROTEAN 3 system, Bio-Rad). For verification of equal protein loading, Ponceau Red staining was performed. Membranes were blocked, then probed with T7 antibody (Novagen) and incubated with a horseradish peroxidase-conjugate of sheep anti-mouse secondary antibody (Amersham) and protein levels were detected by enhanced chemiluminescence (Amersham). Detected band intensity was quantified by densitometric analysis of immunoblots, using Bio-Rad Quantity One analysis software.

Data analysis. For quantitative analysis of Western blots, the optical density of each band was expressed as a percentage of the control value (relative abundance) in the same blot. The combined results from all blots were expressed as the mean relative abundance. Numerical values are reported as mean ± SE (n = number of experiments). Student's t-test was used to test for statistical significance between groups. A value of $p < 0.05$ was considered statistically significant. Statistical analysis of the data was performed by Clampfit 9.0 and Origin 6.0.

Results

Coexpression of Kv1.5 and N-cadherin enhances Kv1.5 current

Oocytes injected with Kv1.5 showed a characteristic outward current with step depolarizations from −80 to +30 mV (Fig. 1A and B). The pulse protocol is depicted in the inset. The outward current was entirely absent in water-injected control oocytes or in oocytes expressing N-cadherin without Kv1.5 (data not shown). In oocytes expressing Kv1.5 the additional expression of N-cadherin resulted in a significantly larger outward current compared to oocytes injected with Kv1.5 alone (Fig. 1B).

N-cadherin does not affect the surface expression of Kv1.5

Further experiments were performed to explore, whether the effect of N-cadherin on Kv1.5 was due to increased channel expression at the plasma membrane. Since three commercially-available Kv1.5 antibodies showed non-specificity, T7-tagged Kv1.5 was expressed in oocytes for analyzing surface expression. Oocytes expressing T7-tagged Kv1.5 displayed currents that were similar

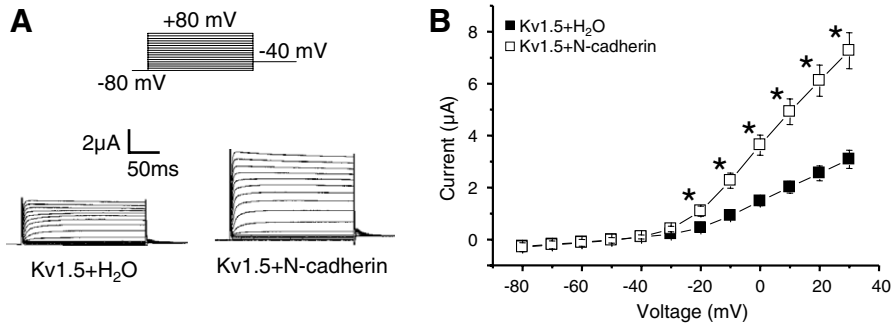

Fig. 1. N-cadherin enhances Kv1.5 currents. Original currents of human Kv1.5 or Kv1.5 coexpressed with N-cadherin (A). The voltage protocol is shown (not to scale), whereby cells were held at −80 mV and in +10 mV increments for 2000 ms were voltage stepped to +80 mV, with 1200 ms repolarizations at −40 mV in between steps. Average current–voltage relationships are shown in (B), and are recorded from batches of oocytes from three different frogs. ■ = Kv1.5 + H_2O, $n = 24$; □ = Kv1.5 + N-cadherin, $n = 22$. * denotes statistical significance ($p < 0.05$) according to Student's t-test analysis.

to untagged Kv1.5 ($n = 5$, Fig. 2A), and tagged-Kv1.5 currents were also increased upon N-cadherin coexpression ($n = 5$, Fig. 2A). Biotin labeling of surface proteins showed that the surface levels of Kv1.5 were not increased upon coexpression of N-cadherin (Kv1.5 + H_2O: 399.2 ± 112.5% mean relative abundance; Kv1.5 + N-cadherin: 378.8 ± 98.0% Δintensity/mm^2, $p = 0.90$, Students' t-test). Fig. 2B shows a representative Western blot of Kv1.5 expression ± N-cadherin. Densitometric analysis showed no significant difference in relative Kv1.5 protein abundance in the plasma membrane between oocytes expressing Kv1.5 alone and oocytes coexpressing Kv1.5 together with N-cadherin (Fig. 2C).

N-cadherin affects the activation, but not inactivation, properties of Kv1.5

The rising phase of activating channel currents was analyzed by single exponential fitting. Activation time constants were significantly different between oocytes coinjected with N-cadherin + Kv1.5 and oocytes injected with Kv1.5 alone (Fig. 3A). N-cadherin coexpression resulted in smaller activation time constants, suggesting that N-cadherin causes faster Kv1.5 activation.

Fitting the current decay after activation at depolarizing potentials to a single exponential function did not yield significant differences in the inactivation of Kv1.5 channels with or without N-cadherin coexpression (Fig. 3B). The time constants of deactivation were analysed by a single exponential fit of tail currents after a 50 ms depolarizing 50 mV pulse. However, at each voltage between −60 and 0 mV, there was no significant difference between the two conditions.

The conductance–voltage relationship G/V was determined at the peak tail currents after the capacitative currents had vanished. The mean G/V curves were fit to a standard Boltzmann function. However, this analysis did not yield any significant differences in the half maximal activation $V_{1/2}$ (Kv1.5 + H_2O: 14.4 ± 4.3 mV; Kv1.5 + N-cadherin: 7.0 ± 2.5 mV; $p = 0.81$, Students' t-test) or slope k (Kv1.5 + H_2O: 0.99; Kv1.5 + N-cadherin: 0.98, $p = 0.64$, Students' t-test).

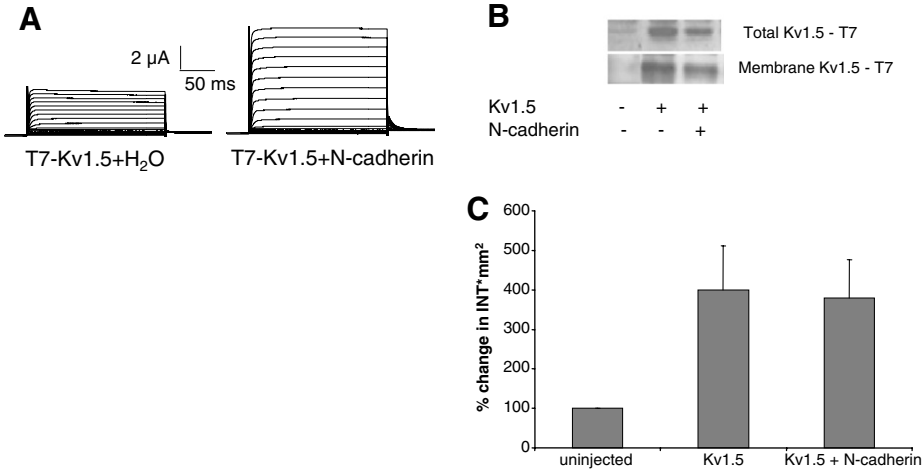

Fig. 2. N-cadherin does not increase surface expression of Kv1.5. Representative T7-tagged Kv1.5 currents with or without N-cadherin coexpression (A). The voltage protocol was described as in Fig. 1. Coexpression of N-cadherin with Kv1.5 does not affect Kv1.5 abundance at the plasma membrane. (B) Representative Western blot showing total and membrane quantities of T7-tagged Kv1.5 (~83 kDa). (C) Graph showing densitometric analysis of the Western blot representing the relative abundance of the Kv1.5 plasma membrane protein ($n = 4$).

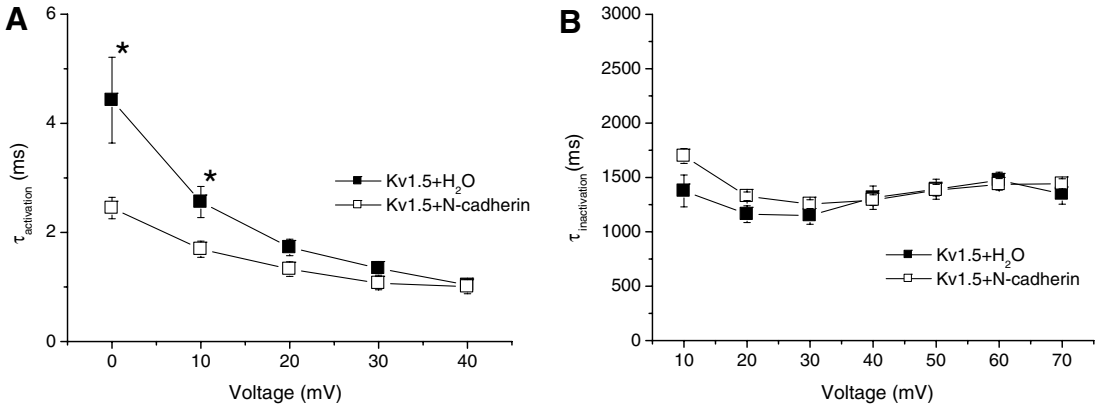

Fig. 3. N-cadherin affects onset of activation, but not inactivation, of Kv1.5. (A) Kv1.5 activation time constants ($\tau_{activation}$) were determined by fitting single exponential functions to the rising phase of currents at each voltage ■ = Kv1.5 + H_2O, $n = 11$; □ = Kv1.5 + N-cadherin, $n = 12$. * denotes statistical significance ($p < 0.05$) according to Student's t-test analysis. (B) Kv1.5 inactivation time constants ($\tau_{inactivation}$) were determined by fitting single exponential functions to the decaying phase of currents at each voltage, ■ = Kv1.5 + H_2O, $n = 6$; □ = Kv1.5 + N-cadherin, $n = 8$.

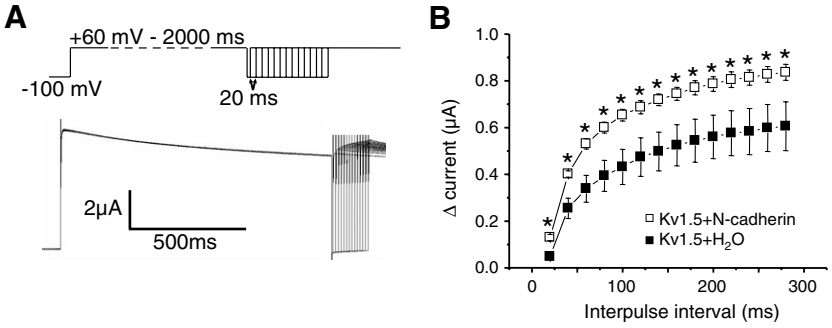

Fig. 4. N-cadherin affects the Kv1.5 recovery after inactivation. Recovery after inactivation was determined according to the voltage protocol shown (A), and the representative current recording is from an oocyte injected with Kv1.5 + H_2O. From a starting potential of −100 mV, cells were subjected to 2 s +60 mV pulses for channel inactivation, and then 20 ms interpulses between −100 and +60 mV. Results (B) are shown as average peak currents over time (Δcurrent between peak current at +60 mV 20 ms pulse and plateau portion after +60 mV inactivating pulse), where ■ = Kv1.5 + H_2O, $n = 5$; □ = Kv1.5 + N-cadherin, $n = 8$. * denotes statistical significance ($p < 0.05$) according to Student's t-test analysis.

N-cadherin decreases recovery time of Kv1.5 after inactivation

For determination of recovery from inactivation, channels were activated and partially inactivated by a 2 s +60 mV pulse, recovered from inactivation by hyperpolarizing interpulses of variable length and subsequently reactivated by a depolarizing pulse to +60 mV (Fig. 4A and B). A representative recording from a Kv1.5 injected oocyte is shown in Fig. 4A. As illustrated in Fig. 4C, the recovery from inactivation is markedly altered in Kv1.5 channels following coexpression with N-cadherin (Fig. 4B). N-cadherin coexpression resulted in significantly increased fractional Kv1.5 recovery from inactivation, compared to Kv1.5 alone.

Discussion

Kv1.5 and N-cadherin have been shown to colocalize in human cardiac myocytes at the intercalated disk regions [26], a finding suggestive for a functional link between the two proteins. However, hitherto no studies have been published on a functional interaction between N-cadherin and Kv1.5. In the present study we show that Kv1.5 channel activity is affected by N-cadherin expression. N-cadherin could enhance Kv1.5 currents by increasing channel cell surface expression and/or by affecting the function of channel proteins. Our results show that N-cadherin alters Kv1.5 channel kinetics, an effect apparently not requiring altered surface expression of the protein. As our experiments have been performed following heterologous expression of Kv1.5, they would not disclose any effect on genomic regulation of Kv1.5 channels.

In our experiments, N-cadherin enhanced Kv1.5 current by channel activation. Specifically, the time constants of Kv1.5 channel activation were significantly faster following coexpression with N-cadherin. On the other hand, coexpression of N-cadherin did not significantly affect inactivation time constants. Moreover, neither tail currents nor channel conductance were significantly affected by N-cadherin coexpression. However, coexpression of N-cadherin was followed by significantly enhanced recovery from Kv1.5 inactivation. N-cadherin affects Kv1.5 channels such that the inactivated state is less preferential, and this trans-

lates cumulatively into a concurrent increased availability of conducting channels.

Kv1.5 is associated with PSD-95/SAP97 [27] and Kv1.5 channels appear to exist in a membrane complex, containing cytoskeletal proteins. For example, caveolin-3 and SAP97 contribute to a structural complex that can regulate Kv1.5 [28]. Accordingly, disruption of the oocyte cytoskeleton with cytochalasin D altered Kv1.5 currents [26]. Moreover, microtubule cytoskeletal disruption with nocodazole was shown to increase Kv1.5 surface expression [29].

Although not through direct physical interaction, the PDZ protein SAP97 was shown to affect Kv1.5 activity, an effect dependent upon the N-terminus of Kv1.5 [30]. The N-terminal T1 domain of voltage-gated K^+ channels is a highly conserved region that has been shown to affect channel gating [31]. Perhaps the N-terminus of Kv1.5 is critical for N-cadherin to exert its effects on Kv1.5.

Both Kv1.5 [32] and N-cadherin [33] have been shown to associate with lipid rafts. In fact, Kv1.5 was the first ion channel reported to be associated with lipid rafts [32]. Lipid rafts are membrane microdomains enriched in cholesterol and sphingolipids, and are implicated in regulating molecular interactions at the cell membrane. Accordingly, lipid rafts may present the membrane organizational structures responsible for regulating N-cadherin and its effects on Kv1.5.

Whatever mechanisms involved, our results provide the intriguing evidence that a classical adhesion molecule has non-adhesion regulatory effects, i.e. the ability to regulate the voltage gated K^+ channel Kv1.5. Cell adhesion has previously been shown to affect K^+ currents [34,35]. Conversely, inhibition of K^+ channels was shown to inhibit cell adhesion [36]. The present observations disclose that N-cadherin and Kv1.5 could participate in this functional link.

In conclusion, we show that the cell adhesion molecule N-cadherin modifies Kv1.5 channel activity, specifically by altering channel activation and recovery from inactivation. These results unravel a novel mechanism of Kv1.5 regulation, which could contribute to the functional link between cell adhesion and excitability.

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