Syntrophin γ2 Regulates SCN5A Gating by a PDZ Domain-mediated Interaction*

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SCN5A encodes the α subunit of the cardiac muscle and intestinal smooth muscle mechanosensitive Na+ channel. Mechanosensitivity in the intestine requires an intact cytoskeleton. We report, using laser capture microdissection, single cell PCR, and immunohistochemistry, that syntrophins, scaffolding proteins, were expressed in human intestinal smooth muscle cells. The distribution of syntrophin γ2 was similar to that of SCN5A. Yeast two-hybrid and glutathione S-transferase pull-down experiments show that SCN5A and syntrophin γ2 co-express and that the PDZ domain of syntrophin γ2 directly interacts with the C terminus of SCN5A. In native cells, disruption of the C terminus-syntrophin γ2 PDZ domain interaction using peptides directed against either region result in loss of mechanosensitivity. Co-transfection of syntrophin γ2 with SCN5A in HEK293 cells markedly shifts the activation kinetics of SCN5A and reduces the availability of Na+ current. We propose that syntrophin γ2 is an essential Na+ channel-interacting protein required for the full expression of the Na+ current and that the SCN5A-syntrophin γ2 interaction determines mechanosensitivity and current availability.

Mechanosensitivity is a level requirement for the survival of an organism (1, 2). At a cellular level, ion channels often serve as the unitary element that underlies mechanosensitivity (3, 4). Mechanosensitive ion channels are of particular relevance in organs constantly submitted to movement, such as the gastrointestinal tract and the heart (5, 6). Contractility in both cardiac muscle and gastrointestinal smooth muscle is initiated by membrane electrical events as a result of changes in ionic conductances (7, 8). In the heart, the upstroke of the action potential is mediated via opening of a tetrodotoxin-insensitive Na+ channel, the α subunit of which is encoded by SCN5A (9). Mutations in SCN5A can result in clinically significant cardiac arrhythmias (8, 10). SCN5A is also expressed in human intestinal circular smooth muscle and the native Na+ current is mechanosensitive (11–13). Mechanosensitivity appears to be dependent on the actin cytoskeleton, since disruption of the actin cytoskeleton by cytochalasin D or gelsolin abolishes mechanosensitivity (13). This suggests that the actin cytoskeleton is required to transmit force to the ion channel. Syntrophins are suggested to be a link between the actin cytoskeleton and membrane-associated proteins including ion channels, enzymes, and receptors, since actin filaments are not known to directly interact with these proteins (14–17). Syntrophins are a multigene family of homologous proteins (18–22). Five syntrophins, α, β1, β2, y1, and y2, have been characterized (22, 23). Each syntrophin is encoded by a separate gene but shares a common domain organization. Each syntrophin contains two tandem pleckstrin homology domains at the N terminus, a single PDZ domain, and a highly conserved C terminus syntrophin-unique region (22, 23). The PDZ domains of syntrophins α, β1, and β2 but not y1 are known to interact with SCN5A and with SCN4A, a skeletal muscle Na+ channel, via the C terminus sequence motif (E/S/T)X (14, 15, 24). The objective of this study was to investigate the interaction between SCN5A and syntrophins in intestinal smooth muscle and the functional consequences of such an interaction on mechanosensitivity. Our hypothesis was that syntrophins couple SCN5A with the actin cytoskeleton, providing a mechanism for mechanical regulation of voltage-dependent ion channel gating.

MATERIALS AND METHODS

Preparation of Single Human Jejunal Circular Smooth Muscle Cells—The Mayo Foundation Institutional Review Board approved the use of human tissue obtained as surgical waste tissue during gastric bypass operations performed for morbid obesity. The method for dissolution of smooth muscle cells from human jejunal circular smooth muscle strips was as previously described (11, 12). Briefly, the mucosa, submucosa, and longitudinal muscle of the jejunum were removed from the specimen by sharp dissection. The circular smooth muscle layer was then cut into small pieces and incubated with enzyme to release single smooth muscle cells used for patch clamp studies and single cell reverse transcription (RT)-PCR.

Poly(A) RNA Isolation and cDNA Library Preparation—Procedures for RNA isolation and the procedure for preparation of cDNA libraries were as described previously (11, 12).

PCR and Single Cell RT-PCR—All PCR amplifications were performed using GeneAmp 2400 PCR systems (PerkinElmer Life Sciences) using standard procedures (12). The protocol for single cell RT-PCR was as previously outlined (12). Spindle-shaped single smooth muscle cells were collected directly into PCR tubes containing tRNA and proteinase K. RT was performed using a mixture of random hexamer and oligo(dt) primers following the instructions of the manufacturer (PerkinElmer Life Sciences). The product of the RT reaction was then amplified by using specific gene primers that were specifically designed to flank a region that contained introns. All PCR products were purified

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and sent to the Mayo Molecular Core Facility for automated DNA sequencing.

**Laser Capture Microdissection**—Surgical tissue was fixed in ice-cold acetone according to the protocol described previously (12). The same number of spots of tissue containing about 1500 smooth muscle cells from the circular muscle layer or the longitudinal muscle layer were collected using the FIK II cell laser capture microdissection system (Arcturus Engineering Inc., Santa Clara, CA) with the 7.5-μm spot size. The caps with collected cells were then immediately placed into sterile 0.5-ml microcentrifuge tubes containing 300 μl of RNA STAT-60 reagent (Tel-TEST Inc., Friendswood, TX) for isolation of total RNA. After washing with 75% ethanol, the RNA pellet was resuspended in nuclease-free water (Ambion Inc., Austin, TX) and used for RT-PCR.

**GST Pull Downs**—Pieces of tissue (approximately 1 × 1 cm) were prepared for immunohistochemistry as previously described (12). Briefly, cryostat sections (12 μm thick) were mounted onto glass slides, air-dried, fixed for 10 min in either cold acetone or 4% paraformaldehyde, and rinsed in phosphate-buffered saline (PBS). Sections were incubated with 10% normal donkey serum and 0.3% Triton X-100 for 1 h to block nonspecific absorption sites and then incubated overnight at 4 °C with anti-syntrophin γ2 rabbit polyclonal antibody (diluted 1:200 in 5% normal donkey serum; a kind gift from Dr. Vincenzo Nigro). The specificity of this antibody for syntrophin γ2 has been previously shown by Piluso et al. (22). After several rinses in PBS, the sections were incubated for 1 h with donkey, anti-rabbit IgG conjugated to Cy3 (Jackson Immunoresearch, West Grove, PA), rinsed in PBS, and coverslipped in glycerol-PBS containing an anti-fade reagent.

**Yeast Two-hybrid Assay**—Yeast two-hybrid assays were performed using HybriZAP-2.1 two-hybrid system (Stratagene, La Jolla, CA). The cDNA fragments encoding the C terminus of SCN5A (amino acids 1915–2015, CTSCN5A) and the C terminus with last 10 amino acids truncated (amino acids 1915–2005, CTSCN5A-10) were amplified by PCR and cloned into EcoRI and SalI restriction sites of the pBD-GAL4 vector to serve as bait in the yeast two-hybrid experiments. Two full-length splice variants of syntrophin γ2, one with an intact PDZ domain and the other lacking the PDZ domain, were inserted into pAD-GAL4 vector (pAD + γ2 and pAD + γ2-PDZ, respectively). The nucleotide sequences of the DNA inserts were confirmed by DNA sequencing analysis to verify that inserts did not contain mutations. The inserts were expressed as fusion proteins with the DNA binding domain and DNA activating domain of GAL4. Several small scale yeast transformation experiments were performed using the lithium acetate method with 40% polyethylene glycol. The plasmids CTSCN5A and CTSCN5A-10 were co-transformed into yeast strains containing the cDNA inserts and served as bait for yeast two-hybrid screens. After transformation, the yeast was plated on selective plates lacking tryptophan, leucine, and histidine to show activation of the GAL4-inducible reporter gene HIS3 through protein-protein interaction. The colonies that grew on the selective plates were either due to the leaky expression of the bait protein or to the specific interaction resulting in expression of the HIS3 gene. To distinguish between leaky expression and specific protein interactions, we used filter lift assays to detect the expression of a second reporter gene, lacZ. The colonies on the selective plate were lifted onto a filter and assayed for β-galactosidase activity using 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside (X-gal) as a substrate.

**GST Pull Downs**—The bacterial expression vector pGEX-5X-1 (Amersham Biosciences) was used to produce a GST fusion protein in E. coli. The C-terminal fragments of SCN5A with and without the truncation were fused in frame into pGEX-5X-1 to produce plasmids pGEX-CTSA and pGEX-CTSA-10. To express GST fusion proteins, pGEX-CTSA and pGEX-CTSA-10 were transformed into E. coli (Stratagene, La Jolla, CA). Each bacterial culture was induced by isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 0.1 mM and allowed to express protein at 28 °C for 4 h. Then the cells were pelleted and resuspended in PBS and 10 mg/ml lysozyme. After incubation for 30 min on ice, protease inhibitor mixture (Calbiochem) was added, and the cells were pelleted for 10 min. To the pellets the DNA was extracted by protein digestion with RNase and DNase. The protein extract was then extracted by centrifugation at 3000 × g for 30 min and incubated with glutathione beads (Amersham Biosciences).

The completed code sequences of the splice variants of syntrophin γ2 with and without the PDZ domain were linked into the expression vector pGEX-5X-1, which tags the FLAG sequence (11). Recombinant plasmids pCMV-γ2 and pCMV-γ2-PDZ were transformed into HEK 293 cells to express the FLAG-tagged syntrophin γ2 proteins. The cells were lysed 24 h later in lysis buffer, and the supernatants were used for GST pull-down experiments. 100 μl (200–300 μg) of HEK 293 cell extracts containing the FLAG-tagged syntrophin γ2 proteins were incubated with 20–25 μl (10 μg) of GST + CTSA/GST + CTSA-10 bead-bound fusion proteins and, as a control, the GST-alone bead-bound fusion protein. After extensive washing with lysis buffer, the samples were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was then incubated with anti-FLAG M2 monoclonal antibody (Sigma). After washing, horseradish peroxidase-conjugated anti-mouse IgG was used. The immunoreactive bands were visualized by ECL according to the manufacturer’s instructions (Amersham Biosciences).

**Expression and Purification of Fusion Protein**—The pGEX-5X-1 plasmid constructs for the PDZ domain of syntrophin γ2 were generated by subcloning at the EcoRI restriction site of the pBD-GAL4 vector. The constructs were verified by sequencing, and the plasmids encoding GST alone and GST plus PDZ were then introduced into BL21 cells for expression. After induction by isopropyl-1-thio-β-D-galactopyranoside, the cell extracts were incubated with glutathione-agarose beads for affinity purification. Following washing, GST and GST plus PDZ were eluted with 10 mM reduced glutathione in 50 mM Tris·HCl (pH 8.0). For patch clamp analysis, the elution was dialyzed in 10 mM HEPES with 50 mM CsCl. The purity of the proteins was determined by SDS-12% polyacrylamide gel electrophoresis. The bands of the fusion proteins were of the expected size with high purity. Protein concentrations were estimated using the Bio-Rad protein assay kit.

**Peptide Synthesis**—A peptide corresponding to the last 10 amino acids (SPDPRDRESIV) of the C terminus of the human SCN5A sequence and a control peptide containing the same amino acids but in random sequence (PRRSVSDEII) were synthesized by the Mayo Peptide Synthesis Facility of the Mayo Proteomics Research Center. The peptides were purified by reverse phase high performance liquid chromatography using a Vydac C-18 column. Purity was >95% as assessed by amino acid analysis and analytical high performance liquid chromatography. Mass weight of the peptide was verified by electrospray ionization mass spectrometry on a Sciex 165B (Concord, Canada).

**Plasmid Constructs and Mammalian Cell Transfection**—The pcDNA3 expression vector (Invitrogen) with human SCN5A (hSCN5A) was used to transfect HEK 293 cells. The plasmids containing the full-length SCN5A minus the last 10 amino acids was made by PCR. The DNAs for syntrophin γ2 were produced by PCR and inserted into the pcDNA3 vector using EcoRI and SalI. All constructs were verified by sequencing. LipofectAMINE™ 2000 Reagent (Invitrogen) was used to transfect green fluorescent protein, pEGFP-C1, (Clontech, Manassas, VA). Transfected cells were identified by fluorescence microscopy and patch-clamped.

**Electrophysiological Recordings**—Whole cell patch clamp recordings were made using standard patch clamp techniques. Whole cell recordings were obtained using Kimble KC-12 glass pulled on a P-97 puller (Sutter Instruments, Novato, CA). Electrodes were coated with BR101 (Dow Corning, Midland, MI) and fire-polished to a final resistance of 3–5 megaohms. Currents were amplified, digitized, and processed using a CyberAmp 320 amplifier, a Digidata 1200, and pCLAMP 8 software (Axon Instruments, Foster City, CA). Whole cell records were sampled at 5 kHz and filtered at 2 kHz with an eight-pole Bessel filter. 70–75% series resistance compensation (lag of 30–40 ms) (to standardize transients), and finally stepped to 40 mV. The interval from the start of one depolarization to the next was 1 s. SCN5A-overexpressed Na+ currents at a −100 mV holding potential were much larger in HEK 293 than in native cells. Therefore, for HEK-293 cell records, cells were held at −80 mV to reduce maximal peak inward current. Transfected cells were then pulsed to the test voltage above the control level as described above. The DNA inserts containing the full-length SCN5A protein were transfected with a pulse protocol where cells held at −100 mV and stepped to −80 mV, stepped to −110 through −60 mV in 5-mV intervals for 3 s to reach a steady state of inactivation, briefly stepped to −110 mV for 10 ms (to standardize transients), and finally stepped to −40 mV. Current was measured at −40 mV. The interval from the start of one depolarization to the next was 4 s.

The pipette solution contained 145 mM Cs+, 20 mM Cl-, 2 mM EGTA, 5 mM HEPES, and 125 mM methane sulfonate for most whole cell recordings. In peptide experiments, the terminal 10-amino acid SCN5A peptide or the control jumbled sequence peptide was added to this solution.
intraocular solution to a concentration of 1 mM. The bath solution contained 149.2 mM Na⁺, 4.7 mM K⁺, 159 mM Cl⁻, 2.5 mM Ca²⁺, and 5 mM HEPES (normal Ringers solution) with an osmolarity of 290–300 mosm. All chemicals other than peptides were obtained from Sigma.

Bath perfusion was used to assess mechanosensitivity as previously described (25, 26). The bath was perfused at 10 ml/min for 30 s to create shear stress and activate the mechanosensitive Na⁺ channel according to a previously established protocol (13).

Data Analysis—Electrophysiological data were analyzed using PCLAMP 8 software, custom macros in Excel (Microsoft, Redmond, WA), or SigmaPlot 2001 for Windows (SPSS Science Marketing, Chicago, IL). Voltages were adjusted for junction potentials using JP-CalcW. Statistical comparisons were performed by a two-tailed paired Student’s t test, and p < 0.05 was used for statistical significance. Tau of inactivation values were determined by fitting a standard two-exponential decay curve between the points at the peak and at 50 ms. Time-to-peak was measured as the difference between the time when the pulse started and the time at maximal peak inward current. Steady state activation and inactivation curves were fitted with a three-paramater sigmoid (Boltzmann) function.

RESULTS

Identification of Syntrophins in Human Jejunal Circular Smooth Muscle Cells—To determine which syntrophins are expressed in intestinal smooth muscle, we used gene-specific primers designed against the five known syntrophins (α, β1, β2, γ1, and γ2) to amplify cDNAs from dissociated human jejunal circular smooth muscle cell libraries. Products of the expected size were detected for syntrophin γ2. Syntrophin γ1 was not present in human jejunal circular smooth muscle (Fig. 1A), consistent with its limited expression to the brain (22). RT-PCR was also carried out on aliquots of freshly dissociated smooth muscle cells and cDNA bands of the expected size for syntrophin α, β1, β2, and γ2 obtained (data not shown), again suggesting that these syntrophins were expressed in intestinal smooth muscle together with the known expression of syntrophins in cardiac and skeletal muscle (22, 23).

To determine the anatomical location of syntrophins within the human intestinal smooth muscle layers, we used laser capture microdissection to collect, separately, smooth muscle cells from the jejunal circular muscle layer and longitudinal muscle layer. Total RNA was extracted from the harvested cells, and reverse transcription was carried out using random primers. An aliquot from the reverse transcription was used for PCR amplification with one specific primer pair for either α, β1, β2, or γ2 in each tube. Single bands of the expected size for syntrophin α and β2 were observed in both circular and longitudinal smooth muscle (Fig. 1B). Syntrophin γ2 was detected only in circular smooth muscle, and β1 was detected only in longitudinal smooth muscle (Fig. 1B). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was amplified from the RT products and used as an internal control.

To further confirm the localization of syntrophin γ2 to the circular muscle layer of the human intestine, we immunolabeled human jejunal sections with a rabbit polyclonal antibody directed against syntrophin γ2 (a kind gift from Dr. Vincenzo Nigro). As shown in Fig. 1C, strong syntrophin γ2 immunoreactivity was observed in the circular smooth muscle layer but
not in the longitudinal muscle layer. Control experiments omitting the primary antibody showed no immunoreactivity in the circular muscle layer (data not shown). Since several different cell types reside in the intestinal smooth muscle layers, single cell RT-PCR was used to localize syntrophins to smooth muscle cells. Smooth muscle cells were dissociated from strips of circular smooth muscle and identified according to their spindle shape. Two or three cells were collected and placed in each tube. Syntrophin α, β2, and γ2 were successfully amplified with gene-specific primer sets designed to span an intron (Fig. 1D) to exclude genomic sequence. The correct size band was recovered from the agarose gel using conventional techniques, and results were confirmed by sequence analysis. There were no products in negative controls (4 µl of bath solution aspirated just above a smooth muscle cell) (Fig. 1D). These results were duplicated in three additional experiments.

Isolation and Characterization of Syntrophin γ2 Splice Variants—Similar to the layer-specific expression of syntrophin γ2, SCN5A is expressed in human intestinal circular smooth muscle cells but does not appear to be strongly expressed in longitudinal cells (12). This cell-specific colocalization between expression of SCN5A and syntrophin γ2 led us to focus on syntrophin γ2. PCR amplification for syntrophin γ2 (Fig. 1A) showed three bands, suggesting different transcripts of syntrophin γ2. To isolate the full-length coding sequence for each transcript, we designed primers to cover the whole open reading frame. RT-PCR amplification using human jejunal muscle tissue produced a 1.7-kb cDNA fragment, which was subcloned into pCR2.1 plasmid vector using the TOPO-TA protocol (Invitrogen). Sequence analysis showed at least five splice variants of syntrophin γ2 (Fig. 2). Splice variant 1 was identical to the published sequence with 17 exons (accession number NM_018968). Splice variant 2 had a 27-bp deletion in exon 9. Splice variant 3 had exons 3–6 deleted with a 222-bp insertion in between exons 11 and 12. Splice variant 4 had a 256-bp insertion with a stop codon between exons 9 and 10. Splice variant 5 had a 46-bp insertion with a stop codon inserted between exons 14 and 15.

Direct Interaction between SCN5A and Syntrophin γ2—To determine whether syntrophin γ2 and SCN5A directly interact in human intestinal smooth muscle and to determine whether the interaction was mediated via the PDZ binding domain on syntrophin, we performed yeast two-hybrid and GST pull-down experiments. The last 100 amino acids of SCN5A with (CTSCN5A) and without (CTSCN5A-10) the last 10 amino acids (the last 10 amino acids include the PDZ binding domain) were inserted into the pBD-GAL4 vector and used as baits (Fig. 3). Splice variant 1 of syntrophin γ2 with an intact PDZ domain and splice variant 3 lacking the PDZ domain were fused into pAD-GAL4 vector and used as prey. In the yeast YRG-2 cells, the reporter gene His was activated only by the interaction between the splice variant 1 of syntrophin γ2 with PDZ domain and the intact C terminus of SCN5A (Fig. 3b). Other pairings of syntrophin γ2 with SCN5A did not activate His, indicating that the last 10 amino acids of SCN5A mediated the binding of SCN5A to the PDZ domain of syntrophin γ2. The specificity of this interaction was confirmed by testing activation of second reporter gene lacZ (Fig. 3c). In addition, syntrophin γ2 and the last 100 amino acids of SCN5A both did not self-activate when transformed into yeast with empty bait or prey vectors (data not shown).

The interaction between the C terminus of SCN5A and the PDZ domain of syntrophin γ2 was verified using GST pull-down assays. Splice variants 1 and 3 of syntrophin γ2 were expressed in HEK 293 cells. Affinity-purified GST, GST plus CT5A, and GST plus CT5A-10, immobilized on glutathione-Sepharose beads, were incubated with cell lysates containing the syntrophin γ2 splice variants 1 and 3. Fig. 4 shows that only GST plus CT5A trapped splice variant 1 of syntrophin γ2 (lane 2) and that there was no interaction between GST plus CT5A with or without the last 10 amino acids (aa) (lanes 5 and 6) and the splice variant 3 of syntrophin γ2, again suggesting a specific interaction between the PDZ domain of syntrophin γ2 and the last 10 aa of SCN5A. GST alone did not bind to either splice variant of syntrophin γ2 (lanes 1 and 4).

The specificity and functionality of the interaction between the C terminus of SCN5A and the PDZ domain of syntrophin γ2 were tested on the native Na⁺ current in human intestinal circular smooth muscle cells. Freshly dissociated cells were patch-clamped, and a 10-aa peptide (1 µm) corresponding to the last 10 aa of SCN5A was introduced via the patch pipette into the cells. The peptide completely abolished the perfusion-induced increase in Na⁺ current. The peptide was allowed to diffuse into the cells for 10 min after breaking in, and the Na⁺ current was activated by perfusion. Perfusion did not increase peak inward Na⁺ current (5 ± 2% increase, n = 8, p > 0.05 compared with preperfusion) (Fig. 5a). In contrast, in control cells without the peptide, perfusion increased peak inward Na⁺

| Published sequence | PDZ domain | PH domain | ATP/GTP binding size |
|--------------------|------------|-----------|----------------------|
| Variant 1          | 1–2        | 3–7       | 8 9 10              | 12–14 15 16 17 |
| Variant 2          | 1–2        | 3–7       | 8 9 10              | 12–14 15 16 17 |
| Variant 3          | 1–2        | 8 9 10 11 | 12–14 15 16 17     |
| Variant 4          | 1–2        | 3–7       | 8 9                 |
| Variant 5          | 1–2        | 3–7       | 8 9 10              | 12–14     |
SCN5A and Syntrophin γ2

FIG. 3. Direct interaction between the PDZ domain of syntrophin γ2 and the last 10 amino acids of SCN5A in vivo. a, schematic diagram of baits and preys used in the yeast two-hybrid system analysis (CTSCN5A, last 100 aa of SCN5A; CTSCN5A-10, C terminus lacking the last 10 aa of SCN5A; Syn-γ2, syntrophin-γ2 splice variant 1 with an intact PDZ domain; Syn-γ2Δ, syntrophin-γ2 splice variant 3 lacking a PDZ domain). b, expression of the reporter gene HIS3. Each pair of constructs as indicated in A was co-transfected into the YRG-2 yeast strain. Yeast transformants were then selected on selective plates and tested for expression of reporter gene HIS3. Strong expression of HIS3 only occurred when the last 10 aa of SCN5A and the PDZ domain of syntrophin γ2 were both present. c, β-galactosidase activity. Colonies that grow on the selective plates were transferred onto the filter papers and assayed for β-galactosidase activity, confirming that SCN5A and syntrophin γ2 interact and that the interaction occurs through the C terminus of SCN5A and the PDZ domain.

FIG. 4. Direct interaction between the PDZ domain of syntrophin γ2 and the last 10 amino acids of the C terminus of SCN5A in vitro. A, the full-length syntrophin γ2 with a PDZ domain (Syn γ21) and the syntrophin γ2 without a PDZ domain (Syn γ22) were transfected into HEK293 cells. Approximately 200 μg of cell lysate was then incubated with GST, GST plus CT5A (last 100 aa of the C terminus of SCN5A), or GST plus CT5A-10 (last 100 aa of the C terminus except for the very last 10 aa) beads. After washing, the proteins bound to the beads were resolved by 10% SDS-PAGE and identified by Western blots using the anti-FLAG antibody as the probe. Specific binding was observed only between GST plus CT5A and syntrophin γ21 (with a PDZ domain). B, lanes 1 and 2 were loaded with 1% of the cell lysates compared with 10% of the extracts for lanes 3 and 4.

Current by 27 ± 3% (data not shown). Introduction of a control jumbled peptide had no effect on this perfusion-induced increase in inward Na⁺ current (27 ± 6% increase in current, n = 6, p < 0.05 compared with preperfusion). Furthermore, introduction of a GST-bound 98-aa sequence (10 nM) corresponding to the PDZ domain of syntrophin γ2 also blocked the perfusion-induced increase in inward Na⁺ current (6 ± 2% increase in current, n = 6, p > 0.05 compared with preperfusion) (Fig. 5b). Introduction of GST alone into the human intestinal smooth muscle cells did not block the perfusion-induced increase in current (19 ± 4% increase in current, n = 8, p < 0.05 compared with perfusion without GST). These results suggest that a direct interaction between the last 10 aa of SCN5A and the PDZ domain of syntrophin γ2 is required to maintain mechanosensitivity of the Na⁺ channel.

Modulation of Gating of SCN5A by Syntrophin γ2—The above data indicate a specific interaction between syntrophin γ2 and SCN5A and that syntrophin γ2 is required for mechanosensitivity of SCN5A. Syntrophins do not appear to be required for the sarcolemmal localization of sodium channels (27, 28). Therefore, to further delineate the functional consequence of the interaction between the two proteins, we transfected HEK293 cells with either SCN5A alone or with SCN5A and syntrophin γ2. Co-transfection of syntrophin γ2 with SCN5A shifted the voltage-dependent activation of SCN5A by 8.5 mV (Fig. 6, a and b). V₅₀ for SCN5A alone was −43.4 ± 0.3 mV (n = 9) and shifted to −34.9 ± 0.3 mV (n = 9), p < 0.05, when syntrophin γ2 was co-transfected with SCN5A. As a result, maximal peak inward Na⁺ current shifted from −23 ± 1 to −15 ± 2 mV. At −40 mV, peak inward Na⁺ current decreased from −737 ± 94 pA to −281 ± 67 pA when syntrophin γ2 was co-transfected with SCN5A (n = 8, p < 0.05). However, maximal peak inward Na⁺ current was unchanged (−1169 ± 174 pA, n = 7 for SCN5A alone; −1163 ± 278 pA, n = 8, p > 0.05 for SCN5A with syntrophin γ2). Time to peak current increased at all voltages tested (Fig. 6c) with a change from 0.87 ± 0.03 to 1.53 ± 0.056 ms at maximal inward Na⁺ current (−20 mV, n = 6, p < 0.05). The slope of the activation curve was also changed (Fig. 6b), with a k value of 5.1 ± 0.2 mV for SCN5A alone and a k value of 6.5 ± 0.2 for
Co-expression of syntrophin γ2 resulted in a slower first tau (fast inactivation) with no change noted for the second tau (slow decay, Fig. 6c, n = 6, p < 0.05). No effect was noted on the kinetics of steady-state inactivation (Fig. 6b); therefore, the net result of the observed changes was a reduction in the overlap of the activation and inactivation relationships, resulting in a reduced window current (29) (Fig. 6b) compared with the window current observed with SCN5A alone. Truncation of the last 10 aa of SCN5A (n = 6, Fig. 7a) or cotransfection of the splice
variant of syntrophin γ2 lacking a PDZ domain (syntrophin γ2α, n = 6, Fig. 7b) had no significant effect on Na⁺ channel gating, suggesting that the effects of syntrophin γ2 on SCN5A current were again mediated by a specific interaction between the PDZ domain of syntrophin γ2 and the last 10 amino acids of SCN5A.

DISCUSSION

The main finding of this study is that mechanosensitivity of the human circular smooth muscle and cardiac Na⁺ channel is dependent on a specific interaction between the PDZ domain of syntrophin γ2 and the last 10 amino acids of the C terminus of SCN5A, the α subunit of both the tetrodotoxin-resistant cardiac muscle and the native intestinal smooth muscle Na⁺ channel. The mechanisms that underlie ion channel mechanosensitivity are complex and vary according to the ion channel studied (3, 30). Potential mechanisms for ion channel mechanosensitivity include a direct interaction between the transmembrane portion of the channel and the lipid bilayer, suggesting that this is an unavoidable consequence of inserting a channel into the membrane (30–32), activation of mechanosensitive signaling cascades that subsequently activate ion channels via phosphorylation or other post-translational modification (3), or force transmission via protein-protein interactions between the channel and the cytoskeleton that alter the channel open probability (3, 4, 6, 33, 34). Our data suggest that for SCN5A, the latter mechanism appears to be a central one for the mechanosensitivity observed upon perturbing the cell’s membrane, since mechanosensitivity of the native Na⁺ channel was completely lost when the interaction between the cytoskeleton and the C terminus of SCN5A was disrupted. The expression of SCN5A and syntrophin γ2 in intestinal muscle was similar, with immunohistochromical and molecular evidence to suggest that both are expressed in human intestinal circular but not longitudinal smooth muscle. The co-expression of SCN5A and syntrophin γ2 suggests that the interaction may be specific to syntrophin γ2 and not be generalizable to all syntrophins with PDZ domains. This is supported by the data from native cells with block of mechanosensitivity when the specific aa sequence of the PDZ domain of syntrophin γ2 is introduced into the cell. The sequences of PDZ domains are known to be highly conserved among all five known syntrophins (22, 23). However, the recently identified syntrophin γ1 has a PDZ domain similar to that of other syntrophins and yet does not bind SCN4A and SCN5A, suggesting different specificity of PDZ domains of syntrophins (24). Moreover, the Na⁺ channel-syntrophin interaction is not necessarily dependent on PDZ domains, since brain Na⁺ channels, which lack the consensus motif E(S/T)X at their C termini, required to bind PDZ domains, still copurified with syntrophin (14), suggesting that multiple interactions occur between syntrophins and Na⁺ channels and that the specific interaction between the C terminus of SCN5A and the PDZ domain of syntrophin γ2 may only be only an absolute requirement for mechanosensitivity.

A link between Na⁺ channel activity and actin cytoskeleton has been proposed previously. Treatment of cardiac myocytes with cytochalasin-D to inhibit actin polymerization reduces peak Na⁺ current and slows inactivation (35, 36). Disruption of the cytoskeleton also alters Na⁺ channel properties in skeletal muscle, epithelial tissue, and leukemia cells (28, 37–39). The data presented here suggest that, for SCN5A, the likely link between SCN5A and the actin cytoskeleton are the syntrophins. Syntrophins localize associated proteins to the membrane by binding with dystrophin (40, 41). In genetically modified mice lacking syntrophin α or the PDZ domain of syntrophin, neuronal nitric-oxide synthase is absent from the sarcolemma, suggesting dependence on syntrophin for sar-
Localization of neuronal nitric-oxide synthase (27, 42). Similarly, the membrane localization of water channel, aquaporin-4, also requires syntrophin (43). Aquaporin-4 is not localized to the membrane when syntrophin is absent. However, syntrophin is not always required for the membrane localization of its associated proteins. The skeletal muscle sodium channel SCN4A is known to bind syntrophin α, β1, and β2, but localization to the membrane does not appear to depend on syntrophins (27, 28). Also, the absence of syntrophin α does not have an apparent effect on the distribution or level of

![Image](image_url)

**Fig. 7.** Effect of truncation of SCN5A and of loss of the syntrophin γ2 PDZ domain on SCN5A kinetics. a and d, whole cell current traces at −40 mV for SCN5A, SCN5A without the last 10 aa cotransfected with syntrophin γ2, and SCN5A co-transfected with syntrophin γ2 without the PDZ domain. b and e, steady-state activation and inactivation curves. c and f, activation and inactivation kinetics. Both truncation of SCN5A and absence of the PDZ domain of syntrophin γ2 resulted in loss of the kinetic changes seen when the full-length SCN5A was co-expressed with syntrophin γ2.
expression of the $\text{Na}^+$ channel. In mdx mice, the distribution of $\text{Na}^+$ channels remains identical despite the lack of dystrophin and reduction of sarcolemmal density of syntrophins (28). These observations suggest that syntrophins do not play a key role in the sarcolemmal localization of $\text{Na}^+$ channel. Rather, the data presented here indicate that syntrophins directly alter SCN5A gating behavior. Thus, syntrophin $\gamma 2$ may be an essential $\text{Na}^+$ channel-interacting protein required to recapitulate the full phenotype of the $\text{Na}^+$ current.

The changes in activation and inactivation kinetics observed on cotransfection of syntrophin $\gamma 2$ with SCN5A would have significant effects on the $\text{Na}^+$ channel whole cell current. A shift in the voltage of activation for SCN5A coupled with slowing of inactivation will result in a smaller contribution of the peak $\text{Na}^+$ current to the action potential. Prolongation of fast inactivation would be expected to increase available $\text{Na}^+$ current over a few milliseconds after the peak, but the reduced window current would tend to reduce late $\text{Na}^+$ current over the window voltage range (29). No effect on slow decay was seen in our study. However, a previous study has reported that the last 8 aa of the C terminus of SCN5A slows inactivation with little effect on activation (44). During the gastrointestinal slow wave the membrane potential of smooth muscle is within the window current of SCN5A suggesting that the channel contributes to the regulation of membrane potential and of intracellular $\text{Na}^+$. Changes in the window current, as seen with syntrophin $\gamma 2$, may alter membrane potential and intracellular $\text{Na}^+$ homeostasis. Mutations in SCN5A are associated with changes in gating behavior and clinical disease (10). Mutations in SCN5A that prolong slow decay result in an enhancement of inward plateau current and a vulnerability to potentially fatal heritable arrhythmias like long QT syndrome subtype 3 and rarely sudden infant death syndrome (45). Mutations that result in a decrease in $\text{Na}^+$ current lead to Brugada’s syndrome and Lenegre disease (10). The changes in SCN5A current kinetics observed on co-transfection of SCN5A in HEK cells with syntrophin $\gamma 2$ may therefore lead to more than one mechanism for cardiac arrhythmias dependent on the relative expression of syntrophin $\gamma 2$ variants. It is presently unclear if syntrophins can directly interact with membrane proteins and the actin cytoskeleton or if dystrophin is always required. However, considering that only 65% of long QT syndrome is genotyped presently on the known long QT-causing genes (46), molecular and functional characteristics of syntrophin $\gamma 2$ described herein makes this $\text{Na}^+$ channel-interacting protein an attractive pathogenic target for unexplained, nongenotyped sudden cardiac death.

In summary, mechanosensitivity of the $\text{Na}^+$ channel encoded by SCN5A requires a specific interaction between the C terminus of SCN5A and the PDZ domain of syntrophin $\gamma 2$. Co-expression of SCN5A and syntrophin $\gamma 2$ results in a smaller $\text{Na}^+$ window current, suggesting the possibility that syntrophins may directly regulate the cardiac muscle and intestinal smooth muscle $\text{Na}^+$ current. These results also raise the possibility that mutations in the PDZ domain of syntrophin $\gamma 2$ or differential expression of the identified splice variants of syntrophin $\gamma 2$ may contribute to clinically significant arrhythmias.

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