Abolished InsP₃R2 function inhibits sweat secretion in both humans and mice

Joakim Klar, …, Katsuhiko Mikoshiba, Niklas Dahl


There are 3 major sweat-producing glands present in skin; eccrine, apocrine, and apoeccrine glands. Due to the high rate of secretion, eccrine sweating is a vital regulator of body temperature in response to thermal stress in humans; therefore, an inability to sweat (anhidrosis) results in heat intolerance that may cause impaired consciousness and death. Here, we have reported 5 members of a consanguineous family with generalized, isolated anhidrosis, but morphologically normal eccrine sweat glands. Whole-genome analysis identified the presence of a homozygous missense mutation in ITPR2, which encodes the type 2 inositol 1,4,5-trisphosphate receptor (InsP₃R2), that was present in all affected family members. We determined that the mutation is localized within the pore forming region of InsP₃R2 and abrogates Ca²⁺ release from the endoplasmic reticulum, which suggests that intracellular Ca²⁺ release by InsP₃R2 in clear cells of the sweat glands is important for eccrine sweat production. Itpr²⁻/⁻ mice exhibited a marked reduction in sweat secretion, and evaluation of sweat glands from Itpr²⁻/⁻ animals revealed a decrease in Ca²⁺ response compared with controls. Together, our data indicate that loss of InsP₃R2-mediated Ca²⁺ release causes isolated anhidrosis in humans and suggest that specific InsP₃R inhibitors have the potential to reduce sweat production in hyperhidrosis.
Abolished InsP$_3$R2 function inhibits sweat secretion in both humans and mice

Joakim Klar,\textsuperscript{1} Chihiro Hisatsune,\textsuperscript{2} Shahid M. Baig,\textsuperscript{3} Muhammad Tariq,\textsuperscript{3} Anna C.V. Johansson,\textsuperscript{1} Mahmood Rasool,\textsuperscript{4} Naveed Altal Maliq,\textsuperscript{2} Adam Ameur,\textsuperscript{1} Kotomi Sugiuara,\textsuperscript{2} Lars Feuk,\textsuperscript{1} Katsuhiro Mikoshiba,\textsuperscript{2} and Niklas Dahl\textsuperscript{1}

\textsuperscript{1}Department of Immunology, Genetics, and Pathology, Science for Life Laboratory, Uppsala University, Uppsala, Sweden. \textsuperscript{2}Laboratory for Developmental Neurobiology, RIKEN Brain Science Institute, Saitama, Japan. \textsuperscript{3}Human Molecular Genetics Laboratory, National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad, Pakistan. \textsuperscript{4}Center of Excellence in Genomic Medicine Research, King Abdulaziz University, Jeddah, Saudi Arabia.

There are 3 major sweat-producing glands present in skin: eccrine, apocrine, and apoeccrine glands. Due to the high rate of secretion, eccrine sweating is a vital regulator of body temperature in response to thermal stress in humans; therefore, an inability to sweat (anhidrosis) results in heat intolerance that may cause impaired consciousness and death. Here, we have reported 5 members of a consanguineous family with generalized, isolated anhidrosis, but morphologically normal eccrine sweat glands. Whole-genome analysis identified the presence of a homozygous missense mutation in \textit{ITPR2}, which encodes the type 2 inositol 1,4,5-trisphosphate receptor (InsP$_3$R2), that was present in all affected family members. We determined that the mutation is localized within the pore forming region of InsP$_3$R2 and abrogates Ca$^{2+}$ release from the endoplasmic reticulum, which suggests that intracellular Ca$^{2+}$ release by InsP$_3$R2 in clear cells of the sweat glands is important for eccrine sweat production. \textit{Itpr2}$^{-/-}$ mice exhibited a marked reduction in sweat secretion, and evaluation of sweat glands from \textit{Itpr2}$^{-/-}$ animals revealed a decrease in Ca$^{2+}$ response compared with controls. Together, our data indicate that loss of InsP$_3$R2-mediated Ca$^{2+}$ release causes isolated anhidrosis in humans and suggest that specific InsP$_3$R inhibitors have the potential to reduce sweat production in hyperhidrosis.

Introduction

Anhidrosis, defined as the absence of perspiration in the presence of an appropriate stimulus such as heat, exercise, or pharmacological agonists, is a rare condition that may be acquired or congenital (1, 2). The causes of anhidrosis or reduced sweating (hypohidrosis), which are heterogeneous, include sweat gland innervation defects (in disorders of the autonomous nervous system) and reduced number of functional sweat glands (in different ectodermal syndromes) (2). Reports of generalized and isolated anhidrosis with normal sweat glands (OMIM 106190) are very few (3, 4).

There are 3 types of sweat glands present in the skin: eccrine, apocrine, and apoeccrine. Of these, eccrine sweat glands have a superior maximum secretion rate (5, 6). Among mammals, humans have the highest proportion of eccrine sweat glands, which provides an advantage for thermal cooling during prolonged exercise (6). The eccrine gland consists of 2 major parts, the excretory duct and the secretory coil. The excretory duct has 2 or 3 layers of epithelial cells and consists of a straight portion leading to the skin surface and a coiled reabsorptive portion. The secretory portion consists of 3 functionally distinct cell types: granulated (dark) cells, parietal (clear) cells, and myoepithelial cells (6, 7). Upon initial stimulation of clear cells of the sweat gland by acetylcholine, intracellular [Ca$^{2+}$] increases and initiates a stepwise process leading to net loss of Na$^+$, Cl$^-$, and water into the glandular lumen (8).

Results

Phenotype of affected family members. The available family members ($n = 10$) consisted of 5 affected individuals (aged 4, 5, 7, 10, and 11 years at time of study), 2 healthy siblings, and 3 parents (Figure 1A). Upon clinical investigation, no abnormal symptoms were observed or reported from family members beyond anhidrosis and severe heat intolerance. In the affected family members, body growth as well as teeth, hair, nails, and skin were normal. Biochemical analysis of serum and urine in affected family members VII:4 and VII:5 (aged 11 and 10 years, respectively) revealed electrolyte levels (Na$^+$, K$^+$, Ca$^{2+}$, Mg$^{2+}$, and Cl$^-$) within normal ranges. S-amyrase levels were also normal. Starch-iodine sweat test (6) confirmed the absence of sweating in affected individuals, and all 5 exhibited abnormal increases in skin and ear canal temperature when exposed to heat (45°C, 45% humidity), accompanied by an abnormal increase in heart rate (Figure 1, B and C). Skin biopsy

Authorship note: Joakim Klar and Chihiro Hisatsune contributed equally to this work.

Conflict of interest: The authors have declared that no conflict of interest exists.

Submitted: May 8, 2013; Accepted: September 11, 2014.

from the forearm of affected family member VII:4 demonstrated normal morphology and number of sweat glands.

Genetic analysis and identification of a candidate mutation. We first performed autozygosity mapping on affected individuals (13) because of the consanguinity and the likely autosomal-recessive inheritance pattern for anhidrosis within this family. The analysis revealed a single homozygous region on chromosome 12p12.1-12p11.22 in all 5 affected individuals (Figure 1A). The region consists of 427 consecutive homozygous SNPs (rs1337853-rs2349565) spanning 31 genes over 3.4 Mb (GRCh37 25,703,471–29,137,928). Segregation of the candidate homozygous region in the family was confirmed with polymorphic microsatellite markers, and linkage analysis resulted in a maximum 2-point logarithm of odds (LOD) score of 3.08.

Figure 1. Genetic analysis, clinical investigation, and InsP₃R sequences. (A) Pedigree of the consanguineous family segregating isolated anhidrosis (black symbols). Affected individuals were homozygous for chromosome 12p marker alleles flanking ITPR2 (black bars). (B) Starch-iodine sweat test at 32°C, demonstrating sweating in a healthy control male (top) and absence of color (due to dry skin) in affected family member VII:4 with anhidrosis (bottom). Both subjects were 11 years of age. (C) Increased temperatures (left; dashed lines, skin surface; solid lines, ear canal) and heart rates (right) at rest over 25 minutes when exposed to 45°C and 45% humidity, in patients (n = 5; black lines) compared with age-matched controls (n = 3; gray lines). P < 0.05; Student’s 2-tailed t test. (D) Left: Protein sequence alignment of the pore region of InsP,R1–InsP,R3 and RYR1. Pore helix and selectivity filter are indicated. Residues of the selectivity filters are highlighted in gray, with the mutated glycine residue (p.G2498) denoted (arrow). Consensus residues correspond to amino acids conserved among all 4 proteins. Right: Interspecies alignment of the pore helix and the selectivity filter domains of InsP,R2 illustrated conservation of residue G2498. Data represent mean ± SD.
Immunostaining of InsP3R2 was positive in the clear cells of the confirmed normal morphology and number of sweat glands. A punch biopsy from the forearm of an affected family member

InsP3R2 in forearm skin biopsies of (A) a healthy control individual and (B) affected family member VII:4. Eccrine sweat glands (boxed regions) are shown enlarged. Control and patient specimens exhibited similar staining: InsP3R2 stained positive in the clear cells (CC), but not the dark cells (DC) (dashed lines). InsP3R2 was also present in cells of the excretory ducts (asterisk) with a concentration in subcellular regions lining the ducts. (C and D) Similar to InsP3R2, S100β staining was positive in the clear cells of the secretory coil of the eccrine sweat gland, but not in the dark cells or the cells of the duct (15). In (C) a control individual and (D) affected family member VII:4. N, nerve end. Original magnification, ×10; ×40 (enlargements). Scale bars: 20 μm.

Targeted enrichment of the 3.4-Mb candidate region was performed on genomic DNA from affected members, followed by sequencing and filtering. The analysis identified a single novel coding variant: c.7492G>A in ITTPR2 (NM_002223.2). The transition results in a predicted glycine-to-serine (p.G2498S) substitution, and it was present in a homozygous state in the 5 affected family members and in a heterozygous state in the 3 parents and 2 healthy siblings available for sampling (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI70720DS1). The glycine residue is highly conserved along the phylogenetic scale from human to zebrafish (PhyloP score, 2.44645; GERP score, 4.43) among the 3 InsP3R subtypes as well as in the closely related ryanodine receptor 1 (RYR1; Figure 1D and ref. 10). The variant — predicted to affect protein function by PolyPhen-2 analysis (HumVar score, 1.00, probably damaging) (14) — was not found in 200 Swedish and 200 Pakistani control chromosomes, nor in 850 exomes that were available in house. Furthermore, the c.7492G>A variant is not present in the latest Exome Variant Server data release (ESP6500SI-V2; http://evs.gs.washington.edu/EVS/).

InsP3R immunohistochemistry in skin biopsies. Histology of a punch biopsy from the forearm of an affected family member confirmed normal morphology and number of sweat glands. Immunostaining of InsP3R2 was positive in the clear cells of the secretory coil of the eccrine sweat gland and was similar between control and patient samples (Figure 2, A and B). In addition, InsP3R2 expression was observed within the cells of the excretory duct and with a concentration in subcellular regions lining the ducts. To investigate the expression of the other InsP3R isoforms in eccrine sweat glands, we stained the skin biopsies for InsP3R1 and InsP3R3. Whereas InsP3R3 showed weak staining in the secretory part and a strong staining in the basal (peripheral) cell layer of the excretory duct (Supplemental Figure 2), we could not detect

expressed WT and mutant InsP3R2 in DT40 chicken B lymphocytes lacking endogenous InsP3R5 (R23-11 cells) (16) and examined their channel properties by Ca2+ imaging. We established 3 independent cell lines expressing WT or mutated InsP3R2 and confirmed the expression of InsP3R2 protein in each stable cell line (Figure 3A). We then stimulated the cells with anti-IgM antibody to activate B cell receptors and intracellular Ca2+ release. In response to IgM stimulation, approximately 80% of cells expressing WT InsP3R2 showed intracellular Ca2+ oscillations (n = 139; Figure 3B), the typical form of InsP3R2-mediated Ca2+ release, consistent with previous studies (17). In contrast, cells expressing p.G2498S mutant InsP3R2 had no detectable Ca2+ response after IgM stimulation (n = 50; Figure 3B). The Ca2+ contents within the endoplasmic reticulum, as measured by passive Ca2+ release after Ca2+ pump inhibitor cyclopiazonic acid (CPA) treatment, were similar in cells expressing WT and p.G2498S mutant InsP3R2 (WT, 100% ± 20.72%, n = 3; p.G2498S, 129.97% ± 55.22%, n = 3; mean ± SD; P = NS, t test). These data strongly suggest that the p.G2498S mutation causes InsP3R2 loss of function.

Itpr2–/– mice exhibit hypohidrosis. To further examine the contribution of InsP3R2 to sweat production, we examined sweat secretion in Itpr2–/– mice, which harbor a targeted disruption of Itpr2, using the starch-iodine assay (18). When pilocarpine was subcutaneously injected into the hind paws of Itpr2–/– mice, individual sweat glands (represented by black dots) appeared within 1 minute, and the number increased in a time-dependent manner, to 78.33 ± 10.92 dots per paw at 20 minutes (mean ± SEM, n = 7; Figure 4A). In Itpr2–/– mice, however, the increase in sweat gland number was significantly attenuated (24.4 ± 2.11 dots per paw at 20 minutes, n = 5; Figure 4A). In addition, the size of each black dot (presumably representing the sweat volume from a single gland) was about half the size in Itpr2–/– versus Itpr2+/– mice (Figure 4B). Similar to our analysis of human sweat glands, immunofluores-
The primary goal of this study was to identify the pathophysiological mechanism leading to congenital generalized and isolated anhidrosis. The study emerged from the investigation of a consanguineous family segregating autosomal-recessive anhidrosis in 5 children. The affected individuals presented with absent eccrine sweat production and with severe and congenital heat intolerance, but no other symptoms from the skin or other ectodermal tissues, such as teeth and hair. The children exhibited normal development and were otherwise healthy. Hence, the clinical picture was suggestive of a gland-intrinsic mechanism.

Genetic analysis identified a unique transition (c.7492G>A) that predicts a missense variant (p.G2498S) in ITPR2, which encodes InsP3R2. The transition was present in a homozygous state in the affected individual and was excluded in control populations.

Figure 3. The p.G2498S mutation abolishes the channel activity of InsP3R2. (A) Expression of WT and mutant p.G2498S mouse InsP3R2 in 3 independent stable clones. (B) Intracellular Ca2+ signals upon IgM stimulation in R23-11 cells expressing WT and p.G2498S mouse InsP3R2 variants. Arrows denote IgM stimulation (M4) at 0.25 μg/ml. Ca2+ signals from 2 independent p.G2498S InsP3R2 clones and 1 WT InsP3R2 clone were analyzed. Representative data (ratio change of Fura-2) from 4 independent experiments are shown. Cells expressing p.G2498S InsP3R2 exhibited no detectable Ca2+ signal in response to IgM stimulation (0%; n = 50 cells). Of WT InsP3R2 cells, 78% showed Ca2+ oscillation, 14% were Ca2+ transient, and 8% exhibited no response (n = 139 cells).

Intracellular Ca2+ release in clear cells of sweat glands is a known critical event for sweat induction (15), and we thus considered the mutated InsP3R2 variant to be a good candidate for the cause of anhidrosis. This hypothesis was supported by our immunostaining of sweat glands from human skin biopsies, which showed distinct expression of InsP3R2 in the secretory portion and in the reabsorbing excretory duct. Both the secretory portion and the ductal part are involved in the net production of sweat; however, the epithelium lining of the sweat duct is not freely permeable to water, which indicates that ductal reabsorption has little effect on secreted sweat volume (19). The missense variant p.G2498S is localized in the pore-forming domain (Pfam, PFO0025; residues 2,336–2,540) of InsP3R2. More specifically, the substitution involves the second glycine in the selectivity filter formed by a core of 5 residues, GGGXG (Figure 1D and refs. 10, 20). In rat InsP3R1 (type I-pore), the G2546 residue corresponds to InsP3R2 G2496 and mutagenesis of InsP3R1 G2546 (i.e., p.G2546A) results in inactivation of the channel activity. That is, p.G2546A mutant InsP3R1 is still able to form homotetramers but unable to mediate Ca2+ release from microsomal vesicles, and it is nonfunctional in a Ca2+ flux assay (21).

We then analyzed p.G2498S mutant InsP3R2 in a chicken cell system devoid of endogenous InsP3R, and the results were consistent with a loss-of-function effect of the p.G2498S mutation. Cells expressing the p.G2498S mutant InsP3R2 showed a complete loss of Ca2+ response upon stimulation, despite Ca2+ stores similar to those of control cells expressing WT InsP3R2. Thus, the abolished intracellular Ca2+ release from the InsP3R2.p.G2498S variant is consistent with previous studies on rat InsP3R1 with a mutation in the corresponding residue (21).

To independently assess the role of InsP3R2 in sweat production, we analyzed eccrine glands in paws of Itp2r2−/− mice. We observed a 3-fold reduction in the number of pilocarpine-responsive sweat glands on Itp2r2−/− mouse paws, and dissected Itp2r2−/− sweat glands showed a significant reduction in Ca2+ response after acetylcholine stimulation compared with those of Itp2r2+/+ mice. Together, these results indicate that InsP3R2 plays a critical role in sweat secretion in both mice and humans. However, the residual sweat production in Itp2r2−/− mice is not fully consistent with the anhidrosis in the human subjects homozygous for the c.7492G>A (p.G2498S) mutation. Our immunohistochemical analysis suggests that InsP3R2 and InsP3R3 are the predominant isoforms expressed in human sweat glands,
and staining of skin biopsies from anhidrotic patients suggested normal levels of the mutated InsP3R2 isoform in sweat glands. Although InsP3R2 is required for normal sweat production in both humans and mice, the phenotypic discrepancy between our patients and the Itpr2–/– model could be explained to some extent by interspecies differences in InsP3Rs expression for sweat gland function. This notion was supported by immunohistochemistry showing that the InsP3R1 isoform was expressed in mouse, but not human, sweat glands as well as the fact that InsP3R1 contributes significantly to the peak height of Ca2+ signals (22). Additionally, we cannot exclude the possibility that the relative level of InsP3R3 is higher in murine versus human clear cells. The expression levels of InsP3R1 and InsP3R3 may thus partially compensate for the loss of InsP3R2 in sweat glands of the Itpr2–/– mice. However, InsP3R1 is not expressed in human clear cells, and expression of InsP3R2 far exceeds that of InsP3R3. Therefore, one possibility is that the InsP3R3 present in human patients is insufficient to compensate for the mutant InsP3R2. Furthermore, the p.G2498S variant of InsP3R2 may have a dominant-negative effect when forming heterotetramers with InsP3R3 in humans. If InsP3R2 is the predominant isoform in human clear cells, the stoichiometry of the expressed InsP3Rs may provide tolerance for quantitative reductions in their channel activity in other tissues of the patients, the level of expressed InsP3R2 mutant proteins may be insufficient to interfere with InsP3R1 and InsP3R3 function. Finally, the different stimuli used to provoke sweat production in the Itpr2–/– mice and human subjects may contribute to the distinct phenotypes. The more physiological induction used in human subjects (i.e., increased temperature) is not comparable to the pilocarpine injections used in mice.

Changes in intracellular [Ca2+] represent a versatile signaling system regulating diverse cellular processes, including cell differentiation and an individual’s metabolism (9). Different combinations of InsP3Rs are required for organ development, for heart (23) and brain (24) function, and for taste perception (25). In line with this, our present data suggest a distinct contribution of the InsP3R2 isoform for exocrine function in salivary and pancreatic glands versus sweat glands. Whereas Itpr2–/– mice exhibited reduced sweat production, they do not show perturbed function of the salivary and pancreatic glands (12). Similarly, the 5 affected family members herein did not present with any abnormal symptoms besides the anhidrosis and severe heat sensitivity. The affected individuals did not report on dry mouth, reduced saliva production, or malabsorption, although minor differences cannot be excluded. Moreover, the growth curves of the patients were within normal ranges. For comparison, Itpr2–/– Itpr3–/– double-knockout mice are hypoglycemic and show growth delay related to dysfunction of the pancreas and the salivary glands (12). Thus, the contribution of InsP3R2 to exocrine function is likely to be different in sweat glands than in salivary and pancreatic glands, possibly resulting from variations in the relative levels of the 3 InsP3R Rs. Additionally, redundant function for the InsP3R Rs may provide tolerance for quantitative reductions in their channel activity in distinct organs and for adequate development and functionality.

In conclusion, we identified the first mutation of InsP3R2 associated with human disease. Our results demonstrated that InsP3R2-mediated Ca2+ release plays an important role in sweat secretion in both humans and mice. Our findings indicate that the InsP3R2 missense mutation p.G2498S underlies the isolated anhidrosis in 5 related patients and that lack of a functional InsP3R2 compromised Ca2+ release, resulting in the absence of sweat production. The human phenotype was modeled in the Itpr2–/– mice, albeit with a milder phenotype corresponding to hypohidrosis. Because eccrine sweating is fundamental for thermoregulation in humans (5), we conclude that InsP3R2 plays a critical role for thermal cooling. Our
findings highlight InsP$_3$R2 as a potential pharmacological target in the treatment of conditions such as hyperhydrosis.

**Methods**

**Clinical samples.** A consanguineous Pakistani family with 5 children affected by severe heat intolerance was referred to the Health Division of NIBGE. Blood and urine samples were obtained from available family members, and punch skin biopsies were taken from 2 affected individuals. Consanguinity was ascertained over several generations, and 4 full siblings and 1 first cousin segregated autosomal-recessive congenital anhidrosis and severe heat intolerance (Figure 1A).

**Sequencing and sequence variant detection.** SNP genotyping was performed on DNA samples from 4 affected family members, using the GeneChip Mapping 250K array (Affymetrix) according to the manufacturer’s protocol. Homozygosity mapping and sorting of genomic regions were performed as described previously with the dedicated software AutoSNPa (13). A cutoff of $>$130 homozygous SNPs was used for selection of candidate regions. Selected regions were further investigated by genotyping all available family members with microsatellite markers. We calculated 2-point LOD scores using the MLINK program of the LINKAGE package (26), assuming autosomal-recessive inheritance, equal male/female recombination rate, full penetrance, and disease allele frequency of 0.00001. Equal allele frequencies of the genotyped markers were used in the calculations. The pedigree was drawn using Cyrillic software (version 2.1.3; Cherwell Scientific Publishing Ltd.), and haplotype analysis was performed manually. A custom enrichment design covering 7M base pairs (NimbleGen Sequence Capture Microarrays; Roche) was used to enrich for the linked region on chromosome 12 (average fold enrichment, 346). Sequencing of the enriched region was performed using the Illumina HiSeq system, and variant detection was performed using LifeScope software (version 2.1; Invitrogen). SNPs and indel data were deposited in GEO (accession no. GSE61122) and additionally stored in an in-house database together with variant annotation information obtained from dbSNP135. Prediction of possible effect on protein function was performed using PolyPhen-2 analysis (14). Exon 53 of ITPR2 (NM_002223.2) was analyzed for the identified variant by bidirectional sequencing of genomic DNA from all available family members using sense (TTGTGTCACGGCACAATTAGA) and antisense (AAAAAGATGTGCTCCTTGAAAA) primers. Sequence reactions were generated using the BigDye Terminator v3.1 Cycle Sequencing Kit (Invitrogen) according to the manufacturer’s protocol and separated on an ABI 3700 instrument (Applied Biosystems).

**Immunohistochemistry.** Histological analysis of skin biopsies was performed after H&E staining. Immunostaining using anti-human S100 (Z0311; Dako), InsP$_3$R1 (HPA016487; Sigma-Aldrich), InsP$_3$R2 (AB9074; Millipore), and InsP$_3$R3 (LC3; ref. 27) antibodies was performed using a DAKO autostainer (Dako). Antibodies were detected using a DAKO Chemmate EnVision kit (Dako). Specificity of the anti-
InsP₃R1 antibody was confirmed using Western blotting and immunocytochemistry of HeLa cells overexpressing InsP₃R1, InsP₃R2, or InsP₃R3 (data not shown).

**Mutagenesis and expression analysis.** The ApaI fragment (2.6 kb) from pBluescript II-C2 construct (28) was cloned into the Apal site of pBluescript II. Using the plasmid as a template, pG2498S mutagenesis was performed using sense (CAGGGCCTAGAATGGGACCGGAGTGTGGATGCTGAG) and antisense (CTCAAGCATTCCCAACTCTCCGTCGCTTTCGTCATTGCGCGCTG) primers and QuickChange Site-Directed Mutagenesis Kit (Agilent Technologies). The mutated Apal fragment was replaced with the Apal fragment of pBluescript II-C2 to construct pBluescript II-C2-G2498S, and the SaI fragment from the vector was cloned into the SaI site of pBacST-neoB. Nucleotide sequences were confirmed by DNA sequencing (Applied Biosystems). Stable clones were established by transfecting the expression vectors into R2311 cells (16), as described previously (28). For expression analysis, 5.0 × 10⁴ cells were directly lysed with a SDS-PAGE sample buffer, separated by SDS-PAGE gel, and probed with anti-InsP₃R, R2 antibody (AB9074; Millipore) by Western blotting.

**Single-cell Ca²⁺ imaging.** Cells were loaded with 5 μM Fura-2/AM (Dojindo Laboratories) for 20 minutes at room temperature in RPMI (Nacalai Tesque Inc.) containing 10% bovine serum. After washing with recording solution (115 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 20 mM HEPES, and 10 mM glucose, pH 7.42), cells were plated on 3.5-cm glass-bottomed dishes. After resting Ca²⁺ level was recorded into the recording buffer using a pair of fine forceps under a stereoscopic microscope. The sweat glands were loaded with both 5 μM Fluo4/AM and 10 μM Fura Red/AM in the recording buffer for 1 hour.

For immunoblotting of mouse sweat gland lysates, the corrected sweat glands from each Itpr2⁻⁻ and Itpr2⁺⁺ mouse paw were lysed with 35 μl SDS sample buffer. After centrifugation at 20,000 g, 15 μl of the lysate was loaded on 6.5% SDS-PAGE. The protein were transferred to PVDF membrane, blocked with PBST containing ECL advance blocking reagent (GE Healthcare), and probed with rabbit anti–insP₃R, R antibody (22) and mouse anti–α-actin antibody (Sigma-Aldrich). After washing with PBST, the membrane was probed with HRP-conjugated anti-rabbit IgG and anti-mouse IgG antibodies. After washing with PBST, the membrane was incubated with HRP substrate, and chemiluminescent signals were developed with LAS-3000 (Fujifilm).

Statistics. Student's 2-tailed t test assuming equal variance was used for statistical analysis. A P value less than 0.05 was considered significant.

**Study approval.** Clinical investigations of patients and family members, sweat tests, temperature measurements, and skin biopsies were carried out in accordance with the Declaration of Helsinki, and the study protocol was approved by the ethics committee of NIBGE. All participating family members and their legal guardians provided written informed consent. Animal studies were approved by the review board at RIKEN Brain Science Institute, and all animals were treated according to the ethical guidelines of the Animal Experiments Committee of RIKEN Brain Science Institute.

**Acknowledgments**

This work was supported in part by the Swedish Research Council (K2013-66X-10829-20-3 and 621-2009-4629) and Swedish Links, Asia (348-2008-6069); Science for Life laboratory Uppsala University and Uppsala University Hospital; Grants-in-Aid for Scientific Research (20220007, to K. Mikoshiba; 24500451, to C. Hisatsune); and The Moritani Scholarship Foundation (to C. Hisatsune). J. Klar is supported by the Swedish Society for Medical Research. We thank all family members who participated in this study. We also thank C. Yokoyama and A.V. Terashima for fruitful discussions; M.W. Sherwood, A. Miyamoto, E. Ebisui, and N. Ogawa for experimental advice and help; all staff of the Support Unit for Bio-Material Analysis RIKEN BSI Research Resources Center; and the Uppsala Genome Center for technical support.

Address correspondence to: Niklas Dahl, Department of Immunology, Genetics and Pathology, Science for Life Laboratory, Uppsala University, BMC, Box 815, 751 08 Uppsala, Sweden. Phone: 46.18.4714859; E-mail: niklas.dahl@igp.uu.se. Or to: Katsuhiko Mikoshiba, Laboratory for Developmental Neurobiology, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako City 351-0198, Japan. Phone: 81.48.467.9745; E-mail: mikosiba@brain.riken.jp.


