Abolished InsP$_3$R2 function inhibits sweat secretion in both humans and mice

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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 major sweat-producing glands present in the skin: eccrine, apocrine, and apoeccrine glands. 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$^{2+}$]$_i$ increases and initiates a stepwise process leading to net loss of Na$, \text{Cl}^-$, and water into the glandular lumen (8).

The present study was undertaken to clarify the pathophysiological mechanisms behind isolated generalized anhidrosis in 5 members of a consanguineous Pakistani family. The segregation of the disease was suggestive of autosomal-recessive inheritance. A genome-wide search for the causative mutation revealed a candidate missense mutation for anhidrosis in $ITPR2$, 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. Itpr2$^{-/-}$ mice exhibited a marked reduction in sweat secretion, and evaluation of sweat glands from 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.

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.
Immunostaining of InsP3R2 was positive in the clear cells of the punch biopsy from the forearm of an affected family member (Figure 2A, and B). Immunoreactivity and cellular localization of 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 InsP3R3 (R23-I1 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-
Intracellular Ca\(^{2+}\) release in clear cells of sweat glands is a known critical event for sweat induction (15), and we thus considered the mutated InsP\(_3\)R2 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 InsP\(_3\)R2 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, PF00520; residues 2,336–2,540) of InsP\(_3\)R2. 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 InsP\(_3\)R1 (type I-pore), the G2546 residue corresponds to InsP\(_3\)R2 G2498, and mutagenesis of InsP\(_3\)R1 G2546 (i.e., p.G2546A) results in inactivation of the channel activity. That is, p.G2546A mutant InsP\(_3\)R1 is still able to form homotetramers but unable to mediate Ca\(^{2+}\) release from microsomal vesicles, and it is nonfunctional in a Ca\(^{2+}\) flux assay (21).

We then analyzed p.G2498S mutant InsP\(_3\)R2 in a chicken cell system devoid of endogenous InsP\(_3\)Rs, and the results were consistent with a loss-of-function effect of the p.G2498S mutation. Cells expressing the p.G2498S mutant InsP\(_3\)R2 showed a complete loss of Ca\(^{2+}\) response upon stimulation, despite Ca\(^{2+}\) stores similar to those of control cells expressing WT InsP\(_3\)R2. Thus, the abolished intracellular Ca\(^{2+}\) release from the InsP\(_3\)R2.p.G2498S variant is consistent with previous studies on rat InsP\(_3\)R1 with a mutation in the corresponding residue (21).

To independently assess the role of InsP\(_3\)R2 in sweat production, we analyzed eccrine glands in paws of Itprr\(^{-/-}\) mice. We observed a 3-fold reduction in the number of pilocarpine-responsive sweat glands on Itprr\(^{-/-}\) mouse paws, and dissected Itprr\(^{-/-}\) sweat glands showed a significant reduction in Ca\(^{2+}\) response after acetylcholine stimulation compared with those of Itprr\(^{+/+}\) mice. Together, these results indicate that InsP\(_3\)R2 plays a critical role in sweat secretion in both mice and humans. However, the residual sweat production in Itprr\(^{-/-}\) 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 InsP\(_3\)R2 and InsP\(_3\)R3 are the predominant isoforms expressed in human sweat glands,
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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 InsP₃R 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 InsP₃R1 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 InsP₃R isoforms would then result in mostly non-functional Ca²⁺ channels. However, 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 isoforms. Additionally, redundant function for the InsP₃R isoforms 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

Figure 4. Decreased sweat secretion in Itpr2⁻/⁻ mice. (A) Pilocarpine-induced sweat response in Itpr2⁺/+ (n = 7) and Itpr2⁻/⁻ (n = 5) mice visualized by the Starch-iodine assay. Representative images of Itpr2⁺/+ and Itpr2⁻/⁻ mouse paws 20 minutes after pilocarpine injection are shown. The number of black dots (arrows) was counted at the indicated times after injection. *P < 0.05, **P < 0.005, Student’s 2-tailed t test. (B) Dot diameter 20 minutes after pilocarpine injection. (C) Immunohistochemistry of InsP₃R2 in sweat glands of Itpr2⁺/+ and Itpr2⁻/⁻ mice. Red, InsP₃R2; green, S100β; blue, DAPI. Data represent mean ± SEM. Scale bar: 20 μm.
findings highlight InsP₃R₂ 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), InsP₃R₁ (HPA016487; Sigma-Aldrich), InsP₃R₂ (AB9074; Millipore), and InsP₃R₃ (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 Apal 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 (CAGGGCCCTAGGAAATGGCAAGGGGATGGATGCTGAAG) and antisense (CTAGCACTTCCCAACTCCTAGCGATCGTACAGTTCCGTCCATGCCATTGCGCTCCGTCG) 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 SalI fragment from the vector was cloned into the SalI site of pBacSTneoB. 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 × 10^5 cells were directly lysed with a SDS-PAGE sample buffer, separated by SDS-PAGE gel, and probed with anti-InsP3, 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 for 2 minutes, cells were stimulated with anti-BCR antibody (M4) at 0.25 μg/ml (16). Fura-2 fluorescent images were analyzed using an inverted microscope (ECLIPSE TE300; Nikon) and a video image analysis system (Argus-50; CA; Hamamatsu Photonics) with excitation filters at 340 ± 10 and 380 ± 10 nm, a dichroic beam splitter at 400 nm, and a bandpass emission filter at 510–550 nm.

Mouse studies. Itpr2−/− mice were described previously (12). Itpr2+/− and Itpr2−/− mice (5 weeks old) were anesthetized by intraperitoneal injection of 36 mg/kg ketamine (Daichi Sankyo) and 16 mg/kg xylazine (Bayer HealthCare). Mouse paws were painted with iodine (5.0% in ethanol; Sigma-Aldrich), then covered with starch solution (0.5 g/ml in ethanol; Sigma-Aldrich), and the peak Fluo4/Fura Red ratio change value was taken as the maximal ratio change of the sweat gland.

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–pan-InsP3R antibody (22) and mouse anti–p-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.

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