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Localization of aquaporin-5 in sweat glands and functional analysis using knockout mice

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Sweat secretion involves the transport of salt and water into the lumen of the secretory coil of the sweat gland. By analogy to salivary and submucosal glands, where fluid secretion is aquaporin-5 (AQP5) dependent, we postulated that aquaporin water channels might facilitate sweat secretion. Immunolocalization with specific antibodies revealed strong expression of AQP5 at the luminal membrane of secretory epithelial cells in sweat glands in mouse paw skin. Novel quantitative methods were developed to compare sweat secretion in wild-type mice and mice lacking AQP5. Total hindpaw sweat secretion was measured by proton nuclear magnetic resonance of sweat-derived ^1H_2O in ^2H_2O solvent, and sweat secretion from individual glands was measured by real-time video imaging of sweat droplet formation under oil. Sweat secretion rates after pilocarpine stimulation did not differ in wild-type mice (0.21 ± 0.03 nl min^{-1} gland^{-1}) *vs.* mice lacking AQP5 (0.19 ± 0.04 nl min^{-1} gland^{-1}). The lack of effect of AQP5 on sweat secretion rate was confirmed by microcapillary collections of sweat from defined regions of mouse paws. Also, as by direct counting of droplets, the number of functional sweat glands was not affected by AQP5 deletion. Sweat gland morphology was similar in wild-type and AQP5 null mice. From sweat coil geometry and gland secretion rate, the rate of fluid secretion was estimated to be 130 nl min^{-1} cm^{-2} of secretory epithelium, substantially lower than that of > 500 nl min^{-1} cm^{-2} in kidney proximal tubules and salivary glands, where active fluid absorption or secretion is aquaporin dependent. These results indicate the expression of AQP5 in sweat gland secretory epithelium, but provide direct evidence against its physiological involvement in sweat fluid secretion in mice.

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Sweat glands contain a secretory coil, where fluid accumulates by active salt transport followed by osmotically driven water transport (Dobson & Sato, 1972; Sato, 1977; Quinton, 1987). The primary fluid secreted into the lumen of the coil is expelled onto the skin surface after transit through a short, water-impermeable duct where additional salt transport occurs. The quantity and/or composition of sweat is abnormal in a number of hereditary diseases such as cystic fibrosis, where the salt concentration is elevated because of defective cystic fibrosis transmembrane conductance regulator (CFTR)-dependent Cl^- transport (Quinton, 1987). In rodents, sweat glands are found mainly on the digits and footpads of the paws, where secretion of a K^+-rich sweat is stimulated by cholinergic agonists (Sato *et al.* 1994; Tafari *et al.* 1997). There is a considerable body of information about the innervation, development and morphology of mouse sweat glands (Sato, 1977; Kennedy *et al.* 1984; Tafari *et al.* 1997).

Recent studies of transepithelial fluid absorption and secretion support the paradigm that aquaporin (AQP) water channels can facilitate near-isosmolar fluid transport. Aquaporins are small integral membrane proteins that provide the principal route for osmotically driven water movement across plasma membranes in many cell types (Borgnia *et al.* 1999; Ishibashi *et al.* 2000; Verkman & Mitra, 2000). The kidney proximal tubule epithelium, which expresses AQP1 at its apical and basolateral plasma membranes, is responsible for near-isosmolar absorption of fluid filtered by the glomerulus. Deletion of AQP1 in mice results in an 80 % reduction in transepithelial osmotic water permeability and a 50 % impairment of fluid absorption (Schnermann *et al.* 1998), producing substantial (~40 mosmol l^{-1}) luminal hypotonicity at the end of the proximal tubule (Vallon *et al.* 2000). The salivary gland contains an acinus into which active salt transport drives water transport by creation of an osmotic gradient. Deletion of AQP5 in the acinar epithelium in mice results in the production of a low volume of hypertonic viscous saliva because of unimpaired salt transport across a relatively water-impermeable acinar epithelium (Ma *et al.* 1999). Similarly, deletion of AQP5 in the acinar epithelium of airway submucosal glands in mice results in impaired secretion of fluid into the upper airways (Song & Verkman,

2001). From these observations we postulated that AQP5 or other aquaporins may facilitate sweat secretion, making aquaporins a potential target for the development of a new class of antiperspirants.

The purpose of this study was to test the hypothesis that aquaporins are involved in sweat secretion. As found in salivary glands (Ma *et al.* 1999; Krane *et al.* 2001; Gresz *et al.* 2001) and airway submucosal glands (Nielsen *et al.* 1997; Song and Verkman, 2001), AQP5 was localized to the luminal membrane of secretory epithelial cells in sweat glands. The functional significance of this finding was investigated by comparing sweat secretion and sweat gland morphology in wild-type mice and transgenic mice lacking AQP5. These studies required the development of novel approaches to measuring sweat secretion in mice, after having found that reported approaches involving individual gland isolation (Sato & Sato, 1978), sweat evaporation (Van Gasselt & Vierhout, 1963; Sato *et al.* 1994), iodine/starch treatment (Tafari *et al.* 1997; Shamsuddin & Togawa, 2000), and impression moulds (Kennedy *et al.* 1984; Vilches *et al.* 1998) were not sufficiently quantitative and/or reproducible for detection of potentially small differences in the knockout mice (see Discussion). Our method for measuring total paw sweat secretion involved proton nuclear magnetic resonance (NMR) of sweat water, and our method for measuring sweat secretion in individual glands involved real-time imaging of sweat droplet accumulation under oil. It was found that despite the strong expression of AQP5 in the secretory epithelium of sweat glands, sweat fluid secretion was not impaired in AQP5 null mice nor were there differences in the number or morphology of sweat glands. The results were interpreted in terms of relatively low rates of transepithelial fluid transport in the sweat gland secretory epithelium compared to other epithelia where fluid transport has been proven to be aquaporin dependent.

METHODS

Transgenic mice

Knockout mice deficient in AQP3 and AQP5 in a CD1 genetic background were generated by targeted gene disruption (Ma *et al.* 1999, 2000*b*). Measurements were made in litter-matched mice (age 6–8 weeks, body weight 25–31 g) produced by intercrossing of heterozygous mice. The investigators were blinded to genotype information in all measurements. Protocols were approved by the University of California, San Francisco Committee on Animal Research. All mice were killed at the end of an experiment, or prior to mouse paw skin collection, by an overdose of pentobarbital.

RT-PCR analysis of aquaporins 1–9 in mouse paw skin

Total RNA was isolated from mouse paw skin, and RT-PCR was performed with sequence-specific sense and antisense oligonucleotide primers for aquaporins 1–9 as described (Song & Verkman, 2001).

Immunocytochemistry and tissue morphology

Immunofluorescence localization of aquaporins in 3–4 μm cryostat sections of paraformadehyde-fixed hindpaw skin was carried out using immunopurified rabbit anti-rat polyclonal antibodies as described previously (Song *et al.* 2001). Sweat gland morphology was examined in toluidine blue-stained plastic-embedded sections. Secretory coil diameter was measured on sections from different mice, with 20–25 coil diameters averaged for each mouse. Because some coils were cut at angles, the largest diameters were measured for those coils that appeared symmetrical with a distinct lumen. Sweat ducts were identified by their deeper blue staining (see Results).

NMR measurement of total sweat gland fluid secretion

Dried plastic vials containing 1 ml deuterium oxide (^2H_2O) (Aldrich, 100 % atom) were maintained in a dry environment (100 % nitrogen blowing in a plastic tent). Mice were anaesthetized with ketamine (40 mg kg^{-1}) and xylazine (8 mg kg^{-1}). A tracheotomy was performed using PE-90 tubing to prevent airway obstruction from salivation in experiments where pilocarpine was administered intraperitoneally. The mouse paw was rinsed with distilled water, dried with nitrogen, and placed in a humidified chamber (35 °C, 100 % humidity). Sweat secretion was stimulated by intraperitoneal injection of pilocarpine (80 mg kg^{-1}). After 10 min, the mouse paw was immersed immediately in a vial containing 0.5 ml anhydrous ^2H_2O and 1 μl [^{1}H]DMSO (as an internal standard) and shaken for 5 s to dissolve/wash all the sweat. The ^2H_2O samples were transferred to NMR tubes in the nitrogen tent, and ^1H_2O concentration was measured using a Varian NMR instrument (AS400) operating at 400 MHz (acquisition parameters: relaxation delay 1 s, pulse 69.2 deg, acquisition time 2 s, 10 repetitions). The integrated NMR signals were determined for the ^1H_2O peak (at 4.46 p.p.m.) and for the [^{1}H]methyl peak of the internal standard [^{1}H]DMSO (at 2.44 p.p.m.) (Flockhart & Pink, 1965; Johnson & Keller, 1969). The ^1H_2O concentration (and hence the amount in sweat) was determined from the ratio of ^1H_2O to [^{1}H]DMSO integrated peaks. For calibration, known amounts of water (0–500 nl) were added to ^2H_2O containing 1 μl [^{1}H]DMSO.

Sweat collection in constant-bore microcapillaries

Mice were anaesthetized with ketamine and xylazine as above. The left or right hindpaw was rinsed with distilled water, then dried with nitrogen, immersed in water-saturated mineral oil in a Petri dish, and pilocarpine (80 mg kg^{-1}, I.P.) was administered. Microcapillary tubing (Drummond Scientific, 1 μl) was pulled into a fine needle shape using a pipette puller (Kopf Instruments) and immersed in oil to collect sweat droplets. The droplets were collected 10 min after pilocarpine injection. Sweat collection took 2–3 min, by which time nearly all sweat was collected without seeing droplets at the surface of the paw. After the collection, the micropipettes were centrifuged to separate oil and sweat, and the total aqueous fluid length was measured to determine sweat volume. In separate experiments in which sweat was collected at 2.5 and 5 min, pilocarpine was injected into the paw skin (30 μl, 1 mg ml^{-1} in phosphate buffered saline (PBS)), the paw was immediately immersed in water-saturated mineral oil, and sweat droplets were collected from 2 to 2.5 min (for 2.5 min study) and from 4.5 to 5 min (for 5 min study). Fifty to sixty droplets were collected from the pad region of the paw.

Imaging of fluid secreted from sweat glands

Mice were anaesthetized with ketamine and xylazine and a hindpaw was rinsed with distilled water and dried with nitrogen. After pilocarpine administration into the paw (30 μl, 1 mg ml^{-1}), the hindpaw was immersed in water-saturated mineral oil. Serial micrographs were taken every 15 s using a CCD camera through a Nikon upright microscope at × 250 total magnification. The diameters of individual droplets were measured as a function of time to compute sweat volume, assuming spherical droplet shape.

RESULTS

To identify aquaporins in mouse sweat glands, RT-PCR was carried out using cDNA isolated from paw skin. Figure 1*A* shows amplification of transcripts encoding AQP3 and AQP5 from paw skin (lanes labelled 'S'). In each case, positive control lanes (labelled 'C') are shown in which the template consisted of cDNAs from tissues known to contain the respective aquaporins. Immunostaining with specific antibodies for AQP3 and AQP5 was performed. Only AQP3 transcript was amplified in paw skin cDNA from AQP5 null mice. AQP5 was mainly localized to the luminal membrane of secretory epithelial cells in sweat coils of wild-type mice, with relatively little expression at the basal membrane and no staining in the duct (Fig. 1*B*, left). Staining was negative in AQP5 null

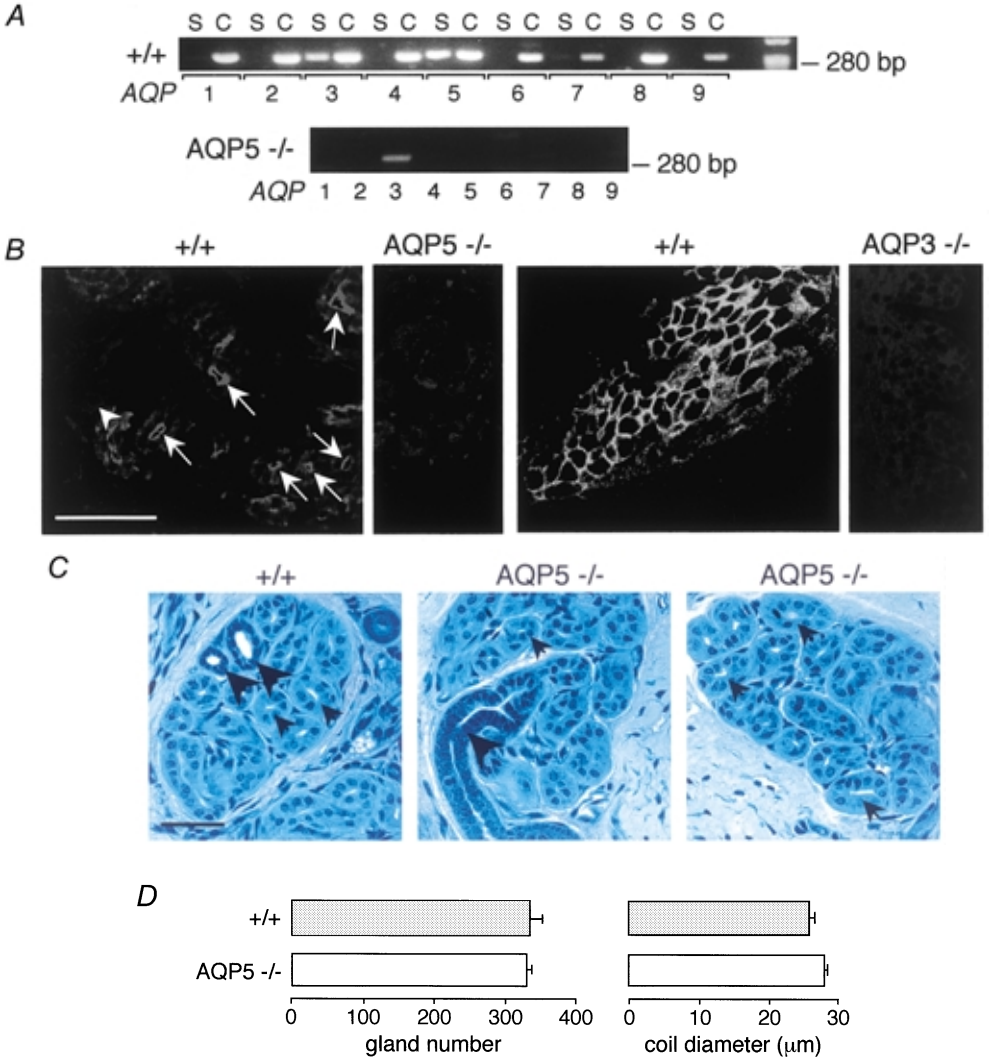

Figure 1. Aquaporin expression and morphological analysis of mouse sweat gland

A, RT-PCR analysis of aquaporin expression in full-thickness hindpaw skin of wild-type and AQP5 null mice. Transcripts for portions of the coding sequences of indicated mouse aquaporins were PCR amplified using specific primers. Lanes labelled 'S' correspond to amplifications carried out using skin cDNA as a template, and lanes labelled 'C' correspond to amplifications carried out using a mixture of cDNAs from brain, lung, liver and kidney, which contain all mouse aquaporins. *B,* immunofluorescence of paw skin with immunopurified AQP3 and AQP5 antibody showing localization of AQP5 protein at the luminal membrane of secretory epithelial cells in sweat coil (left) and AQP3 protein in epidermal keratinocytes (right). White arrows: coils, white arrowhead: duct. Staining was negative in paw skin of AQP3 or AQP5 null mice (narrow panels). Scale bar: 50 μm. *C*, sweat gland/duct morphology assessed in thin plastic-embedded sections of hindpaw skin from wild-type (left) and AQP5 null (middle and right) mice. Arrows point to sweat coils, and arrowheads to sweat ducts. *D,* quantitative analysis of sweat coil diameter (determined from sections as in *C*) and sweat gland number (determined by counting sweat droplets under oil after pilocarpine stimulation). Data shown as means ± S.E.M. for measurements on 5 mice.

mice. AQP3 was expressed exclusively in epidermal keratinocytes in paw skin of wild-type mice, with no staining in AQP3 null mice (Fig. 1*B*, right). AQP3 has been localized in previous studies to mammalian epidermis (Frigeri *et al.* 1995; Matsuzaki *et al.* 1999), where it plays an important role in hydration of the stratum corneum (Ma *et al.* 2002). Comparative morphological and functional studies were therefore carried out on wild-type and AQP5 null mice.

Figure 1*C* shows representative stained plastic sections of dermis of the mouse paw showing sweat glands with sweat ducts (arrowheads) and coils (arrows) indicated. Ducts were identified by their deeper blue staining as seen for a duct cut in longitudinal section (middle panel). In a genotype-blinded evaluation, there were no qualitative morphological differences in sections from wild-type *vs.* AQP5 null mice. Figure 1*D* shows a quantitative analysis of sweat coil diameter and total number of functional sweat glands per paw. Sweat coil diameters were measured from photographs of plastic sections and the number of functional sweat glands was measured by counting fluid droplets under oil after pilocarpine stimulation.

Initial functional measurements of total sweat were made by a proton NMR method in which sweat ^1H_2O was measured in a ^2H_2O solvent. This assay is highly sensitive for detection of ^1H_2O. In these experiments an internal standard [^{1}H]DMSO was added to normalize NMR spectra. Figure 2*A* (left) shows NMR spectra of ^2H_2O containing different amounts of ^1H_2O. Figure 2*B* shows a linear relationship between relative ^1H_2O/[^{1}H]DMSO peak areas and ^1H_2O concentration. For sweat collections, mouse hindpaws were placed in a humidified chamber for 10 min and then immersed briefly in the ^2H_2O solvent to dissolve sweat droplets. Without pilocarpine stimulation, 70 nl of ^1H_2O was collected, which represents a combination of condensate and diffusional water exchange (Fig. 2*B*). There was a significantly increased ^1H_2O content when mice were pre-treated with pilocarpine, but no difference was observed in the AQP5 null mice. Therefore AQP5 deletion did not grossly affect sweat secretion, and so methods with greater sensitivity and time resolution were applied to detect potentially subtle differences in rates of sweat secretion.

Figure 3*A* shows photographs of mouse hindpaws at 5 min after pilocarpine administration. Sweat fluid droplets were seen at the skin surface, particularly in the regions of footpads and digits as reported previously (Kennedy *et al.* 1984; Tafari *et al.* 1997). All droplets in a defined region of skin were rapidly collected at a specified time using constant-bore microcapillaries with a drawn tip. Figure 3*B* summarizes the collected sweat fluid volumes of total glands at 2.5, 5 and 10 min after pilocarpine stimulation. There was no significant effect of AQP5 deletion. No sweat fluid was collected for identical experiments performed without pilocarpine in wild-type or AQP5 null mice.

For improved time resolution, a real-time imaging method was used to follow the increase in sweat droplet diameter after intradermal hindpaw pilocarpine administration to induce prompt sweat secretion. Images were taken with the paw under mineral oil using a × 25 long working distance objective to follow the growth of a set of sweat fluid droplets at the same time. Figure 4*A* shows representative images of individual sweat droplets from wild-type and AQP5 null mice, showing increasing

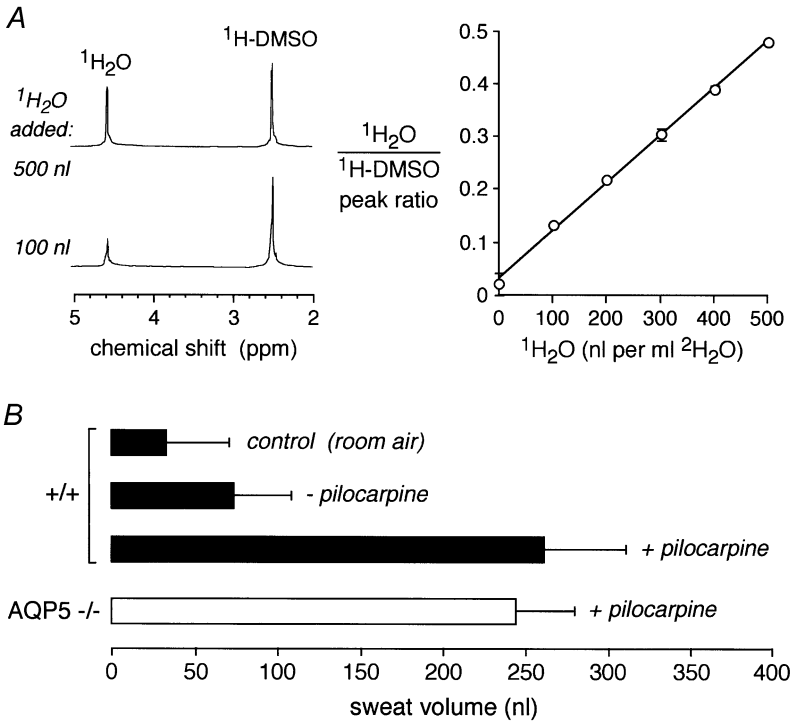

Figure 2. Proton nuclear magnetic resonance measurement of total sweat secreted from mouse hindpaws

A , left, representative NMR spectra of ^2H_2O containing [^{1}H]DMSO and indicated quantities of ^1H_2O; right, calibration of the ratio of integrated peak intensities of ^1H_2O *vs.* [^{1}H]DMSO (mean ± S.E.M., $n = 5$) as a function of the amount of added ^1H_2O. *B*, sweat collections. After anaesthesia, tracheotomy and pilocarpine administration, hindpaws were placed in a humidified atmosphere for 10 min during which time sweat droplets accumulated without evaporation. Droplets were collected by brief immersion of hindpaws in ^2H_2O containing [^{1}H]DMSO as an internal standard. Total sweat (mean ± S.E.M., $n = 5$) was not significantly different between wild-type and AQP5 null mice.

diameter over time, from which droplet volume was computed assuming a spherical shape. Figure 4*B* (left) shows the growth of representative individual droplets, which was approximately linear to at least 5 min and similar in wild-type and AQP5 null mice. Figure 4*B* (right) summarizes the averaged growth of sweat droplets, showing no significant effect of AQP5 deletion.

DISCUSSION

The purpose of this paper was to test the hypothesis that aquaporin water channels are involved in sweat secretion. As postulated, AQP5 protein was expressed strongly on the apical surface of secretory epithelial cells in sweat coils. AQP5 was found previously to be expressed in several secretory glands including salivary glands, airway submucosal glands and lacrimal glands, as well as in alveolar type I epithelial cells (Nielsen *et al.* 1997; Dobbs *et al.* 1998; Ma *et al.* 1999; Borok *et al.* 2000; Ma *et al.* 2000*a*; Moore *et al.* 2000; Krane *et al.* 2001; Song & Verkman, 2001). Because previous phenotype studies of aquaporin null mice have shown that the tissue-specific expression of an aquaporin does not indicate physiological significance (Verkman *et al.* 2000), functional studies of sweat secretion were carried out in wild-type and AQP5 null mice. Quantitative measurement methods were developed to be able to detect small differences in the rate of sweat secretion as discussed further below. Experiments were carried out using maximal agonist stimulation of sweat secretion based on reports that cholinergic agents such as pilocarpine elicit the strongest sweat response in adult rodents (Sato, 1977; Sato *et al.* 1994; Vilches *et al.* 1998). The rationale for inducing maximal sweat secretion was to allow detection of subtle differences in wild-type *vs.* AQP5 null mice, since the effects of reduced epithelial water permeability are predicted to be seen best at high rates of active, isosmolar fluid transport. The principal finding was that although AQP5 is expressed in sweat glands, neither sweat gland morphology nor function was affected by AQP5 deletion in mice. These results contrast with data for salivary glands (Ma *et al.* 1999; Krane *et al.* 2001) and airway submucosal glands (Song & Verkman, 2001), where cholinergic-stimulated fluid secretion was markedly impaired in AQP5 null mice.

Several methods to measure sweat secretion from mouse paw were initially tested before selecting the NMR, microcapillary collection and video imaging methods reported

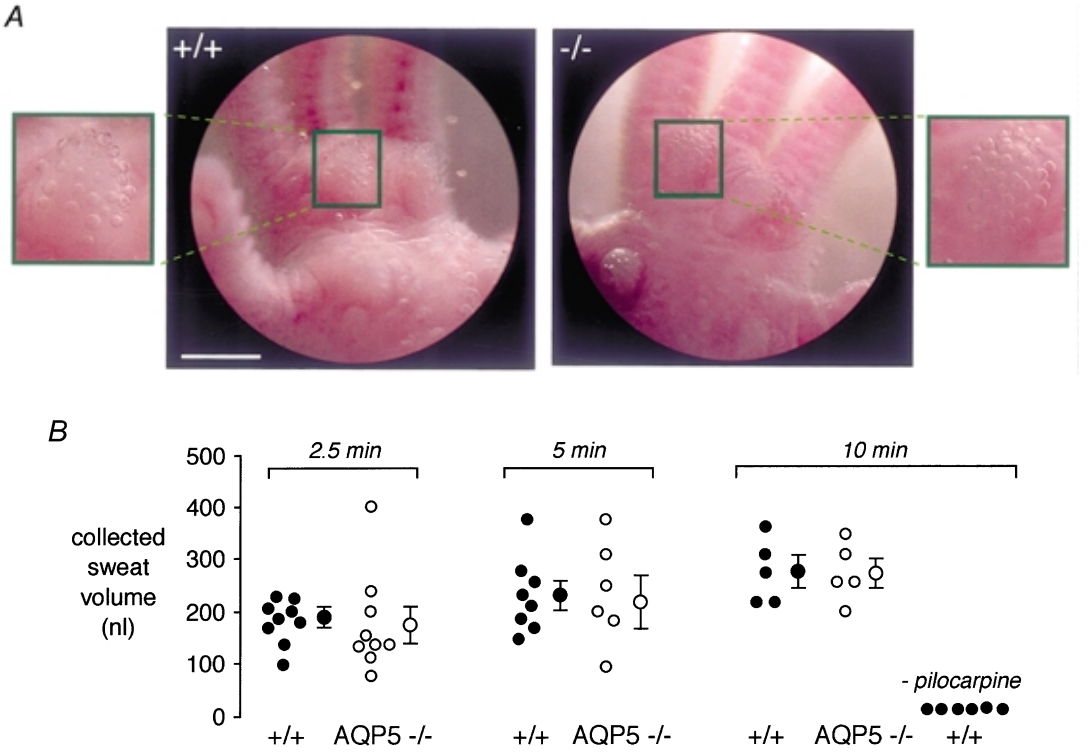

Figure 3. Measurement of sweat secretion by microcapillary fluid collection

Hindpaws were covered with oil to visualize the appearance of sweat droplets after pilocarpine administration. *A*, low magnification photographs of sweat droplets from wild-type (left) and AQP5 null (right) mouse at 5 min after pilocarpine administration. *B*, collected sweat volumes from 5–9 mice at indicated times after pilocarpine administration for wild-type (●) and AQP5 null (○) mice. Data from individual mice are shown with means ± S.E.M. Differences are not significant. Where indicated a 10 min sweat fluid collection was carried out in mice treated identically but not given pilocarpine.

here. Silicone impression methods have been widely used to count sweat droplets and estimate droplet size (Vilches *et al.* 1998; Tian *et al.* 2000). However, the problem acknowledged with this method for quantitative measurements is that considerable uncertainty is introduced by the gradual hardening of the impression material during sweat secretion. We found that video imaging permits direct determination of gland droplet volumes continuously and without perturbing the skin surface. Sweat secretion was also measured in initial studies using a Meeco moisture analyser (Kerr Manufacturing Co., Romulus, MI, USA), as reported previously (Van Gasselt & Vierhout, 1963; Sato *et al.* 1994). However, we found that the characteristics of the contact with the moisture collection cup (pressure, size, area of skin) strongly influenced the results. Although pilocarpine-stimulated sweat secretion could be measured, there was considerable variability in the data precluding the detection of differences of less than a factor of two. We also were not satisfied with the reproducibility and accuracy of the iodine–starch method (Tafari *et al.* 1997; Shamsuddin & Togawa, 2000; Nejsum *et al.* 2002), which is useful mainly for qualitative estimation of the number of glands that secrete fluid containing amylase. This method, which provides information about sites of amylase secretion (but not fluid secretion), relies on generation and subjective assessment of blue colour after amylase catalysis of the iodine–starch reaction.

The NMR method developed here for measurement of total paw sweat secretion relies on the ability to detect small quantities of sweat ^1H_2O in a ^2H_2O (deuterium oxide) solvent. The complementary microcapillary collection method permits direct sampling of sweat secreted from a defined region of skin. We found no differences in sweat secretion rates in wild-type *vs.* AQP5 null mice by the three complementary methods. We conclude that AQP5 deletion in mice does not impair sweat fluid secretion or the number of functional sweat glands.

The lack of effect of AQP5 deletion on sweat secretion contrasts with previous findings in salivary and airway submucosal glands where AQP5 deletion impairs fluid secretion (Ma *et al.* 1999; Krane *et al.* 2001). We propose that the relatively slow rate of sweat secretion is responsible for the lack of effect of AQP5 deletion. The average rate of sweat secretion in mice found here and in previous studies (Sato *et al.* 1994) is ~0.2 nl min^{-1} $gland^{-1}$. Estimating a total epithelial surface area of 0.0015 cm^2 for the sweat coil from published morphological studies (Sato & Sato, 1978; Sato *et al.* 1994) and data presented here, the area-normalized rate of sweat fluid secretion is ~130 nl min^{-1} μm^{-2}. This value is substantially less than that of 500 nl min^{-1} μm^{-2} in the kidney proximal tubule where fluid absorption is AQP1 dependent (Schnermann *et al.* 1998); the estimated value was even higher in salivary glands (Ma *et al.*1999). For comparison, the maximally stimulated rate of alveolar fluid clearance was 16 nl min^{-1} cm^{-2}, and it was shown that AQP5 deletion, which reduces alveolar water permeability 10-fold, did not affect alveolar fluid clearance (Ma *et al.* 2000*a*). Aquaporins and high water permeability may not be required for slow rates of active fluid transport (per unit area) because the basal (aquaporin-independent) water permeability is adequate to permit osmotic equilibration after active vectorial salt transport.

Since the submission of our manuscript, Nejsum *et al.* (2002) reported localization of AQP5 primarily at the apical membrane of the sweat gland secretory coil in mice, in agreement with our results, as well as in the initial part of

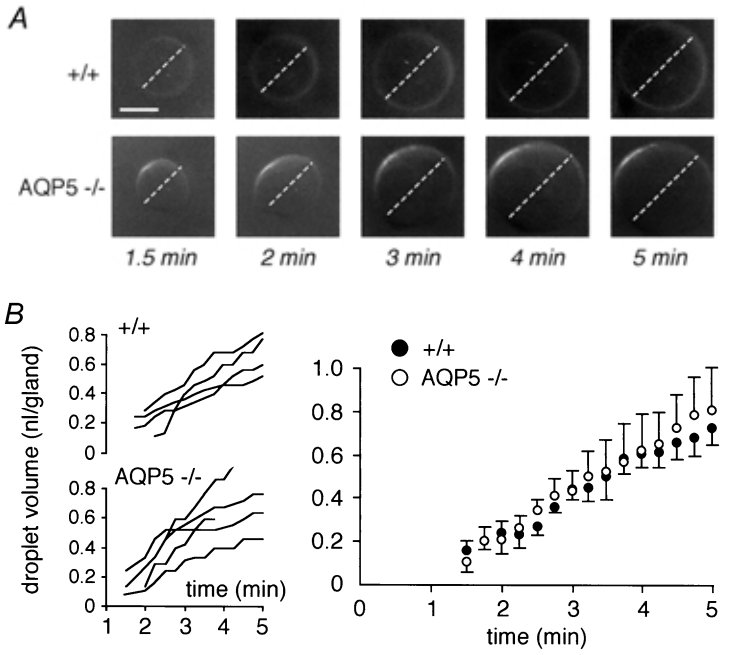

Figure 4. Sweat secretion from individual sweat glands measured by real-time video imaging

Hindpaws were covered with oil to visualize the appearance of sweat droplets after pilocarpine administration. *A*, representative series of micrographs showing expanding individual fluid droplets secreted from hindpaw sweat glands after pilocarpine stimulation in wild-type and AQP5 null mice. *B*, left, increasing volume in representative individual sweat droplets; right, averaged fluid volumes (mean ± S.D., 6–8 droplets measured in 6 mice of each genotype) secreted at indicated times after subcutaneous pilocarpine administration. Differences are not significant.

the sweat duct in mice and the duct in humans. Although they reported that sweat gland morphology was similar in wild-type and AQP5 null mice, in agreement with the finding here, they found impaired sweat secretion in AQP5 null mice using the iodine-starch method at a single time point. Sweat fluid secretion was not evaluated in their study. Differences in methodology or mouse strains (CD1 here, 129SVJ in Nejsum *et al.*) could account for the different conclusions. Side-by-side comparison using the same mouse strains and methods will be required to address this issue.

In conclusion, our results provide direct evidence against functionally important involvement of AQP5 in sweat fluid secretion in mice. Because of differences in sweat gland morphology and physiology in humans *vs.* rodents, the conclusion that AQP5 does not participate in sweat secretion in humans cannot be made with certainty. Further, although we believe it to be unlikely, the possibility cannot be excluded that other, as yet unidentified, aquaporins are coexpressed with AQP5 in sweat glands such that AQP5 deletion does not greatly reduce osmotic water permeability.

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