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Dynamic Analysis of Histamine-Mediated Attenuation of Acetylcholine-Induced Sweating via GSK3β Activation

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Sweating has been associated with the exacerbation of atopic dermatitis (AD) in diverse ways. Acetylcholine (ACh)-mediated sweating is known to be attenuated in AD, but its cause remains obscure. To address this issue, the impact of histamine on ACh-induced sweating was evaluated. Sweating was measured by counting the number of active sweat pores by the starch–iodine reaction and dynamic optical coherence tomography; sweat was visualized using two-photon excitation fluorescence microscopy in mice and the quantitative sudomotor axon reflex test in humans. Both histamine receptor antagonists and H1 receptor (H1R)-knockout (KO) mice were used to determine methodological specificity. Histamine demonstrably inhibited ACh-induced sweating in both mice and humans via H1R-mediated signaling. In sweat glands, ACh inactivated glycogen synthase kinase 3β (GSK3β), a kinase involved in endocytosis and secretion, whereas simultaneous stimulation with histamine activated GSK3β. Results of two-photon excitation fluorescence microscopy confirmed the dynamic motion of sweat and sweat glands after ACh treatment, showing that simultaneous stimulation with histamine altered their dynamic properties. These results indicate that histamine inhibits sweat gland secretions by blocking ACh-induced inactivation of GSK3β. Histamine-mediated hypohidrosis might be involved in the mechanism of abnormal skin dryness in patients with AD.

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INTRODUCTION

Disturbances in the homeostatic function of the skin evoke a variety of dermatoses. Sweating is involved in the maintenance of skin homeostasis, with antimicrobial (Rieg *et al*, 2005; Schittek *et al*, 2008) and moisturizing effects (Eishi *et al*, 2002), and in the regulation of skin surface pH (Schmid-Wendtner and Korting, 2006). Recent reports also suggest that impaired sudomotor function contributes to the pathogenesis of atopic dermatitis (AD) (Imayama *et al*, 1994; Eishi *et al*, 2002; Kitaba *et al*, 2011; Shiohara *et al*, 2011; Kijima *et al*, 2012), and agree that deterioration of sudomotor function is found in patients with AD. These results indicate that reduction of sweating might impair moisturization and protection against infections of the skin. However, the mechanism of aberrant sweating in AD remains unknown.

There are likely two hypotheses: the first is sweat duct obstruction by abnormal keratinization (Sulzberger *et al*, 1947; Papa and Kligman, 1966) and the second is abnormal sudomotor function of sweat glands (Kijima *et al*, 2012). Because not all AD patients with anhidrosis develop histopathological sweat duct obstruction, we hypothesize that certain inflammatory mediators might reduce the function of sweat glands. A potential candidate for this hypothesis was histamine, which signals through a seven-transmembrane receptor, as well as an acetylcholine (ACh) receptor (Du Buske, 1996; Simons and Simons, 2011). It is well known that antihistamines also have anticholinergic properties (Meltzer, 1990; Du Buske, 1996; Murota and Katayama, 2011). Thus, we evaluated the effects of histamine on ACh-mediated sweating in animals.

Assessment of sudomotor function varies in methodology and has been evaluated by visualization of the sweat response by the iodine–starch reaction (Nejsum *et al*, 2002) or optical coherence tomography (OCT) (Ohmi *et al*, 2009).

Here, we evaluated the impact of histamine on ACh-mediated sweating using a multimodal approach *in vivo*, and discuss the possible involvement of sudomotor dysfunction. Moreover, in this study, we attempted to visualize the sweat response using two-photon excitation fluorescence

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Abbreviations: ACh, acetylcholine; AD, atopic dermatitis; ANOVA, analysis of variance; GSK3α/β, glycogen synthase kinase 3α/β; H1R, histamine type-1 receptor; KO, knockout; M3R, muscarinic 3 receptor; OCT, optical coherence tomography; QSART, quantitative sudomotor axon reflex test

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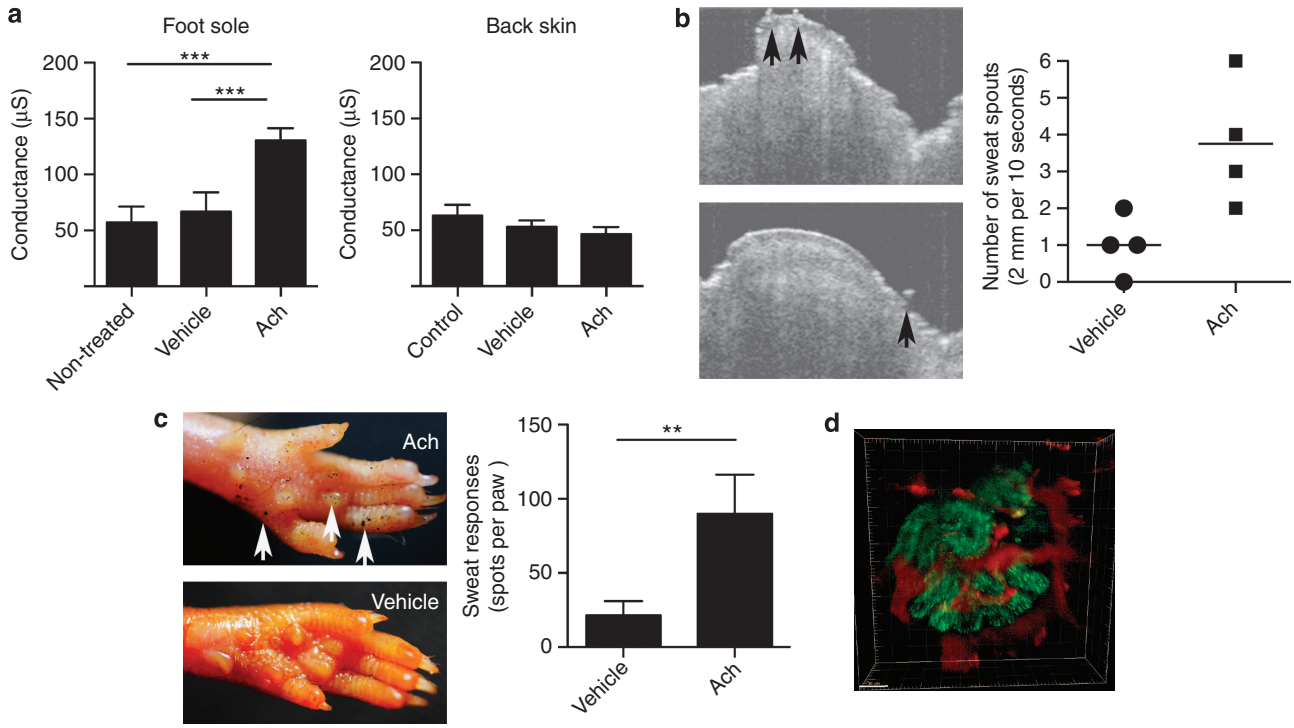


Figure 1. Multimodal approach to assess sudomotor response to acetylcholine (Ach) *in vivo*. (**a**) The degree of stratum corneum hydration was assessed with conductance measurements. Left: foot sole, right: back skin. Statistical analysis was performed by one-way analysis of variance, Bonferroni's multiple comparison test ($n = 5$–6). (**b**) Dynamic images of sweating visualized by optical coherence tomography. Graph data show the quantification of the images ($n = 5$). (**c**) The result of counting the number of iodine–starch reaction–positive sweat pores. $^{**}P = 0.003$, unpaired *t*-test ($n = 5$–7). Arrows indicate the positive spots. (**d**) Observations with two-photon microscopic analysis allowed a complete view of sweat glands. Green (FITC): gross cystic disease fluid protein (sweat gland); red (Texas Red): dextran (microvessels).

microscopy and succeeded in obtaining the dynamic images of sweat glands after sudomotor stimulation.

RESULTS

Validation of evaluation methodologies for ACh-mediated sweating in mice

To demonstrate the validity of assessment of sudomotor function in mice, four different methods were performed. First, skin hydration was evaluated by the impedance method based on the idea that sweat will moisten the skin surface (Figure 1a). Skin hydration significantly increased on the ACh-treated foot sole, but not on ACh-treated, sweat gland–free back skin. The dynamic image of sweating obtained by OCT revealed the increased sweat spout from the sweat duct on the ACh-treated foot sole (Figure 1b and Supplementary Figure 1 online). The sudomotor reaction to ACh was also confirmed by counting the number of iodine–starch reaction–positive sweat pores (Figure 1c). These results indicate that our multimodal evaluations can adequately assess the sudomotor reaction to ACh *in vivo*. Furthermore, two-photon microscopic analysis revealed the three-dimensional structures of sweat glands and microvessels visualized using anti-gross cystic disease fluid protein antibody and intravenously administered red fluorescent dextran, respectively (Figure 1d).

Effects of histamine on ACh-mediated sudomotor function

Histamine was administered simultaneously with ACh, and sudomotor response was examined in mice. First, the measurement results of skin hydration were increased by stimulation with ACh alone, but there was no apparent increase after coadministration of histamine with ACh (Figure 2a). In the observation record of OCT, the number of active sweat glands in ACh-treated mice decreased after coadministration of histamine (Supplementary Figure 1B and C online). High numbers of sweat pores positive for the iodine–starch reaction by ACh treatment decreased after coadministration of histamine (Figure 2b). To confirm which type of histamine receptor has a crucial role in the suppression of sudomotor function, antagonists for histamine receptors (i.e., H1 receptor (H1R), H2R, and H4R) were administered intraperitoneally (Figure 2b). Pyrilamine and fexofenadine, H1R antagonists, decreased the ACh-induced sweating response and prevented the histamine-mediated inhibition of ACh-induced sweating. In contrast, both H2 and H4 receptor antagonists markedly reduced ACh-induced sweating, but did not affect the histamine-mediated inhibition of ACh-induced sweating (Figure 2b). H1R-knockout (KO) mice showed no apparent inhibition by histamine of increased skin hydration (Figure 2c) and of increased number of iodine–starch reaction–positive sweat pores by Ach treatment (Figure 2d).

Localization of ACh and histamine receptors in the microenvironment of the eccrine sweat gland

Next, to resolve the question of the unknown mechanism of histamine-mediated inhibition of sweating, the localization of

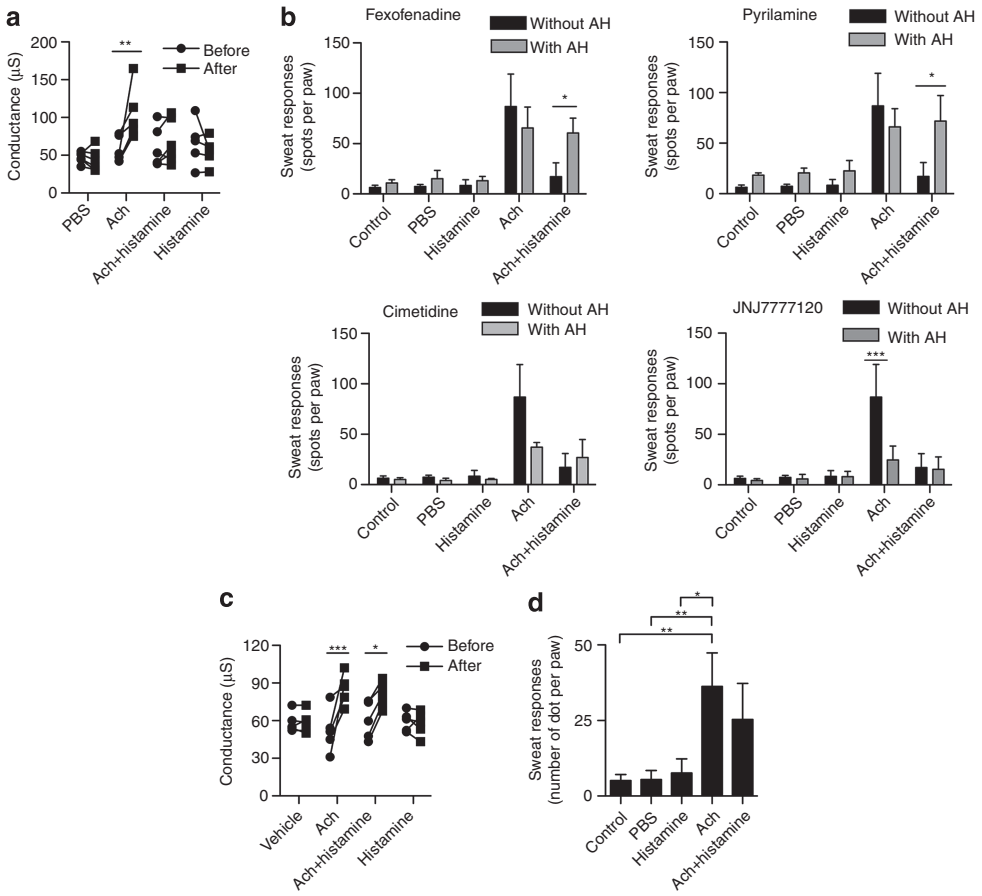


Figure 2. The effects of histamine (His) on sudomotor function. (**a**) The degree of stratum corneum hydration was assessed with conductance measurements before (black circles) and after (black square) acetylcholine (Ach) administration with or without His. $^{**}P<0.01$, two-way analysis of variance (ANOVA), Bonferroni's multiple comparison test ($n=5$–6). (**b**) The result of counting the number of iodine–starch reaction–positive sweat pores with or without administration of various antihistamines. Antagonists for H1R, H2R, and H4R were fexofenadine or pyrilamine, cimetidine, and JNJ7777120, respectively. AH, antihistamine. $^{***}P<0.001$, $^{*}P<0.05$, two-way ANOVA, Bonferroni's multiple comparison test ($n=5$–7). (**c**) The degree of stratum corneum hydration in H1R-KO mice was assessed with conductance measurements before (black circles) and after (black square) Ach with or without His. $^{***}P<0.001$, $^{*}P<0.05$, two-way ANOVA, Bonferroni's multiple comparison test ($n=5$). (**d**) The results of counting the number of iodine–starch reaction–positive sweat pores in H1R-KO mice. $^{**}P<0.01$, $^{*}P<0.05$, one-way ANOVA, Bonferroni's multiple comparison test ($n=5$–6).

histamine or ACh receptors in the microenvironment of the sweat gland was determined by immunohistochemical staining (Figure 3 and Table 1). The muscarinic 3 receptor (M3R) was chosen as the ACh receptor, because ACh receptors in mouse eccrine sweat glands are predominantly M3R (Vilches *et al*, 1995). As a result, sweat glands expressed H1, H2, H4 and M3 receptors. Protein gene product-9.5-positive peripheral nerve fibers were co-stained with antibody against the H1, H2, and M3 receptors, but myoepithelial cells did not stain with antibodies against the H1, H2, H4, and M3 receptors. These results suggest that there might be cross talk between two distinct pathways downstream of histamine and ACh receptors in sweat glands and nerve fibers.

Histamine blocks phosphorylation of glycogen synthase kinase 3β activated by ACh stimulation

A phosphorylation antibody array using whole-skin lysates was used to examine the extent of cross talk between the histamine and ACh signaling pathways (Figure 4a). We found two candidate signal transducers, including glycogen synthase kinase 3α/β (GSK3α/β) and AMP-activated protein kinase α1, which were differentially phosphorylated by ACh and histamine treatment (Figure 4a). GSK3α/β and AMP-activated protein kinase α1 were phosphorylated by ACh alone, but not by ACh plus histamine or histamine alone. Among these molecules, we focused on GSK3β, which is thought to be involved in cellular membrane transport and trafficking (Adachi *et al*, 2009, Rexhepaj *et al*, 2010). Such phosphorylation dynamics of GSK3β were also observed in western blotting analysis (Figure 4b). Immunohistochemical staining of phosphorylated GSK3β was performed to confirm whether this cross talk occurred in eccrine sweat glands. As a result, phosphorylation of GSK3β was observed in sweat glands of ACh-treated skin, but not in ACh- and histamine-treated skin (Figure 4c).

Histamine reduces the frequency of sweat secretion

As GSK3β is inactivated by phosphorylation, the adiaphoretic effect of histamine was assumed to be due to decreased

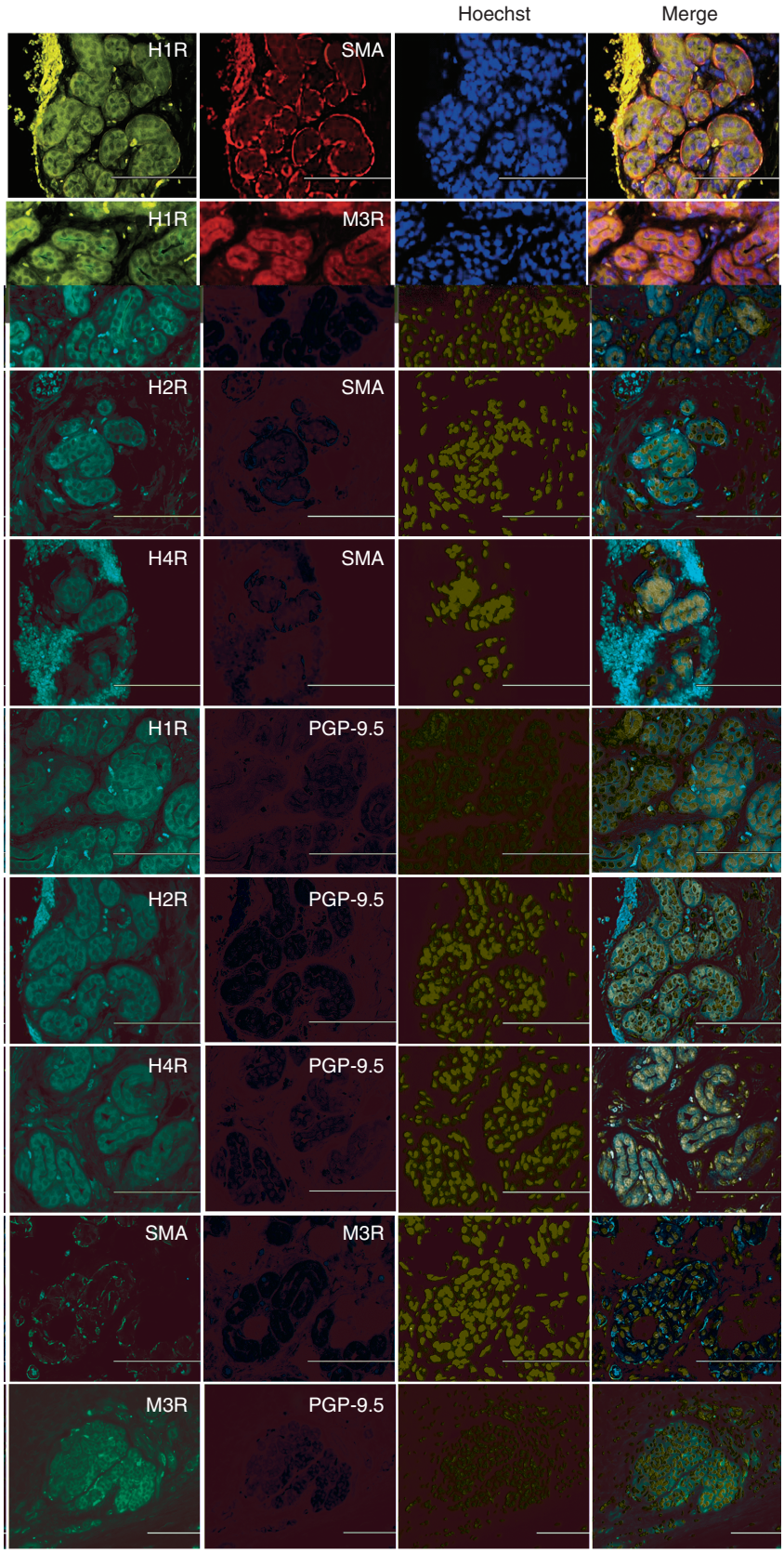


Figure 3. Localization of H1, H2, H4, and M3 receptors in the sweat gland microenvironment. Skin specimens derived from mice hindpaws were immunohistochemically stained with antibodies against histamine receptors, SMA, protein gene product-9.5 (PGP-9.5), and M3 receptor. Bar = 100 μm.

secretion of sweat by activation of GSK3β. To confirm this hypothesis, the dynamic imaging of sweat glands in mouse hindpaws was observed using two-photon microscopy. In this attempt, three-dimensional images of sweat glands and their configuration were obtained. After 15 minutes of intraperitoneal ACh administration, the sweat glands became distended and contracted, followed by the appearance of optically less-transparent areas, seen as 'dark' spots (Figure 5a and b and Supplementary Figures 2A, B and 3A online). Although we could not definitely identify the entity of the opaque spots, we reasonably assumed that these spots are derived from the change in local pH, that is, acidification due to sweating (fluorescent intensity of fluorescein is known to be critically dependent on the pH of the environment (Ohkuma and Poole, 1978)).

In contrast to ACh administration, simultaneous intraperitoneal administration of histamine decreased the number of opaque spots (Figure 5a–c and Supplementary Figures 2C and 3B online).

Table 1. Localization of H1R, H2R, H4R, and M3R on eccrine sweat gland

	H1R	H2R	H4R	M3R
Sweat gland	+	+	+	+
Myoepithelial cell	−	−	−	−
Nerve fiber	+	+	−	+

Abbreviations: H1, 2, and 4R, type 1, 2, and 4 histamine receptor, respectively; M3R, muscarinic 3 receptor.

Histamine decreases the ACh-induced, axon reflex–mediated sweating volume in humans

The inhibitory effects of histamine on ACh-mediated sweating in humans were assessed by quantitative sudomotor axon reflex test (QSART) (Figure 6). When histamine was iontophoretically applied with ACh simultaneously, QSART-measured results were significantly decreased compared with results from stimulation solely with ACh.

DISCUSSION

The present study has partly revealed the mechanisms of anhidrosis in AD using multiple assessments, showing that the inhibitory effect of histamine on Ach-induced sweating occurred via phosphorylation (inactivation) of GSK3β downstream of the H1R.

First, it was surprising that histamine has effects other than induction of allergic inflammation. It has been reported that histamine affects many kinds of cells, which express histamine

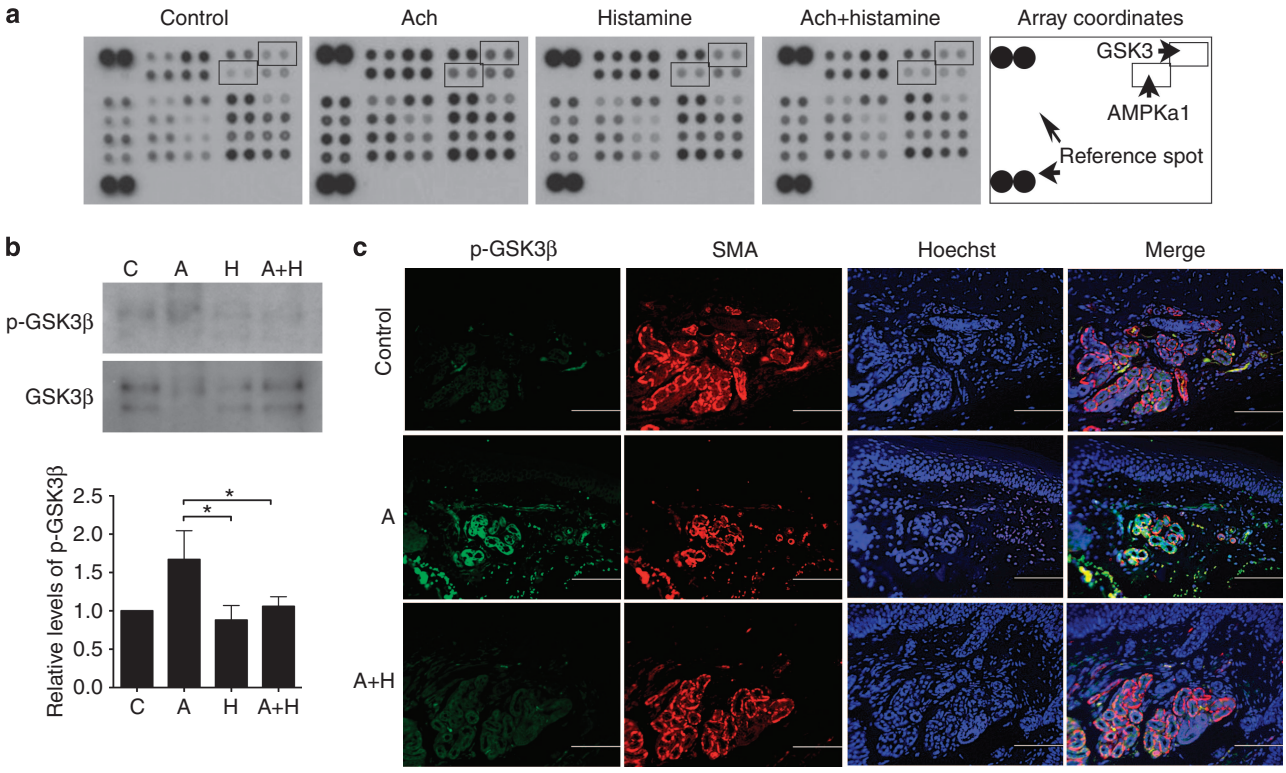


Figure 4. Cross talk between acetylcholine (Ach)- and histamine-mediated signal transduction. (**a**) Phosphorylation profiles of kinases and protein substrates in Ach- or His-treated skin were analyzed with a phosphokinase antibody array. (**b**) Western blot analysis for phosphorylated GSK3β (p-GSK3β) and GSK3β. The graph shows the relative levels of p-GSK3β (mean ± SD). Signal intensity was determined by densitometry. Relative phosphorylation was calculated and normalized against total amounts of GSK3β and quantified against the signal intensity of the control group. *$P<0.05$, two-way analysis of variance, Bonferroni's multiple comparison test ($n=3$). A, acetylcholine; A + H, acetylcholine + histamine; C, control; H, histamine. (**c**) Skin specimens derived from hindpaws stimulated with ACh with or without histamine were immunohistochemically stained with anti-p-GSK3β and anti-SMA antibodies. Bar = 100 μm.

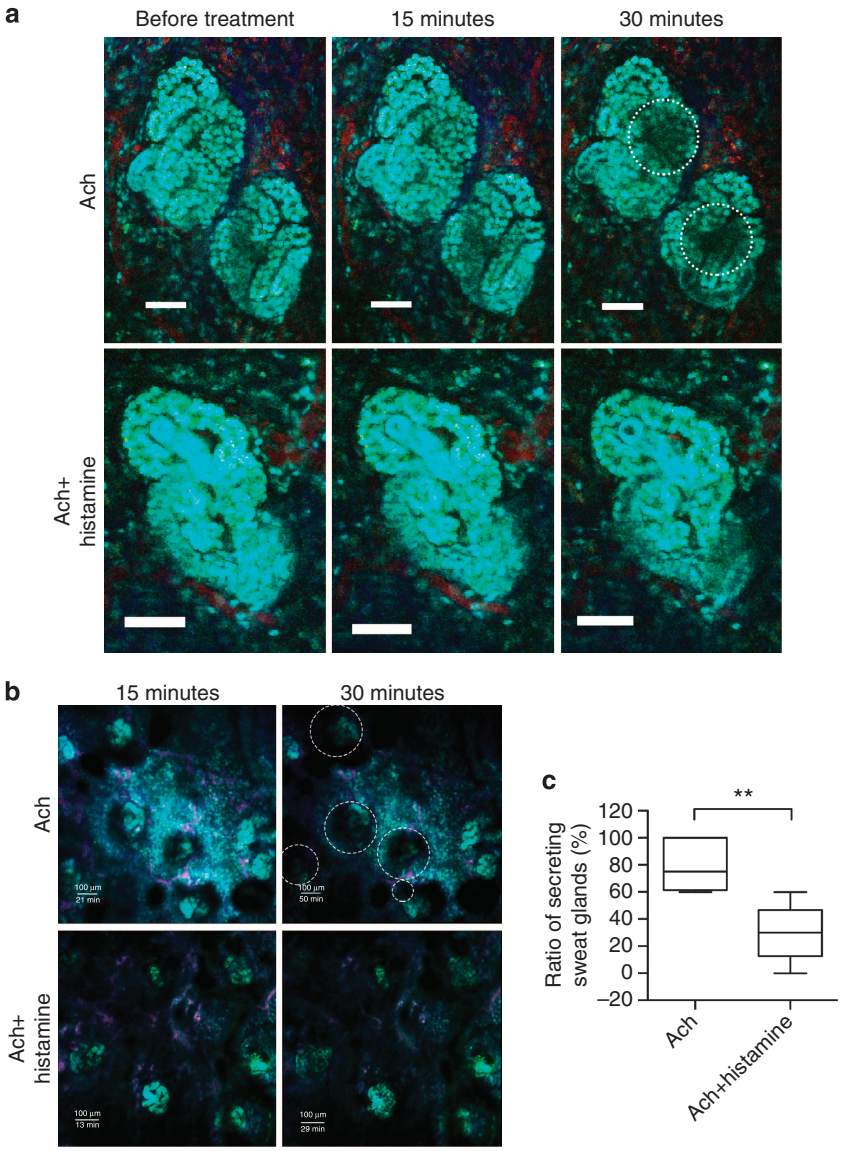


Figure 5. Dynamic imaging of sweat glands by two-photon microscopic analysis. (**a**) Acetylcholine (ACh) or ACh and histamine (ACh + histamine) were intraperitoneally injected. Subsequently, the motion of the sweat glands was recorded for 30 minutes. Photos before treatment, 15 minutes, and 30 minutes after observation are presented. Circles with a dotted line show the opaque spots that possibly suggest sweating. Bar = 50 μm. (**b**) The number of low-density spots per field per 30 minutes was counted. Bar = 500 μm. Circles with a dotted line enclose the low-density spots. (**c**) The ratio of the number of sweat glands associated with or without low-density spots is presented. $^{**}P<0.01$, unpaired *t*-test ($n=5$).

receptors. Previous studies also revealed that histamine is involved in (1) waking mechanisms (Lin *et al*, 1988), cognitive functioning (Gengo and Manning, 1990), and regulating the level of food intake by affecting neuronal activity (Masaki *et al*, 2004); (2) innate immune responses (Ertam *et al*, 2007; Metz *et al*, 2011); and (3) induction of chemokines, cytokines, and adhesion molecules on various types of cells (Fujikura *et al*, 2001; Giustizieri *et al*, 2004; Murota and Katayama, 2011). However, as far as we know, the effects of histamine on the sweating response have not been reported.

Recent reports shed light on the possible involvement of decreased sweating in the pathogenesis of AD (Eishi *et al*, 2002; Shiohara *et al*, 2011; Kijima *et al*, 2012; Sugawara *et al*, 2012), but the mechanisms of hypohidrosis in AD remain obscure. The pathogenic involvement of histamine in AD has been discussed in past articles, as histamine was thought to contribute to early events in AD (Akdis *et al*, 2006; Vanbervliet *et al*, 2011). The results of our study suggest that histamine might also impair skin homeostasis via decreasing sweating volume. Decreased sweating is also found in patients with urticaria, especially the cholinergic type (Itakura *et al*, 2000; Kobayashi *et al*, 2002). Thus, it might be said that sweating dysfunction may contribute to histamine-related skin disorders.

Furthermore, we found that H1Rs had a crucial role in histamine-mediated inhibition of ACh-induced sweating. Administration of H1R antagonists in wild-type mice attenuated the inhibitory effects of histamine on ACh-induced

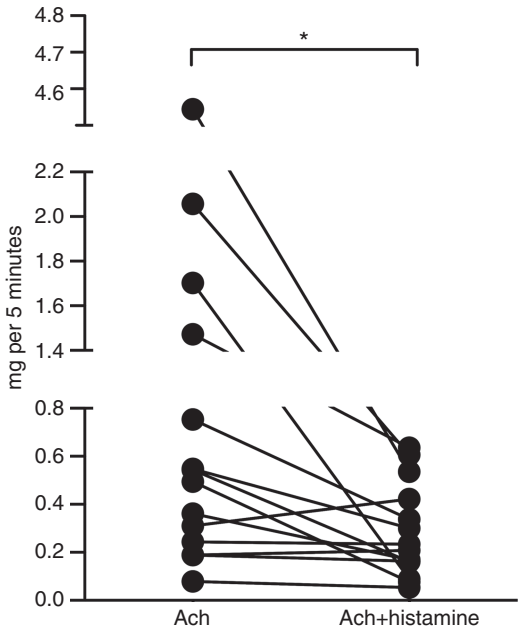


Figure 6. Histamine reduced axon reflex–mediated sweating volume in humans. The effect of simultaneous administration of histamine on quantitative sudomotor axon reflex test was assessed in the same individual ($n=13$). *$P<0.05$, paired *t*-test. Ach, acetylcholine.

sweating; similarly, H1R-KO mice showed diminished inhibition by histamine. As H1Rs are expressed in secretory sweat glands and nerve fibers, histamine might affect both tissues. These results indicate that H1R antagonists might have potential therapeutic effects in allergic patients with anhidrosis. Unexpected results were obtained from the administration of cimetidine and JN7777120; we found that these compounds decreased ACh-induced sweating but did not increase the low numbers of sweat pores positive for the iodine–starch reaction. These results suggest that an H2R or H4R antagonist might hold promise for treating hyperhidrosis. However, at present, we cannot explain the mechanisms of such phenomena; thus, further investigation is needed to clarify this issue.

We investigated the cross talk between the signaling pathways downstream of histamine and ACh, and found potential signal transducers that were differently activated by histamine or ACh. GSK3β is a Ser/Thr kinase with pleiotropic functions, including glycogen metabolism, regulation of the cell cycle, cell survival and differentiation, and inflammation (Doble and Woodgett, 2003; Jope and Johnson, 2004; Wang *et al*, 2011); it is phosphorylated in ACh-stimulated sweat glands. As GSK3β is also known to participate in the regulation of both cellular membrane transport and trafficking (Adachi *et al*, 2009; Rexhepaj *et al*, 2010), it is possible that GSK3β could be involved in sweat secretion (Supplementary Figure 4 online). When necessary, ACh causes the inactivation (phosphorylation) of GSK3β, stimulating sweat secretion. Histamine might inhibit ACh-induced sweat secretion by leading to the dephosphorylation (activation) of GSK3β. Previous reports about the function of GSK3β in glomerular filtration provide collateral evidence for this hypothesis. Mice with aberrantly activated GSK3β (gsk^{KI}) develop increased body temperature, food and water intake, glomerular filtration rate, urinary flow rate, urine osmolarity, urinary Na+ and K+, urea excretion, and proteinuria (Boini *et al*, 2008; Boini *et al*, 2009). Thus, it is easy to assume that activation of GSK3β might be involved in sweat secretion and glomerular filtration. This idea is supported by the dynamic images of sweat glands with two-photon microscopic analysis. To our knowledge, the real-time motion of sweat glands was previously unreported. The present study also revealed the inhibitory effects of histamine on GSK activation. As certain antihistamines affect glomerular filtration and renal function (Chansel *et al*, 1982; Abboud, 1983; Radke *et al*, 1985), histamine might affect the properties of secretory glands throughout the body.

AMP-activated protein kinase α1, a key regulator of intracellular fatty acid metabolism, is activated by increasing intracellular AMP levels (Liu *et al*, 2006); it is also inactivated by histamine administration. Unfortunately, although we could not confirm the function of AMP-activated protein kinase α1 in sweat glands, there is still the possibility that AMP-activated protein kinase α1 is involved in ACh-induced cAMP accumulation in sweat glands (Sato and Sato, 1983; 1987). Such attractive result should be confirmed elsewhere.

Dysfunctional sweating responses are not only problems for allergic dermatoses but also big problems for many types of diseases, including idiopathic anhidrosis or hyperhidrosis. Although these diseases strongly impair patients' quality-of-life, as well as activities of daily living, it should be said that there are no promising therapeutic methods for these diseases. We believe that this report may provide the basis of understanding the mechanisms involved and contribute to formulating therapeutic strategies for diseases involving sudomotor dysfunction.

MATERIALS AND METHODS

Animals

Ten- to twelve-week-old female C57BL/6J (C57BL/6) mice (10–12 weeks of age, weighing 20–30 g) were purchased from Clea (Osaka, Japan). H1R-KO (Hrh1 KO) mice were purchased from Oriental Bio Service (Kyoto, Japan). Mice were maintained in our pathogen-free animal facility. All animal care was in accordance with the institutional guidelines of Osaka University.

Preparation of reagents

ACh (pilocarpine hydrochloride; Junsei Chemical, Tokyo, Japan) and histamine (histamine diphosphate; Wako Pure Chemical Industries, Osaka, Japan) were dissolved in phosphate-buffered saline on the day of the experiment. Iodine (Sigma-Aldrich) was dissolved in ethanol (5% w/v) and starch (Wako Pure Chemical Industries) was diluted in mineral oil (nacalai tesque) (0.5% w/v).

In vivo sweat response

ACh-induced sweating was measured by both counting the spots in the starch–iodine reaction and measuring skin conductance to evaluate the number of active sweat glands and sweat volume, respectively.

The starch–iodine reaction was carried out using a slightly modified method from a previous report (Nejsum *et al*, 2002). Mice were anesthetized with intraperitoneal injection of a mixture of three drugs, 0.3 mg kg^{-1} domitor, 5 mg kg^{-1} vetorphale, and 4 mg kg^{-1} dormicum. Mouse hindpaws were painted with 3.5% iodine in

ethanol and 10% starch solution in mineral oil. Subsequently, subcutaneous injection with 20 μl phosphate-buffered saline (vehicle), 50 μg/20 μl ACh, and 120 μg/20 μl histamine was performed. The control group was the nontreated group. Each group consisted of 3–6 mice. Two minutes after injection, the treated hindpaws were photographed, and black spots caused by the amylase reaction (i.e., iodine and starch response) were counted.

Optical coherence tomography

Because there is a large refractive index difference on the wall of the spiral lumen between the sweat and keratinous epidermal tissues, the eccrine sweat ducts in the stratum corneum of the epidermis are clearly recognized by a spiral lumen with a high reflective light intensity. The dynamic OCT images of Ach-induced sweating in the *x–z* plane were obtained from B-mode OCT. The administration methods for vehicle, ACh, and histamine were the same as in the starch–iodine reaction test.

Two-photon excitation fluorescence microscopy

Intravital multiphoton imaging of sweat glands in the skin was performed using previously described protocols (Ishii *et al*, 2009) with modifications. Mice were anesthetized with isoflurane (Escain; 2.0% vaporized in 100% oxygen). Skin was observed with an inverted multiphoton microscope (A1R-MP; Nikon, Tokyo, Japan) driven by a Ti:Sapphire laser (Chameleon Vision II; Coherent, Santa Clara, CA) tuned to 800 nm and an inverted microscope equipped with multi-immersion objectives (CFI-Plan-Fluor, ×20/N.A. 0.75; Nikon). Sweat glands were visualized by anti-M3 antibody staining, and blood vessels were visualized using Texas Red-labeled 70-kDa dextran. To detect second harmonic-generated (ch1), FITC (ch2), Red (ch3), and Far Red (ch4) emission signals, 492 nm short pass, 500/50 nm, 562/25 nm, and 601/56 nm bandpass filters were used, respectively. Raw imaging data were processed with Volocity three-dimensional image analysis software (PerkinElmer, Waltham, MA). Sweat glands, microvessels, and nuclei were stained with FITC-conjugated anti-gross cystic disease fluid protein antibody or anti-aquaporin5 antibody, Texas Red-conjugated 70-kDa dextran, and Hoechst 33342, respectively.

Phosphorylation antibody array

Skin samples of mice hindpaws were obtained immediately after subcutaneous injection of Ach with or without histamine, or vehicle. Samples were lysed in buffer containing phosphatase inhibitor. The Phospho-Kinase-Array kit (proteome profiler antibody array; R&D Systems, Minneapolis, MN) was used according to the manufacturer's instructions.

Histamine antagonism

For histamine antagonism, 15 minutes before subcutaneous injection of ACh or histamine, mice were injected intraperitoneally with pyrilamine (700 μg per body), fexofenadine hydrochloride (600 μg per body), and cimetidine (400 μg per body).

Quantitative sudomotor axon reflex test

This study was approved by the Institutional Review Board of Osaka University hospital (ID: UMIN000009247). Healthy volunteers were enrolled in this study after obtaining a written informed consent and providing written and oral information to the study physicians. This study was performed in adherence with the guidelines of the Declaration of Helsinki.

An examination was performed on the basis of the method established by Lee *et al* (2009) (Kijima *et al*, 2012). Briefly, the subjects were asked to remain quiet for 60 minutes before undergoing QSART in a hospital outpatient clinic at constant temperature (20 °C) and humidity (60%). The sweat volume–measuring capsule and digital analyzer of measured sweat volume (SKINOS SKD-2000, Nagano, Japan) were used in QSART. ACh (100 mg ml^{-1}) with or without histamine (0.1 ~ 1% w/v) was iontophoretically applied to the skin from the outer compartment, whereas the glands of the skin in the central compartment of the capsule were activated indirectly via an axon reflex sweating. The axon reflex sweating volume was measured during the 5 minutes of iontophoresis. Data for DIR sweating were obtained over the subsequent 5 minutes.

Histological analysis

Paraffin-embedded samples of hindpaw skin were cut to 4-μm thick sections. The antibodies used were rabbit anti-M3 (Abcam, Cambridge, UK), goat anti-H1R, -H2R, and -H4R (all from Santa Cruz Biotechnology, Santa Cruz, CA), anti-protein gene product-9.5 (Chemicon; AB1761), and anti-SMA (Santa Cruz Biotechnology) antibodies. Secondary antibodies used were anti-rabbit and anti-goat conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA), and anti-rabbit and mouse antibodies conjugated to Alexa Fluor 555 (Invitrogen). Images of immunolabeled sections were captured with a BZ-8000 microscope (Keyence, Osaka, Japan).

Western blot analysis

Skin samples were crushed in liquid nitrogen and solubilized at 4 °C in lysis buffer (0.5% sodium deoxycholate, 1% Nonidet P40, 0.1% sodium dodecyl sulfate, 100 μg ml^{-1} phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate, and protease inhibitor cocktail). The protein extracts (20 μg each) were analyzed with an anti-phospho-GSK3α/β antibody (no. 9331, Cell Signaling Technology, Danvers, MA). An anti-GSK3α/β antibody (no. 5676; Cell Signaling Technology) was used as a control. Protein bands were detected using the ECL Plus kit (GE Healthcare, Buckinghamshire, UK). GSK3β was detected as two bands around 45 kDa. The intensity of the bands was quantified by using NIH image J software.

Statistic analysis

Prism5 software (GraphPad Software, La Jolla, CA) was used for statistical analysis. Statistical significance was examined by unpaired *t*-test or Bonferroni's multiple comparison test. Graph bars represent the mean ± SD. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, and $^{****}P<0.0001$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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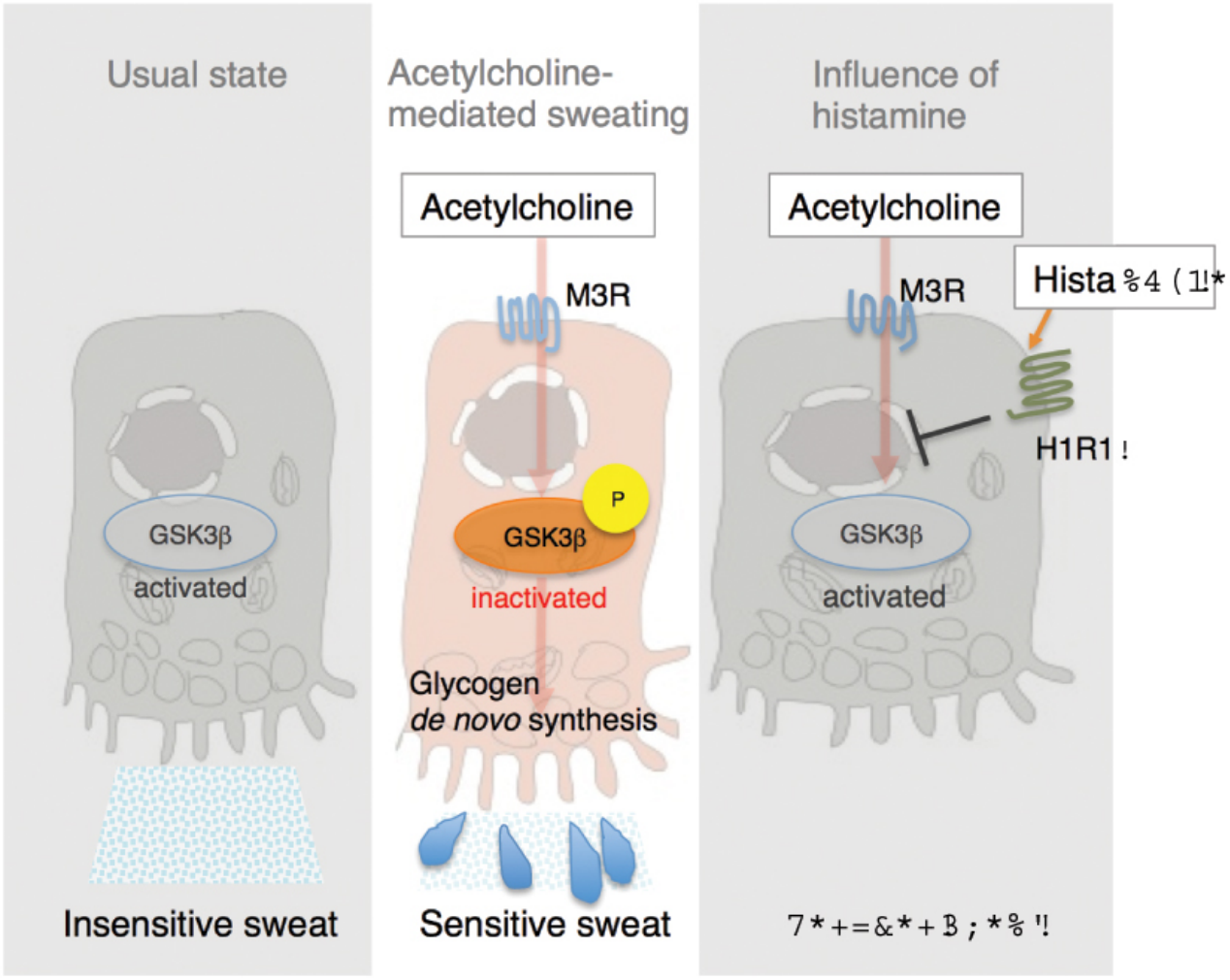
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Usual state
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Supplemental Figure Legends

Figure 1. In vivo imaging of sweating from the skin surface of the hindpaw. A. Before ACh stimulation. B. After ACh stimulation. C. After ACh and histamine stimulation.

Figure 2. Dynamic image (high magnification) of a sweat gland after i.p. injection of ACh. **A., and B.** Stained with anti GCDFP antibody and anti-AQP5 antibody, respectively (green). **C.** Dynamic image (high magnification) of a sweat gland after i.p. injection of ACh and histamine. Bar indicates 50 μm. Green (FITC): GCDFP or AQP5 (sweat gland). Red (Texas Red): dextran (microvessels).

Figure 3. A. Dynamic image (low magnification) of a sweat gland after i.p. injection of ACh. **B.** Dynamic image (low magnification) of a sweat gland after i.p. injection of ACh and histamine. Bar indicates 100 μm.

Figure 4. Schema of the hypothetical function of GSK3β in sweat gland cells. M3R: muscarinic receptor. H1R: histamine type-1 receptor. P: phosphorylated.