Functional Vanilloid Receptor-1 in Human Submandibular Glands

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ABSTRACT

Vanilloid receptor-1 (VR1) was originally found in the nervous system. Recent evidence indicates that VR1 is also expressed in various cell types. We hypothesized that VR1 exists in the human submandibular gland (SMG) and is involved in regulating salivary secretion. VR1 mRNA and protein were expressed in human SMGs and a human salivary intercalated duct cell line. VR1 was mainly located in serous acinar and ductal cells, but not in mucous acinar cells. Capsaicin, an agonist of VR1, increased intracellular free calcium, enhanced phosphorylation of extracellular signal-regulated kinase, and induced the trafficking of aquaporin 5 (AQP5) from the cytoplasm to the plasma membrane. These effects were abolished by pre-treatment with the VR1 antagonist capsazepine. Furthermore, capsaicin cream applied to the skin covering the submandibular area increased salivary secretion. These findings indicated that a functional VR1 is expressed in the human SMG and is involved in regulating salivary secretion by mediating AQP5 trafficking.

KEY WORDS: capsaicin, submandibular gland, vanilloid receptor-1.

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INTRODUCTION

Vanilloid receptor-1 (VR1) is a ligand-gated non-selective cation channel that can be activated by heat and acid, as well as by capsaicin (Caterina *et al.*, 1997; Tominaga *et al*., 1998). Although VR1 was previously found only in neural cells, recent evidence suggests that a functional VR1 is expressed in various types of cells, including bladder and bronchial epithelial cells (Veronesi *et al.,* 1999; Birder *et al.*, 2001), keratinocytes (Inoue *et al.*, 2002), dental pulp (Miyamoto *et al.*, 2005), mononuclear cells (Saunders *et al.*, 2007), and synovial fibroblasts (Engler *et al.*, 2007). Activation of the peripheral VR1 by capsaicin increases the level of intracellular free calcium ($[Ca^{2+}]_i$) and modulates diverse physiological functions other than nociceptive transduction (Birder *et al*., 2002; Zhu *et al.*, 2007; Taylor *et al.*, 2008).

The regulation of salivary gland secretion is a complex process. Fluid and electrolyte secretion is primarily evoked by the action of acetylcholine on muscarinic cholinergic receptors and norepinephrine on α-adrenoceptors, whereas protein secretion is mainly evoked by adrenergic agonists acting through β-adrenoceptors (Melvin *et al.*, 2005). Moreover, secretion of saliva is regulated by various peptides through the related receptors under physiological conditions (Ekström, 1999).

VR1 is expressed in the rat tongue and palate, and capsaicin application to the apex of the tongue or the palatinal mucosa increases salivation (Dunér-Engström *et al.*, 1986). We found that VR1 was expressed in the rabbit submandibular gland (SMG), and that capsaicin promoted salivary secretion from the SMG (Zhang *et al*., 2006). On the basis of these findings, we hypothesized that activation of VR1 may be a novel pathway in regulating the salivation of SMGs. The present study characterized the expression and distribution of VR1 in human SMGs. Moreover, we investigated the functions of VR1 in regulating intracellular signal molecules and saliva secretion.

MATERIALS & METHODS

Reagents and Antibodies

Capsaicin and capsazepine were purchased from Sigma (St. Louis, MO, USA). Capsaicin cream (0.075%) was purchased from Medicis Pharmaceutical Corp. (Phoenix, AZ, USA). Antibodies to VR1, extracellular signal-regulated kinase (ERK 1/2), phosphorylated-ERK (p-ERK), actin, and aquaporin 5 (AQP5) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Fluo-3/AM was purchased from Biotium (Hayward, CA, USA).

EMLA (containing 2.5% each of lidocaine/prilocaine) was purchased from AstraZeneca (Shanghai, China).

Human Participants and Ethics

We collected saliva from 30 female and 30 male healthy volunteers (mean age: 41 yrs). Participants with a history of oral and maxillofacial diseases or systemic disease were excluded. We collected SMG samples from 11 individuals (ages 22-73 yrs; seven males) undergoing functional neck dissection for primary oral squamous cell carcinoma without irradiation and chemotherapy. The research protocol was approved by the Peking University Institutional Review Board, and all participants signed an informed consent document for sample collection. All glandular tissues used were confirmed to be histologically normal.

SMG Tissue and Cell Preparation

The SMG samples obtained from surgery were transported to the laboratory within 30 min in 4°C Krebs-Ringer Hepes (KRH) solution (containing, in mmol/L, 120 NaCl, 5.4 KCl, 1 $CaCl_2$, 0.8 $MgCl_2$, 11.1 glucose, 20 Hepes, pH 7.4), aerated with 95% O_2, and then minced into small pieces (0.5 mm^3). For cell preparation, SMG tissue was minced in Ca^{2+}-free KRH solution on ice, then digested with 100 units/mL of collagenase (Worthington, Lakewood, UK) and 1% BSA for 60 min as reported previously (J Li *et al.,* 2006).

RT-PCR

Total RNA from SMGs was purified with the use of Trizol (Invitrogen, Carlsbad, CA, USA). VR1 amplification from cDNA involved 1 cycle at 94°C for 5 min, then 30 cycles of 94°C for 30 sec, 59°C for 30 sec, and 72°C for 30 sec. The primers were based on the cDNA sequence of human VR1 (GenBank Accession No. NM080706.2, 2875-3019). The sense and antisense primers for VR1 and actin (used as an internal control) were 5′-TTTCAGGCAGACACTGGAAGA-3′ and 5′-TTGAAGACCTCAGCGTCCTCT-3′; 5′-TCCTCCCTGGAGAAGAGCTA-3′ and 5′-TCAGGAGGAGCAATGATCTTG-3′, respectively. The amplification products were visualized on 1.5% agarose gel with ethidium bromide.

Immunoblotting

The tissues or cultured cells were homogenized in lysis buffer as described previously (YM Li *et al*., 2006). The homogenate was centrifuged at 1000 *g* for 10 min at 4°C, and the supernatant was collected. The concentration of protein was measured by the Bradford method. Equal amounts of protein (80 μg for VR1 expression and 40 μg for ERK-phosphorylation) were separated by 9% SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% non-fat milk, probed with primary antibodies (anti-VR1 antibody, 1:500 diluted, anti-ERK/p-ERK antibody, 1:2000 diluted), then incubated with horseradish-peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized by enhanced chemiluminescence reagent (Pierce Biotechnology, Rockford, IL, USA).

Immunofluorescence

Frozen sections (10 μm) of SMGs and fixed pcDNA-AQP5-transfected HSG cell line cells were incubated at 4°C overnight with primary antibody (1:200), then secondary antibody (1:500) for 2 hrs at 37°C. Nuclei were stained with 4,6-diamidino-2-phenylindole. Fluorescence images were captured by confocal microscopy (Leica TCS SP2, Wetzlar, Germany). Normal goat IgG was used as a negative control.

Measurement of $[Ca^{2+}]_i$

Cells were loaded with the Ca^{2+}-sensitive fluorescent probe fluo-3/AM (4 μmol/L) in KRH solution for 30 min at 37°C and stimulated with capsaicin. The change in $[Ca^{2+}]_i$ level was recorded by confocal microscopy. Excitation was performed at 488 nm, and the emission signals were collected through a 515-nm barrier filter. Images were taken every 5 sec and used for quantitative measurement (Leica TCS NT, Wetzlar, Germany).

Plasmid Construction

The human AQP5 cDNA (Proteintech, Chicago, IL, USA) was inserted at the *Hind* III (upper) and *Bam*H I (lower) sites of pcDNA 4.0 (Invitrogen) with the synthesized primers 5′-CCCAAGCTTGGGATGAAGAAGG-3′ and 5′-GAGGATCCTCAGCGGGTGGT-3′ to yield the plasmid pcDNA-AQP5. The correct construction of cloning plasmids was confirmed by sequencing.

Human Salivary Intercalated Duct Cell Line (HSG) Culture and Transfection

HSG cell line cells were cultured in Dulbecco's modified Eagle's medium with 5% (v/v) fetal bovine serum, 100 U/mL penicillin, and streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Transfection of HSG cell line cells with pcDNA-AQP5 was performed with the use of LipofectAMINE (Invitrogen) according to the manufacturer's protocol. The transfected cells were selected in medium containing 400 μg/mL zeocin (Invitrogen).

Measurement of Salivary Secretion

Participants were instructed not to eat, drink, or smoke for 60 min before saliva collection at 9:00~11:00 a.m. Participants swallowed to clean the residual saliva in the oral cavity, then put a dry cotton roll at the parotid ductal papilla to absorb saliva from the parotid gland. Participants sat in a relaxed position with the head in a slightly forward-inclined position, allowing saliva to accumulate on the floor of the mouth. Capsaicin cream (0.075%) was applied to the skin covering the SMG. Saliva was continuously collected for 5 min before and at 10, 30, and 60 min after capsaicin stimulation by means of a micropipette connected to a suction device, as described previously (Fox *et al*., 1985). The collected saliva was transferred to an Eppendorf tube that was pre-weighed ($Weight_1$) and re-weighed after collection ($Weight_2$). Saliva flow rate (mg/min) = ($Weight_2$−$Weight_1$)/5 min.

Data Analysis

Data are expressed as mean ± SD. Statistical analysis involved one-way ANOVA, then *post hoc* tests for multiple-group comparison with the use of SPSS v11.5 (SPSS Inc., Chicago, IL, USA). A $P < 0.05$ was considered statistically significant.

RESULTS

Expression and Distribution of VR1 in the Human SMG

VR1 mRNA and protein were detected in SMG tissue, isolated SMG cells, and HSG cell line cells (Figs. 1A, 1B). VR1 protein was widespread in both acini and ducts in SMGs (Fig. 1D). In serous acini, VR1 was located in the cytoplasm and membrane of the cells, whereas in mixed acini, VR1 was mainly located in serous cells and was barely detected in mucous cells (Fig. 1C).

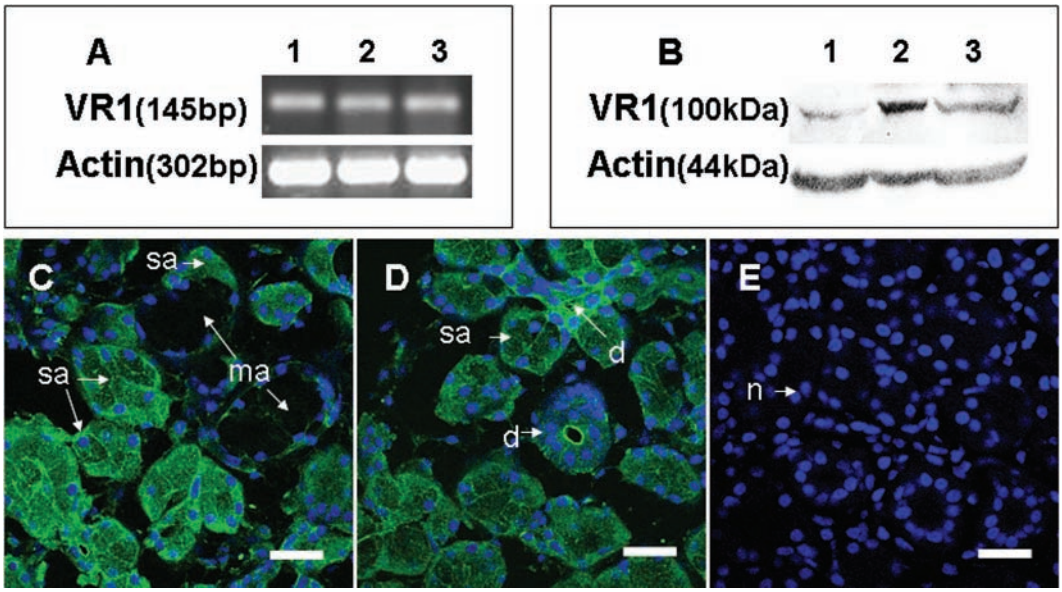

Figure 1. Expression of VR1 in the human submandibular gland (SMG). **(A)** Expression of VR1 mRNA was detected by RT-PCR, with β-actin as an internal control. **(B)** Expression of VR1 protein was detected by immunoblotting. Protein (80 μg) was separated on a 9% SDS-PAGE, and VR1 immunoreactivity was detected with polyclonal anti-VR1 antibody. Lane 1: SMG. Lane 2: Isolated primary cells from SMG. Lane 3: Human salivary intercalated duct cell line (HSG) cells. **(C, D)** Representative immunofluorescence images of VR1 in SMGs. SMG sections were immunostained with anti-VR1 antibody, then incubated with Alexa fluor-linked anti-goat IgG (green). Nuclei (blue) were labeled with 4,6-diamidino-2-phenylindole. **(E)** Normal goat IgG was used as a negative control. N, nucleus; sa, serous acinar cells; ma, mucous acinar cells; d, duct. Bar: 20 μm.

Effect of Capsaicin on $[Ca^{2+}]_i$ and ERK1/2 Phosphorylation

In isolated SMG cells, 1 μmol/L capsaicin did not change $[Ca^{2+}]_i$ levels. At 5 μmol/L, capsaicin caused a small increase, whereas at 10 μmol/L, it evoked a rapid and significant increase in $[Ca^{2+}]_i$ levels. Pre-treatment with capsazepine, a VR1 antagonist, abolished the capsaicin-induced elevation in levels of $[Ca^{2+}]_i$ (Fig. 2A).

Capsaicin significantly increased ERK phosphorylation in cultured SMG tissue (Fig. 2B). ERK was rapidly phosphorylated at 1 min, peaked in level at 5 min, and returned to the basal level at 30 min after capsaicin (1 μmol/L) treatment (Fig. 2C). The level of total ERK1/2 was not changed. Pre-treatment with capsazepine abolished the capsaicin-induced ERK1/2 phosphorylation (Fig. 2D). These results indicated that capsaicin increased $[Ca^{2+}]_i$ level and activated ERK1/2 through VR1.

Effect of Capsaicin on AQP5 Trafficking

AQP5 could not be detected in the original cells of the HSG cell line, but was widespread in the pcDNA-AQP5-transfected cells (Figs. 3A-3C). The level of AQP5 was increased in the membranes after stimulation with capsaicin (20 μmol/L) for 1, 5, and 10 min (Figs. 3D-3F), then was redistributed into the cytoplasm after 20 min (Fig. 3G). Pre-treatment with capsazepine abolished the capsaicin-induced AQP5 trafficking (Fig. 3H). Treatment with 1, 10, 20, and 50 μmol/L capsaicin for 5 min also confirmed that capsaicin induced AQP5 translocation from the cytoplasm to the membrane (Appendix). However, pre-treatment with the ERK upstream kinase inhibitor U0126 or PD98059 (Sigma, St. Louis, MO, USA) could not block the capsaicin-induced AQP5 trafficking (Appendix).

Effect of Capsaicin on Saliva Secretion from SMGs

In 60 participants tested for saliva flow, the flow rate was increased at 30 min after capsaicin cream was applied to the skin covering the SMG. The salivary flow rate was particularly increased in males and participants aged 20 to 40 yrs old (Table). The increased percentage in capsaicin-induced salivary secretion was similar between males and females, but was high in young participants (20-39 yrs). Since the dermal application of capsaicin may cause a burning sensation, we used the superficial anesthetic EMLA cream on the same area 60 min before capsaicin treatment in 10 participants (five females). EMLA relieved the burning sensation, but did not interfere with capsaicin-induced increase in salivation (data not shown).

DISCUSSION

In this study, we found VR1 mRNA and protein expressed in human SMGs. Capsaicin increased the level of $[Ca^{2+}]_i$, enhanced ERK1/2 phosphorylation, and induced the trafficking of AQP5 in a VR1-dependent manner. This suggested that the mechanism of capsaicin promoting saliva secretion *via* VR1 in human SMGs might involve AQP5 trafficking.

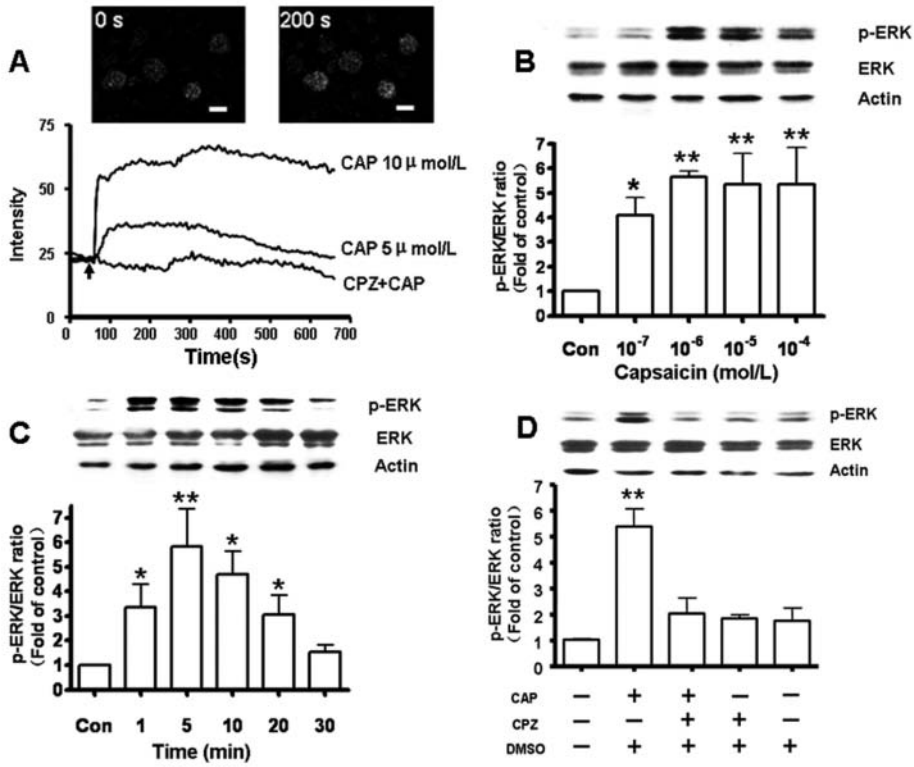

Figure 2. Effect of capsaicin on intracellular Ca^{2+} mobilization and ERK1/2 phosphorylation. **(A)** Fluorescence image of fluo-3AM-loaded cells before (0 sec) and after stimulation with capsaicin (10 μmol/L) for 200 sec. $[Ca^{2+}]_i$ dynamics in the individual cell with 5 and 10 μmol/L capsaicin stimulation. CPZ+CAP: pre-treatment with VR1 antagonist capsazepine (10 μmol/L) for 10 min, then stimulation with 10 μmol/L capsaicin. Arrow denotes the time of capsaicin application. Bar: 10 μm. **(B)** SMG tissues were incubated with different doses of capsaicin for 5 min. **(C)** SMG tissues were incubated with 1 μmol/L capsaicin for the indicated times. **(D)** SMG tissues were pre-incubated with or without 10 μmol/L capsazepine for 30 min, and then stimulated with 1 μmol/L capsaicin for 5 min. The protein (40 μg) extracts were separated by SDS-PAGE and immunoblotted with an antibody specific for p-ERK1/2 or total ERK1/2. Membranes were re-probed with antibody against actin to ensure equal loading. CAP, capsaicin; CPZ, capsazepine; DMSO as the vehicle control. Data are mean ± SD of 4 independent experiments. * $P < 0.05$ and ** $P < 0.01$ compared with control.

VR1 was originally demonstrated only in neural cells. However, non-neural expression of VR1 has recently become the focus of much attention. In the present study, the expressions of VR1 mRNA and protein were detected in human SMG. Possible interference of VR1 in connective and neural tissues could be excluded by the examination of VR1 expression in cells isolated from SMG and from the HSG cell line.

In rabbit SMGs, VR1 is mostly localized in the basolateral membrane of duct cells (Zhang *et al.*, 2006); however, in human SMGs, we found VR1 mainly in serous acinar cells and ducts, but not in mucous acinar cells. The difference in VR1 distribution may be due to the different species used: Approximately 80% of the intralobular tissue of adult rabbit SMGs consists of seromucous acini (Ahlner and Lind, 1993), whereas serous acinar cells are predominant in human SMG acini (Scott, 1979).

The function and intracellular pathways of VR1 activation in human SMGs are unknown. As a non-selective cation channel, capsaicin-induced VR1 activation causes a dose-dependent influx of Ca^{2+} in neurons (Wood *et al.*, 1988). In salivary acinar cells, an increase in $[Ca^{2+}]_i$ level is the primary signal for fluid secretion (Melvin *et al.*, 2005). Our results showed that capsaicin increased $[Ca^{2+}]_i$ levels in isolated SMG cells, but the increase was abolished by the VR1 antagonist capsazepine; thus, capsaicin could regulate intracellular calcium signaling *via* VR1 in human SMGs.

ERK plays a critical role in intracellular signal transduction. Capsaicin activating the ERK pathway has been demonstrated in dorsal root ganglia (Tang and Nakata, 2008) and in human hepatoma cells (Joung *et al.*, 2007). Capsaicin-induced proliferation mediated by the ERK pathway was reported in androgen-sensitive prostate cancer cells (Malagarie-Cazenave *et al.*, 2009). Furthermore, activation of ERK is involved in leptin- induced arachidonic acid release and prostaglandin E_2 generation in salivary gland acinar cells (Slomiany and Slomiany, 2008). However, ERK is not involved in isoproterenol-induced mucin secretion in rat sublingual glands (Slomiany and Slomiany, 2004). Our results showed that capsaicin induced ERK phosphorylation *via* VR1 in human SMGs.

AQP5 plays an important role in the rapid movement of water in salivary glands (Ma *et al.*, 1999). Activation of muscarinic receptors causes the trafficking of AQP5 in rat parotid tissues (Ishikawa *et al.,* 1998). We found that capsaicin induced AQP5 trafficking in AQP5-transfected HSG cell line cells in a time- and dose-dependent manner. Capsazepine, but not U0126 or PD98059 (ERK upstream kinase inhibitors), abolished capsaicin-induced AQP5 trafficking, which suggests that the effect was mediated *via* VR1, but not ERK. AQP5 trafficking induced by capsaicin might explain, at least in part, the secretory mechanism mediated by VR1. However, the downstream molecules of ERK in mediating the effect of capsaicin in SMGs need further investigation.

We previously demonstrated that arterial infusion of capsaicin increased salivary secretion in isolated rabbit SMGs (Zhang *et al.*, 2006). However, evaluating the direct effect of capsaicin on salivary secretion from human SMGs is difficult. Application of capsaicin to the tongue induced salivary secretion (Dunér-Engström *et al.*, 1986). We found that the dermal application of capsaicin promoted salivary secretion *in vivo,* and the effect elicited by capsaicin might depend on age and the intrinsic glandular function, but not on sex. Furthermore, a superficial anesthetic could not block the capsaicin-induced salivation increase, which

suggests that the effect of capsaicin was not mediated by a nociceptive response. Since lipophilic capsaicin can pass through cellular membranes and thus act on intracellular proteins (Jung *et al.*, 1999), our results suggest that the capsaicin-induced increase in salivation was initiated by the direct activation of VR1 in SMGs. However, capsaicin activating epidermal VR1 for the indirect stimulation of salivation could not be excluded.

In summary, we found a functional VR1 expressed in human SMGs. The effects of capsaicin on increases in $[Ca^{2+}]_i$ levels, ERK phosphorylation, AQP5 trafficking, and salivary secretion in SMGs were VR1-dependent. Activation of VR1 is a novel mechanism regulating the function of human SMGs, which may lead to a new strategy for therapy of salivary gland dysfunction.

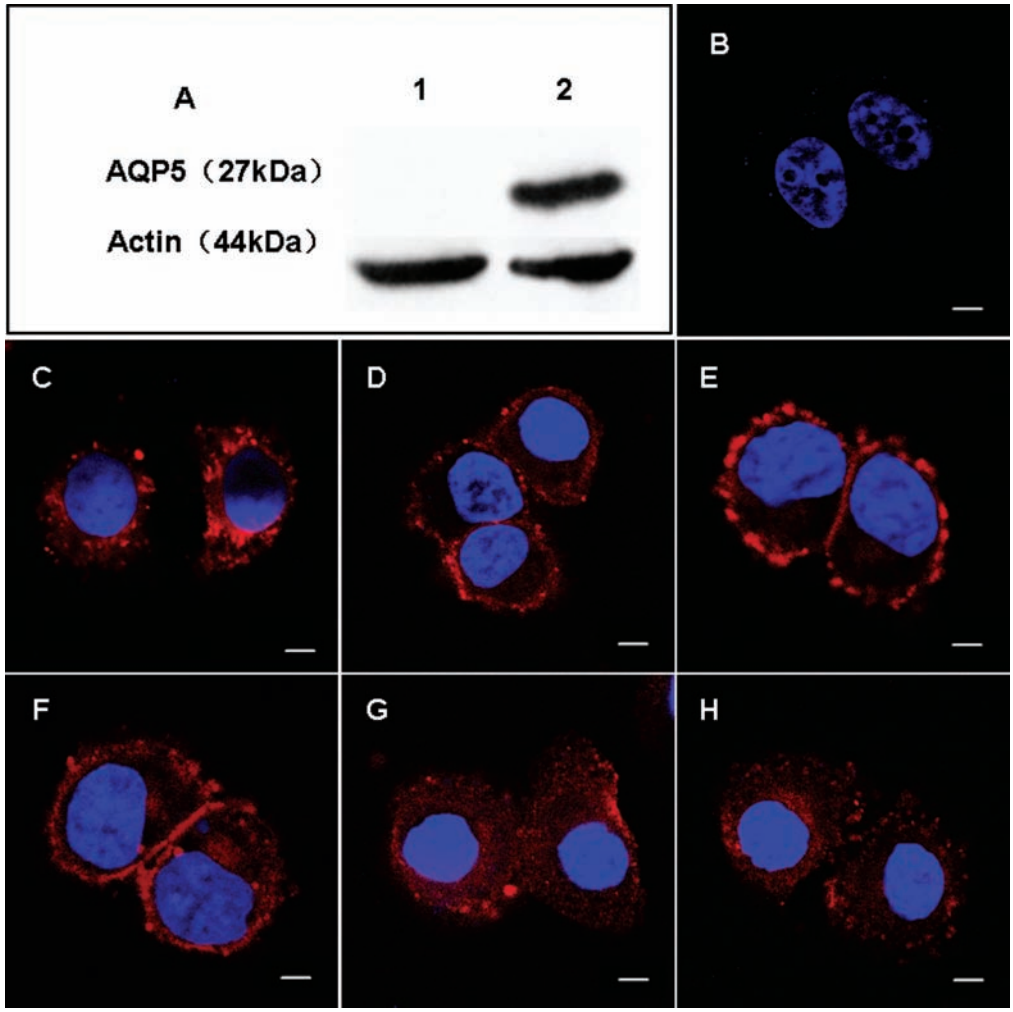

Figure 3. Effect of capsaicin on AQP5 trafficking in AQP5-transfected HSG cell line cells. **(A)** AQP5 expression in HSG cell line cells. Lane 1: HSG cell line cells. Lane 2: pcDNA-AQP5-transfected HSG cell line cells. **(B)** Representative immunofluorescence image of HSG cell line cells without AQP5 transfection. Nuclei (blue) were labeled with 4,6-diamidino-2-phenylindole. **(C)** Representative immunofluorescence image of AQP5 (red) in unstimulated AQP5-transfected HSG cell line cells. Capsaicin (20 μmol/L) treatment for 1 min **(D),** 5 min **(E),** 10 min **(F),** and 20 min **(G)** in pcDNA-AQP5-transfected HSG cell line cells. **(H)** Pre-treatment with 10 μmol/L capsazepine for 30 min, then stimulation with 20 μmol/L capsaicin for 5 min. Bar: 5 μm.

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Table. Effect of Capsaicin on Salivary Flow Rate in 60 Individuals

	Salivary Flow Rate (mg/min)					
		Sex		Age (yrs)		
Time (min)	All Participants (n = 60)	Female (n = 30)	Male (n = 30)	20-39 (n = 30)	40-60 (n = 15)	> 60 (n = 15)
0	170 ± 83	147 ± 79$^{\#}$	193 ± 82	211 ± 66††	167 ± 89†	91 ± 44
10	172 ± 88	146 ± 81$^{\#}$	197 ± 89	214 ± 70††	169 ± 93†	89 ± 52
30	196 ± 99*	171 ± 95*$^{\#}$	220 ± 99*	252 ± 83*††	181 ± 90*†	97 ± 49
60	170 ± 87	142 ± 82$^{\#}$	192 ± 87	213 ± 68††	164 ± 93†	88 ± 48

Data are mean ± SD. Capsaicin cream (0.075%) was applied to the skin of both sides of the submandibular area. Salivary secretion was collected for 5 min before and after capsaicin stimulation. *$P < 0.05$ compared with the baseline (0 min) for each column. $^{\#}P < 0.05$ compared with males. $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$ compared with participants > 60 yrs. Ratio of females to males in each age group: 15:15 (20-39 yrs), 7:8 (40-60 yrs), and 8:7 (> 60 yrs).

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