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Expression and characteristics of vanilloid receptor 1 in the rabbit submandibular gland ☆

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Abstract

Vanilloid receptor 1 (VR1) is a polymodal receptor originally found in sensory neurons of the central nervous system. Recent evidence indicates that VR1 is also expressed in non-neuronal tissues. We report here endogenous expression of VR1 in rabbit submandibular gland (SMG) and its possible role in regulating saliva secretion based on: (i) the expression of VR1 mRNA and protein detected in SMG; (ii) VR1 was mainly localized in the basolateral membrane of duct cells and the cytoplasm of acinar cells and also in cytoplasm of primary cultured neonatal rabbit SMG cells; (iii) stimulation of neonatal rabbit SMG cells with capsaicin induced a significant increase in intracellular calcium, and capsazepine, a VR1 antagonist, abolished this increase; (iv) infusion of capsaicin via the external carotid artery to isolated SMG increased saliva secretion of the gland. These findings indicated that VR1 was expressed in SMG and appeared to play an important role in regulating saliva secretion.

Keywords: Vanilloid receptor; Submandibular gland; Capsaicin; Saliva secretion; Calcium; Rabbit

Vanilloid receptor 1 (VR1) is a ligand-gated selective cation channel expressed in nociceptors. It can be activated by capsaicin (the main pungent ingredient in hot chilli peppers), heat (>43 °C), and low pH, followed by membrane depolarization and the opening of cation-selective ion channels [1,2]. Many reports suggest that VR1 participates in the regulation of acute pain and inflammation [3–7]. Significant Ca^{2+} entry induced by capsaicin resulted in cell injury and desensitization to other noxious stimuli in neurons, which makes it a promising therapeutic tool to mitigate neuropathic pain under pathological conditions [8]. Originally VR1 was found only in sensory neurons of the central nervous system. Recently, reports have shown the presence of VR1 in various non-neuronal cells, such as mast cells [9], skin epidermal keratinocytes [10], and urinary bladder epithelial cells [11]. Activation of these peripheral VR1 receptors by capsaicin induced intracellular Ca^{2+} ($[Ca^{2+}]_i$) increase, and may play a critical role in the regulation of several physiological functions other than nociceptive transduction.

The submandibular gland (SMG) is an exocrine organ which secretes saliva and macromolecules under the regulation of the autonomic nerve system. It has been proposed that the secretory function of the SMG is primarily regulated through the complex action of α-adrenoceptors,

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β-adrenoceptors, muscarinic-cholinergic receptors, and peptidergic receptors [12,13]. In addition, there are reports that the application of capsaicin to the tongue or palate reproducibly caused salivation [14], but up to date the presence and localization of VR1 in SMG have not been studied and the evidence for the involvement of VR1 in the physiological regulation of SMG secretion is also limited.

This study investigated the expression and characteristics of VR1 in the SMG. Data obtained from RT-PCR, Western blotting, and immunohistochemistry supported the presence of VR1 in the rabbit SMG. Furthermore, the possible role of VR1 in regulating the concentration of $[Ca^{2+}]_i$ was investigated and direct evidence that capsaicin infusion of the isolated rabbit SMG increased saliva secretion of the gland was obtained. These data therefore support the proposition that VR1 is functionally expressed in SMG.

Materials and methods

Reagents. Capsaicin and capsazepine purchased from Sigma–Aldrich (St. Louis, MO) were dissolved in dimethylsulfoxide (DMSO) to make a concentration of 50 mM and diluted to their final concentration with Krebs–Ringer Hepes (KRH) solution containing 120 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 0.8 mM $MgCl_2$, 11.1 mM glucose, and 20 mM Hepes (pH 7.4). Antibody to VR1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to actin was purchased from Oncogene (Cambridge, MA). The calcium-sensitive indicator fluo-3/AM was purchased from Biotium (Hayward, CA).

Reverse-transcriptase polymerase chain reaction (RT-PCR). Healthy New Zealand rabbits (weighing 2–2.5 kg) were used in this study. The animal experiments were approved by the Committee of Animal Research, Peking University Health Science Center and were in accordance with the Guidance of the Ministry of Public Health for the care and use of laboratory animals. Rabbits were anesthetized with pentobarbital sodium (50 mg/kg weight) and the SMG was rapidly removed and frozen in liquid nitrogen. Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA preparations were treated with DNase I to remove contamination by genomic DNA before RT-PCR. cDNA was prepared from 4 μg of total RNA with M-MLV reverse transcriptase (Promega, Madison, WI) and primed with oligo(dT). VR1 was amplified from 10 μl cDNA as follows: 1 cycle at 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s. The primers were based on the cDNA sequence of rabbit VR1[15] (GenBank Accession No. AY487342) with β-actin as an internal control. The primers for VR1 were sense (5′-ATGGGCGACCTGGAGT TCAC-3′) and antisense (5′-TTGATGATGCCCACGTTGGT-3′) corresponding to nucleotides 2154–2173 and 2484–2503, respectively. The primers for actin were sense (5′-ATCTGGCACCACACCTTCTACAA TGAGCTGGCG-3′) and antisense (5′-CGCACTACTCCTGCTTGCTG ATCCACATCTGC-3′). The amplification products were visualized on 1.5% agarose gel by use of ethidium bromide and sequenced to confirm identity.

Immunoblotting. The SMG tissues were homogenized with a polytron homogenizer in lysis buffer containing 150 mM NaCl, 50 mM Tris–HCl, pH 7.2, 1 mM ethylenediamine tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.1% SDS, and 0.1% sodium deoxycholate. The homogenate was centrifuged at 1000*g* at 4 °C for 10 min and the supernatant was collected. The concentration of proteins was measured by Lowry's method. Crude protein extracts (40 μg) were separated by 9% SDS–PAGE and transferred to polyvinylidene difluoride membrane. The membrane was blocked with 5% non-fat milk and then probed with anti-VR1 antibody followed with horseradish peroxidase-labeled secondary antibody, the signal developed with enhanced chemiluminescence (Amersham Biosciences Inc, Piscataway, NJ) and exposed to X-OMATTM films (Kodak, Rochester, New York). The density of the bands was scanned and quantified by LEICA550IW image analysis system.

Immunohistochemistry. SMGs were fixed with 10% formalin for 2 h at room temperature. Five micrometer sections of the glands were immunostained according to the ABC method [16]. Briefly, sections were incubated with VR1 antibody (1:200) for 2 h at 37 °C followed by biotinylated secondary antibody (1:200) for 30 min at 37 °C and avidin-biotin peroxidase was visualized by diaminobenzidine. Sections of rabbit dorsal root ganglion (DRG) were used as positive controls. Negative controls were incubated with goat IgG replacing the primary antibody or with antibodies that were pre-absorbed with the immunizing control peptide at 10^{-4}–10^{-6} M. In addition, frozen sections were cut and fixed for 10 min with prechilled acetone. The sections were incubated at 4 °C overnight with VR1 antibody (1:200) followed by FITC-labeled secondary antibody (1:300) for 1 h at 37 °C. Nuclei were stained with ethidium bromide. Fluorescence images were captured with a confocal microscope (Olympus Fluoview FV500, Japan).

Cell culture and identification. Primary cultures of rabbit SMG cells were prepared by enzymatic digestion according to the method [17] with minor modifications. Briefly, neonatal (1-day-old) rabbits were anesthetized with sodium pentobarbital (50 mg/kg weight). The SMGs were excised and dissected free of connective tissue, rinsed twice with ice-cold phosphate-buffered saline (PBS), cut into small pieces, and digested in medium containing 0.1% of pancreatin and 0.025% of collagenase for 20 min in a shaking water bath at 37 °C. The digestion was terminated and washed twice with Dulbecco's modified Eagle's media (DMEM) containing 5% fetal bovine serum (FBS) and centrifuged at 1000*g* for 5 min. The cells were then resuspended in DMEM containing 15% FBS and filtered through a single layer of nylon bolting cloth (150 mesh). The filtrate was cultured in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h. The cells were stained with cytokeratin (cytokeratins 17 and 20, Dako cytomation, Glostrup, Denmark) and confirmed of epithelial origin.

Immunocytochemistry. SMG cells were cultured on glass coverslips for 2 days. These cells were rinsed twice with PBS, fixed with 4% (w/v) paraformaldehyde in PBS for 10 min, permeabilized with 10% Triton X-100 for 10 min, washed with PBS, and then blocked with 5% horse serum for 1 h. The cells were incubated with anti-VR1 antibody diluted 1:200 for 2 h and washed for three times followed by incubating with FITC conjugated anti-goat IgG at 1:1000 dilution for 1 h. After washing with PBS for three times, the cells were mounted on glass slides and examined by a confocal microscope.

Measurement of $[Ca^{2+}]_i$. Cells were loaded with the Ca^{2+}-sensitive fluorescent probe fluo-3/AM (4 μM) in KRH solution with or without calcium for 20 min at 37 °C [18] and viewed with a confocal microscope. Ca^{2+}-free KRH solution was prepared with 0.2 mM EGTA. Capsaicin was added to the media to test the effect of capsaicin on $[Ca^{2+}]_i$. When necessary, pretreatment with capsazepine was performed at 10 μM for 10 min before addition of capsaicin. Excitation was performed at 488 nm and the emission signals were collected through a 515 nm barrier filter. Confocal images were taken every 7 s and used for quantitative measurement.

Effects of capsaicin on saliva secretion in isolated rabbit SMG. The functional consequences of infusion of capsaicin into the isolated rabbit SMG were measured according to the method described earlier [19]. In brief, rabbits were anesthetized by sodium pentobarbital (50 mg/kg weight). The SMG was isolated and perfused through a polyethylene cannula placed in the external carotid artery. The main excretory duct was cannulated for collection of the saliva. KRH solution was warmed to 37 °C, bubbled with 95% O_2: 5% CO_2, and perfused at a rate of 3 ml/min from a constant-infusion pump. After equilibration for at least 30 min, different concentrations of capsaicin were introduced into the perfusion buffer and perfused into the gland for 30 min. The secretion of the gland was measured as the length of moist filter paper (35 mm × 5 mm) in 5 min.

Data analysis. Data are presented as means ± SD. The statistical analyses were performed using unpaired Student's *t* test and *P* values <0.05 were considered significant.

Results

The expression of VR1 mRNA and protein in rabbit SMG

The expression of VR1 mRNA in rabbit SMG was detected with specific primers for rabbit VR1 (Fig. 1A). The PCR products were observed at the expected size of 350 bp in the gel and further confirmed by sequencing. cDNA sequence of the PCR product was found to be identical to the reported sequence of VR1 (GenBank Accession No. AY487342, 2154–2503). To further evaluate VR1 protein expression within the rabbit SMG, crude protein extract from SMG was examined by immunoblotting. Immunoreactive VR1 with a molecular mass of approximately 95 kDa was detected (Fig. 1B), corresponding to the published data [10].

Localization of VR1 protein in rabbit SMG and primary cultured SMG cell

As shown in Fig. 2, VR1 protein was detected in the basolateral region of duct cells (arrow d) in rabbit SMG. The intensity of the reaction seen in the acinar cells (arrow a) was lower than that of the duct cells, although the localization was mainly in cytoplasm (Fig. 2A). The distribution of VR1 in SMG was confirmed again using immunofluorescence (Fig. 2B). Since VR1 was abundant in DRG, rabbit DRG was used as positive control. Immunoreactivity on rabbit DRG sections showed typical diffuse cytoplasm staining in small DRG neurons (Fig. 2C). To further demonstrate the location of VR1 in SMG cells, primary cultured neonatal rabbit SMG cells were used to detect the VR1 distribution. Fluorescence images showed that VR1 was demonstrated in the cytoplasm of the cells (Fig. 2D).

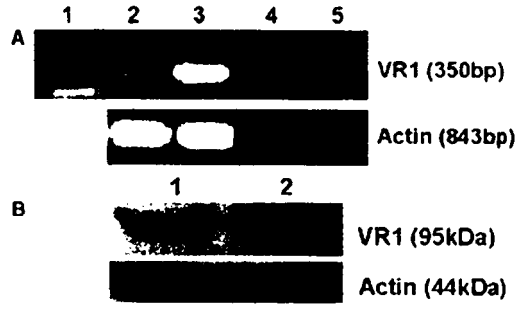

Fig. 1. Expression of VR1 in the rabbit submandibular gland (SMG). (A) VR1 mRNA expression was detected by RT-PCR. Lane 1, DNA marker; lane 2, rabbit SMG; lane 3, rabbit dorsal root ganglia (DRG); lanes 4 and 5, SMG and DRG water control. PCR products were separated on 1.5% agarose gel and visualized with ethidium bromide. (B) VR1 protein expression was detected by immunoblotting. Lane 1, rabbit SMG; lane 2, rabbit DRG. Approximately 40 μg of protein was separated on a 9% SDS-polyacrylamide gel electrophoresis and VR1-immunoreactivity was determined using a polyclonal anti-VR1 antibody. The blot is a representative of five separate experiments with similar results.

Effects of capsaicin on Ca^{2+} mobilization in primary cultured SMG cell

$[Ca^{2+}]_i$ is rapidly increased following the application of capsaicin to the cultured SMG cells. A typical example is shown in Fig. 3. An initial small peak was shown at about 50–60 s and followed by a sustained plateau after the addition of 5 μM capsaicin (Fig. 3C). The elevation of $[Ca^{2+}]_i$ was abolished by preincubation with 10 μM of the VR1 antagonist, capsazepine (Fig. 3D). To evaluate the role of extracellular Ca^{2+} in the capsaicin-induced Ca^{2+} increase, SMG cells were treated with capsaicin in Ca^{2+}-free solution. As illustrated in Fig. 3E and F, $[Ca^{2+}]_i$ fluctuated in SMG cells after exposure to 5 μM capsaicin and the capsaicin-induced $[Ca^{2+}]_i$ elevation was attenuated by pretreatment with 10 μM capsazepine in the absence of extracellular Ca^{2+}. The solvent alone did not affect $[Ca^{2+}]_i$ (trace not shown).

Effects of capsaicin on saliva secretion in isolated SMG

The isolated rabbit SMG was used to examine capsaicin-stimulated saliva secretion in the intact gland. Infusion of capsaicin from the external carotid artery significantly increased saliva secretion of the isolated SMG from 2 to 3 min after the drug was introduced into the SMG and sustained about 30 min. The effects of different concentration of capsaicin on the secretion of SMG are shown in Fig. 4. Pre-treatment with 10 μM VR1 antagonist capsazepine for 30 min abolished the increased secretion of SMG induced by capsaicin. Treatment with capsazepine alone did not affect the SMG secretion. These results indicated that capsaicin could stimulate saliva secretion in SMG via VR1 activation.

Discussion

We report here for the first time that VR1 is present in the rabbit SMG. We further determined that capsaicin stimulated salivary secretion by activation of VR1 in SMG cells. In addition, the elevation $[Ca^{2+}]_i$ was involved in the effect of capsaicin on SMG cells.

Originally, VR1 was thought restricted to primary afferent neurons and to neurons of the central nervous system [3], but there is growing evidence for its additional expression in non-neuronal cells such as mast cells [9], skin epidermal keratinocytes [10], urinary bladder epithelial cells [11], the epithelium of the stomach [20], and the palate [21]. These observations indicate that VR1 is more broadly distributed than previously thought. In the present study, the expressions of VR1 mRNA and protein were detected in the rabbit SMG. The VR1 protein was localized mainly in the basolateral plasma membrane of SMG ductal cells. Since the SMG sample used in this study contains various cell populations i.e. connective and neural tissues in addition to epithelial cells, we further detected the VR1 distribution in primary cultured SMG cells to confirm that

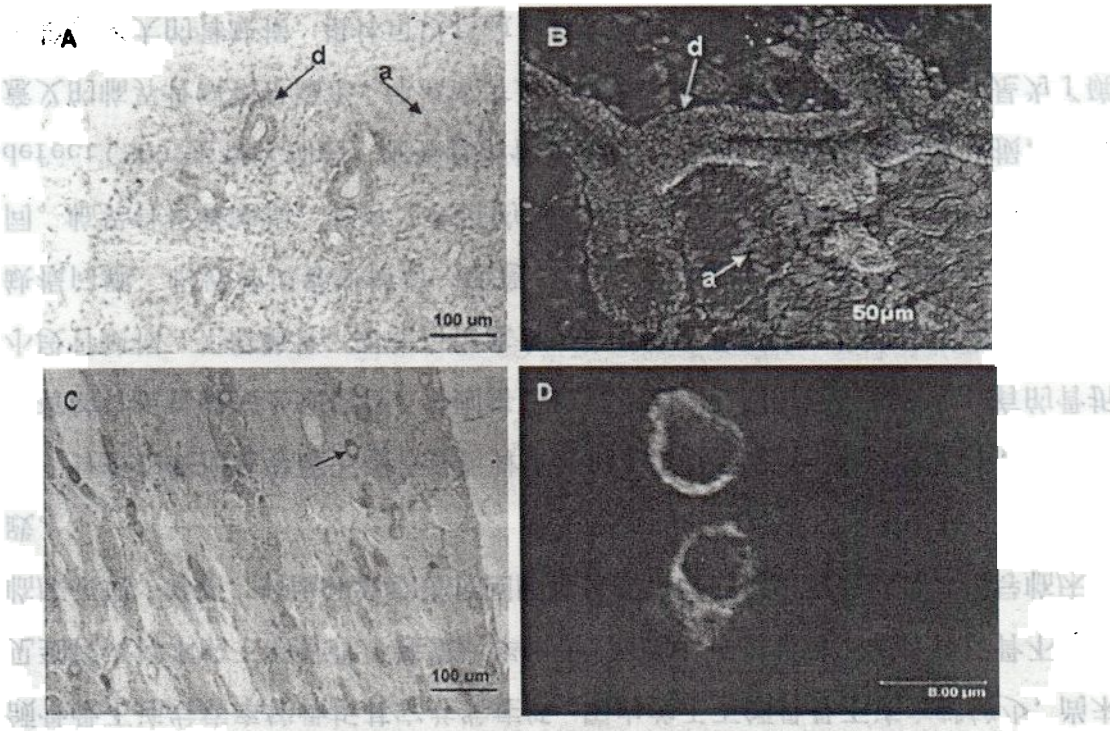

Fig. 2. Localization of VR1 protein by immunohistochemistry. (A) Immunoperoxidase labeling (VR1) in rabbit SMG, VR1 protein was detected in the basolateral region of duct cell (shown by the arrow marked d). The intensity of the reaction seen in acinar cells (shown by the arrow marked a) was lower than that of duct cells, although the localization was mainly in cytoplasm. (B) Immunofluorescence labeling (VR1) in rabbit SMG. (C) Rabbit DRG was used as a positive control. Immunoreactivity on rabbit DRG sections showed typical diffuse cytoplasm staining in small DRG neurons shown by the arrow. (D) Localization of VR1 in neonatal rabbit SMG cells. Cells were fixed and reactivity to anti-VR1 antibody was determined using a FITC-linked anti-goat IgG. Fluorescence was detected in the cytoplasm of cells. Figure is a representative of five separate experiments with similar results. (Bars: A and C, 100μm; B, 50μm; D, 8 μm).

VR1 was present in epithelial cells in SMG. The results showed that VR1 fluorescence was mainly in the cytoplasm. Some studies have shown that extensive expression of VR1 was found in endoplasmic reticulum of small DRG neurons [22] and in the plasma membrane and sarcoplasmic reticulum of skeletal muscle [23].

VR1, also referred to as transient receptor potential channel-vanilloid subfamily member 1 (TRPV1), is a distant relative of the transient release potential (TRP) family of store-operated calcium channels. Members of the TRP family have been proposed to mediate entry of extracellular Ca^{2+} into cells in response to depletion of $[Ca^{2+}]_i$ stores. In cultured trigeminal ganglion neurons, capsaicin causes a marked calcium accumulation in cells and this serves as an initial step to activate biochemical pathways ultimately leading to VR1 desensitization [24]. As in neurons, activation of peripheral VR1 with capsaicin results in $[Ca^{2+}]_i$ increase, which may play an important role in the regulation of some physiological functions. In human keratinocyte, activation of the cells by capsaicin induced calcium influx and resulted in a dose-dependent expression of cyclooxygenase-2, an increased release of interleukin-8 and prostaglandin E2, which contributed to the induction of inflammation during noxious cutaneous stimulation [10]. In bladder epithelial (urothelial) cells, exogenously vanilloids increased intracellular Ca^{2+} and evoked nitric oxide release through VR1 [11]. With salivary acinar cells, an increase in the level of $[Ca^{2+}]_i$ is the primary fluid secretion signal. Agonist-activated Ca^{2+} entry in salivary cells follows the emptying of intracellular Ca^{2+} stores and plays a key role in determining the amplitude of the sustained elevated cytosolic Ca^{2+} signals [25]. By far, the most potent natural stimulus for salivation is activation of muscarinic receptors. It was found that salivation was coupled with the elevation of $[Ca^{2+}]_i$ when carbacol bound to muscarinic receptors [12]. Since oral treatment with capsaicin could cause reproducible salivation, it was interesting to examine whether capsaicin induces any effects on calcium mobilization in SMG cells. In primary cultured neonatal rabbit SMG cells, capsaicin caused a small Ca^{2+} transient peak followed by a sustained plateau in the presence of extracellular Ca^{2+} and it also triggered Ca^{2+} mobilization in the absence of extracellular Ca^{2+}, both elevations of $[Ca^{2+}]$ were abolished by capsazepine. These results suggested that capsaicin induced the elevation of $[Ca^{2+}]_i$ in SMG cell through VR1. Furthermore, Ca^{2+} mobilization induced by capsaicin resulted not only from Ca^{2+} influx but also from intracellular Ca^{2+} stores. The pharmacological characteristics of this Ca^{2+} mobilization response in SMG cells are very similar to those described in DRG neurons, where

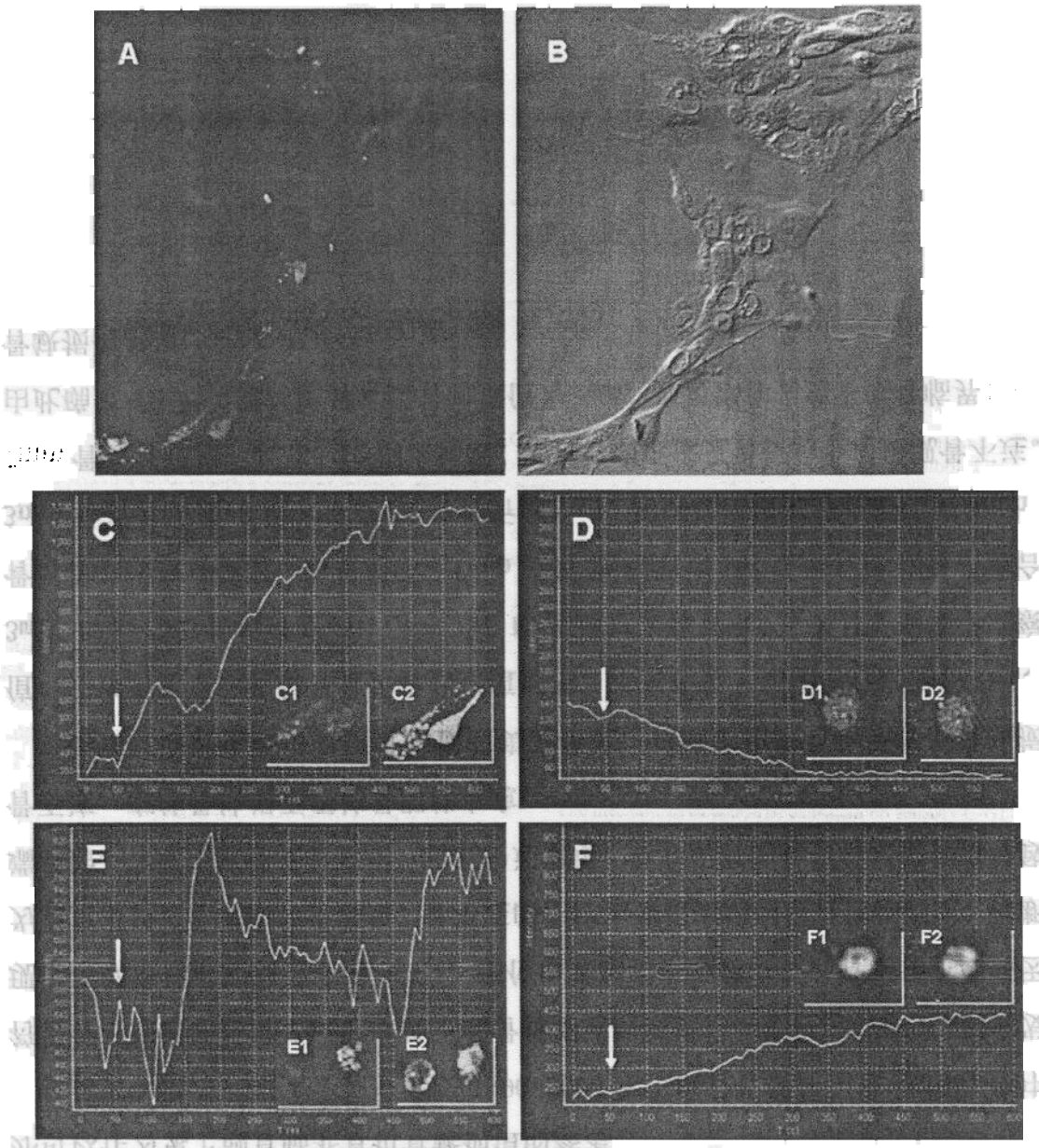

Fig. 3. Capsaicin-induced $[Ca^{2+}]_i$ changes in neonatal rabbit SMG cells. (A) Fluorescence image of fluo-3AM loaded cells. (B) Transmitted light image of fluo-3AM loaded cells. (C) $[Ca^{2+}]_i$ dynamics at the individual cell level in the presence of extracellular Ca^{2+} when the cells were treated with 5 μM capsaicin. (D) $[Ca^{2+}]_i$ dynamics at the individual cell level in the presence of extracellular Ca^{2+} when the cells were pretreated with 10 μM capsazepine followed by 5 μM capsaicin treatment. (E) $[Ca^{2+}]_i$ dynamics at the individual cell level in the absence of extracellular Ca^{2+} when the cells were treated with 5 μM capsaicin. (F) $[Ca^{2+}]_i$ dynamics at the individual cell level in the absence of extracellular Ca^{2+} when the cells were pretreated with 10 μM capsazepine followed by 5 μM capsaicin treatment. Arrows denote the application time of capsaicin. Each trace is a representative of five separate experiments. Insets are the fluorescence images of the cells before (C1, D1, E1, and F1) and after (C2, D2, E2, and F2) the capsaicin stimulation.

VR1 activation caused Ca^{2+} mobilization both from intracellular Ca^{2+} stores and Ca^{2+} influx [22]. Recently, it has been reported that capsaicin could induce the leakage of calcium from sarcoplasmic reticulum when those VR1 expressed on the sarcoplasmic reticulum in skeletal muscle cells were activated [23]. These results indicate that no matter where it is, plasma membrane or intracellular membrane, VR1 participates in the cellular Ca^{2+} modulation. Although the precise role of VR1 in the genesis of Ca^{2+} signals in SMG cells is far from well understood, their localization in both of plasma membrane and cytoplasmic membrane suggests that it plays a role in the regulation of Ca^{2+} signal to maintain the physiological function of SMG.

We have also shown the direct evidence that capsaicin activates VR1 receptor resulting in promoting the saliva

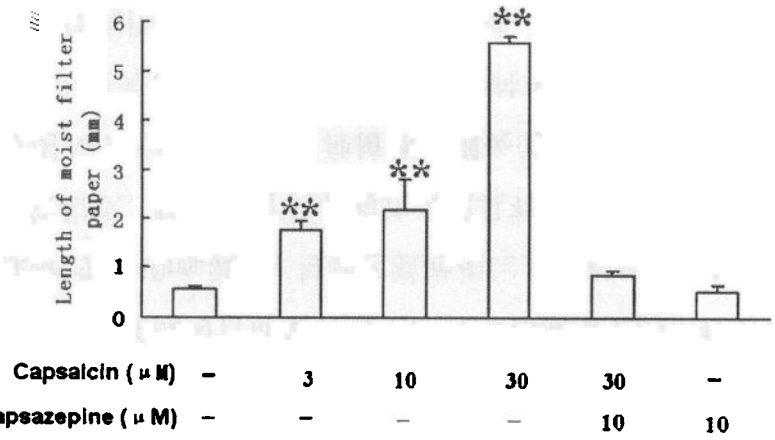

Fig. 4. Effects of capsaicin on saliva secretion of the isolated SMG. The SMG was isolated and perfused through a polyethylene cannula placed in the external carotid artery. The main excretory duct was cannulated for collection of saliva. KRH solution was warmed to 37 °C, bubbled with 95% O_2: 5% CO_2, and perfused at a rate of 3 ml/min with a constant-infusion pump. After equilibration for at least 30 min, different concentrations of capsaicin were introduced into the perfusion buffer and perfused into the gland for 30 min. Saliva secretion by the gland was measured as the length of moist filter paper (35 mm × 5 mm) in 5 min. The vanilloid specificity of the capsaicin-induced responses was determined by using 10 μM capsazepine (CPZ) as vanilloid antagonist. Data are presented as means ± SD for four separate experiments. $^{**}P < 0.01$ compared with the control.

secretion by infusion capsaicin to the isolated SMG. This finding provided additional support of the functional expression of VR1 in rabbit SMG.

In conclusion, the present findings indicate that VR1 is expressed in the rabbit SMG and capsaicin stimulates saliva secretion through VR1 in SMG cells. The elevation of $[Ca^{2+}]_i$ may contribute to the salivary secretion induced by capsaicin in SMG cells. These findings may pave a route for further understanding the mechanism of saliva secretion and lead to novel strategy for therapy of salivary gland dysfunction.

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