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Expression of vanilloid receptors in rat gastric epithelial cells: role in cellular protection

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Abstract

Vanilloid receptors subtype 1 (VR1), a nonselective cation channel responsive to capsaicin, protons, and noxious heat, has been recently identified in not only neural but also non-neural cells. In the present study, we demonstrated the peripheral expression of VR1 in gastric mucosal epithelial cells and investigated the role of the receptor in cellular protection. The rat gastric mucosal epithelial cell line was used. The expression of VR1 was examined by Western blotting and RT–PCR. Cell damage was induced by immersion in 10% ethanol or acid (pH 4.0) for 30 min, and cell viability was determined by MTT assay. Capsaicin or resiniferatoxin was added 30 min before the challenge with ethanol or acid, while capsazepine or ruthenium red (a VR1 antagonist) was added simultaneously with capsaicin. The distinct expression of VR1 protein and mRNA was detected in rat gastric mucosal epithelial cell line as well as in the rat stomach and spinal cord by Western blotting and RT–PCR, respectively. The cDNA sequence of the PCR product was found to be almost identical to that of the authentic VR1 (99.8%) when the product was subcloned and sequenced. On the other hand, the cell damage induced by ethanol or acid was dose-dependently prevented by pretreatment with capsaicin. The protective effect of capsaicin was mimicked by resiniferatoxin and almost totally abolished by co-addition of capsazepine or ruthenium red. These findings suggest that VR1 is expressed peripherally in gastric mucosal epithelial cells and plays a cellular protective role.

Keywords: VR1 (vanilloid receptor subtype 1); RGM-1; Capsaicin; Resiniferatoxin; Gastric mucosal epithelial cells; Cellular protection

1. Introduction

Capsaicin, the main ingredient of hot chili peppers, selectively activates the peripheral terminal of sensory C and Aδ fibers, generally referred as polymodal nociceptors, and evokes a sensation of tingling and burning pain [1,2]. Vanilloid receptor subtype 1 (VR1), a receptor responsible for capsaicin action, has been cloned by Caterina *et al*. [3] from rat dorsal root ganglia and recognized as a common molecular target for protons and noxious heat (>43°) as well as vanilloid compounds [3,4]. Several vanilloid receptor homologues have been also cloned and demonstrated to be located in not only neural but non-neural cells as well [5].

Capsaicin protects the gastric mucosa against several injuries in animals and humans [6–9]. It is generally considered that this effect of capsaicin results from the activation of sensory afferent neurons in the stomach and is mediated by various physiological functions, such as mucosal blood flow [10–13], mucus secretion [14], and bicarbonate secretions [15]. In addition, Nozawa *et al*. [16] have recently provided direct evidence for the presence of VR1 in the stomach by immunohistochemistry and by the binding of [^{3}H] resiniferatoxin binding, an ultrapotent capsaicin analogue. These findings led us to speculate that the effect of capsaicin in the stomach would be attributable to the activation of VR1 in capsaicin-sensitive afferent neurons. However, several papers have recently shown the presence of VR1 in other peripheral cells, such as a human bronchial epithelial cell line [17], skin epidermal keratinocytes [18,19], and urinary bladder epithelial cells [20]. Thus, VR1 expressed peripherally

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might also be involved in the regulation of some physiological functions, although it remains unknown whether the receptor is expressed peripherally in the gastric mucosa.

In the present study, we demonstrated the existence of VR1 in a cultured rat gastric epithelial cell line (RGM-1) and investigated the role of VR1 in cellular protection against damage induced by ethanol or acid.

2. Materials and methods

2.1. Reagents and animals

Capsaicin was purchased from Wako, resiniferatoxin and capsazepine from Sigma, and ruthenium red from Daiichi Kagaku. Capsaicin, RTX, capsazepine, and ruthenium red were prepared in 99.9% ethanol and serial dilutions made in D-MEM. Male Sprague–Dawley rats (200–240 g) were purchased from Nippon Charles River. The animals were fed standard rat chow and tap water *ad libitum*. The experimental procedure using rats was approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

2.2. Cells and cell culture

A RGM-1 was provided from Riken Cell Bank. RGM-1 cells were cultured in a mixture of Ham's F12 medium and Dulbecco's modified minimum essential medium (D-MEM), supplemented with 20% fetal bovine serum, 100 μg/mL of streptomycin and 100 U/mL of penicillin in a humidified atmosphere of 5% CO_2 at 37°.

2.3. Western blot analysis

RGM-1 cells, whole rat stomach and rat spinal cord were homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride, 32 mM sucrose, 1 mM dithiothreitol, 10 μg/mL of soybean trypsin inhibitor, 10 mg/mL of leupeptin, and 2 μg/mL of aprotinine. Then, the homogenized samples were centrifuged at 100,000 *g* for 1 hr at 4°. The supernatant was removed, and the pellet was resuspended in the homogenized buffer. The protein concentration of the suspension was determined using a BCA protein assay kit (Pierce). The samples (10 μg/lane) were then electrophoresed on 10% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes (PROTRAN, Schleicher & Schuell). The membranes were incubated with anti-VR1 antibody (Santa Cruz Biotechnology) and treated with horseradish peroxidase-conjugated anti-goat IgG (Santa Cruz Biotechnology). The immune complexes were visualized using the enhanced chemiluminescence detection system (Perkin-Elmer Life Sciences).

2.4. Reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA from RGM-1 cells, rat stomach, and rat spinal cord were extracted using Sepasol RNA I (Nacalai Tesque). Total RNA primed by oligo(dT)20 was reverse-transcribed with a first strand cDNA synthesis kit (Rever-Tra Ace α, TOYOBO) after treatment with DNase I (TOYOBO) to remove contamination of genomic DNA. The PCR primers of rat VR1 were sense (5′-TCA-TGGGTGAGACCGTCAACAAG-3′) and antisense (5′-T-GGCTTAAGGGATCCCGTATAAT-3′) corresponding to nucleotides 2122–2144 and 2549–2527, respectively, of the published rat VR1 sequence [3], giving rise to a 428-bp PCR product. An aliquot of the RT reaction product served as a template in 30 cycles with 0.5 min of denaturation at 95° and 1 min of extension at 68° using the Advantage 2 polymerase mixture (CLONTECH) on a thermal cycler (TAKERA TP-240). A portion of the PCR mixture was electrophoresed in 1.8% agarose gel in TAE buffer (40 mM Tris, 2 mM EDTA, and 20 mM acetic acid; pH 8.1), and the gel was stained with ethidium bromide and photographed. The PCR product was subcloned into pGEM-T Easy vector (Promega), and its sequence was determined by the dideoxy chain termination method with a BigDye terminator v3.0 Cycle Sequencing kit (Biosystems) using an automatic DNA sequencer (ABI Prism 310 Genetic Analyzer; Biosystems). The sequence of the PCR product was analyzed using the BLAST program (NCBI).

2.5. Induction of cell damage

The cells were cultured at 2×10^4 in a 96-well flat-bottomed microplate (FALCON, Becton Dickinson Laboware), which was coated with collagen type I (Cellmatrix, Nitta Gelatin) for 48 hr and used for the experiment after reaching confluence. The culture medium was aspirated, and the cells were treated with fresh culture medium containing capsaicin (10^{-9} to 10^{-6} M) or resiniferatoxin (10^{-9} to 10^{-12} M) for 30 min, with or without capsazepine (10^{-6} or 10^{-5} M) or ruthenium red (10^{-6} or 10^{-5} M). Then, the cells were washed with normal phosphate-buffered saline (PBS: pH 7.4). Cell damage was induced by treatment for 30 min with either D-MEM (without fetal bovine serum) containing 10% ethanol or acidified PBS (pH 4.0). After aspiration of the solution, the cells were washed twice with D-MEM and subjected to a viability assay.

2.6. Evaluation of cell viability

A colorimetric assay was performed using 3-(4,5-dimethlyl-2 thiazoyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Nacalai Tesque) in 50 μL of culture medium. In brief, 10 μL of MTT solution (5 mg/mL in PBS) was

first added to the culture medium, and the cells were then incubated in 5% CO_2 at 37° for 4 hr. Subsequently, 150 μL of 0.04 mol/L HCl in isopropanol was added, and the mixture was left at room temperature for 18 hr. The absorbance of color change was determined using a microplate reader (SME-3400, IWAKI) at 590 and 620 nm (as a reference). The $A_{590} - A_{620}$ was taken as the index of cell viability. The absorbance change taken from the wells of normal cultured cells (untreated with 10% ethanol or acidified PBS) was used as the 100% viability value. The percentage of viability was calculated with the formula as follows:

$$\frac{(A_{590} - A_{620})_{\text{sample}}}{(A_{590} - A_{620})_{\text{normal}}} \times 100.$$

2.7. Statistical analysis

Experimental results are expressed as means ± SE from five cultured wells in the same experiment. The statistical analyses were performed using a two-tailed unpaired *t*-test and Dunnett's multiple comparison test, and values of $P < 0.05$ were considered to be significant.

3. Results

3.1. Expression of VR1 protein and mRNA in RGM-1

In Western blot analysis, the immune complex with the specific anti-VR1 antibody was detected in the membrane fraction of RGM-1 as well as in the rat stomach and spinal cord, although the amount expressed was lower in RGM-1 than that of the stomach and spinal cord (Fig. 1). Similarly, the distinct expression of VR1 mRNA was detected in RGM-1 cells as well as in the stomach and spinal cord by RT–PCR using specific rat VR1 primers (Fig. 2). In addition, the cDNA sequence of the PCR product was found to be almost identical to that of the authentic VR1 (427/428 bp; 99.8%) when the product was subcloned and sequenced.

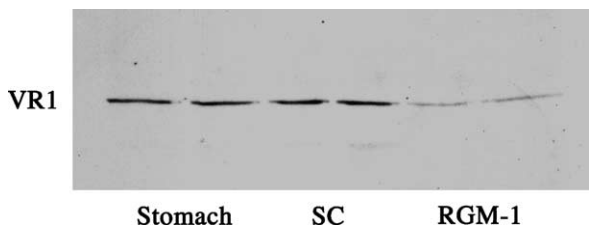

Fig. 1. Western blot analysis for VR1 protein in RGM-1, whole rat stomach and rat spinal cord. Each band of RGM-1 cells was obtained from respective different passages of cells, while each band of the stomach and spinal cord was obtained from two different animals. The expression of VR1 protein (around 95 kDa) was detected in the membrane fraction of RGM-1 as well as in the stomach and spinal cord detected from by Western blotting, using specific anti-VR1 antibody.

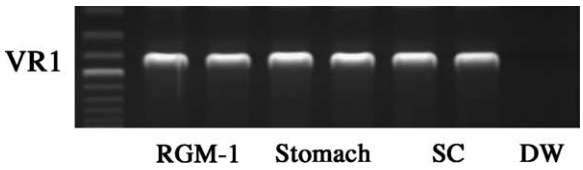

Fig. 2. RT–PCR analysis for VR1 mRNA in RGM-1, whole rat stomach and rat spinal cord. Each band of RGM-1 cells was obtained from respective different passages of cells, while each band of the stomach and spinal cord was obtained from two different animals. The expression of VR1 mRNA was detected in RGM-1 as well as in the stomach and spinal cord by RT–PCR, using specific primers for rat VR1 mRNA. When the product was subcloned and sequenced, it was found that the sequence of the PCR product was almost identical to that of rat VR1 mRNA (427/428 bp; 99.8%).

3.2. Effects of capsaicin and resiniferatoxin on cell damage

The treatment with 10% ethanol in D-MEM for 30 min induced cell damage, the cell viability decreasing to 69.5 ± 2.8% in comparison with normal (D-MEM alone). Although capsaicin (10^{-9} to 10^{-6} M) alone did not affect the viability of RGM-1 cells (data not shown), the pretreatment with this agent concentration-dependently prevented the decrease in cell viability in response to 10% ethanol, the inhibition being 42.3, 59.3, 69.5, and 90.2%, respectively, and a significant effect was observed at the concentration of 10^{-9} M or higher (Fig. 3A). Resiniferatoxin (10^{-12} to 10^{-9} M) also had a protective effect against the ethanol-induced cell damage, the inhibition being 32.5, 51.8, 69.2, and 81.3%, respectively (Fig. 3B). A significant effect of resiniferatoxin was observed at the concentration of 10^{-11} M, which was around 100–500 times less than that of capsaicin.

On the other hand, the treatment with acidified PBS (pH 4.0) for 30 min also induced cell damage, the cell viability decreasing to 62.0 ± 4.2% in comparison with normal PBS (pH 7.4). Pretreatment with either capsaicin or resiniferatoxin prevented the cell damage induced by acid, in a concentration-dependent manner, and a significant effect was obtained by both agents at the same dose levels as those in case of ethanol-induced cell damage, the inhibition at each concentration of capsaicin and resiniferatoxin being 41.3, 63.7, 69.5, 81.6%, and 27.9, 64.5, 78.7, 71.3%, respectively (Fig. 4A and B).

3.3. Effects of VR1 antagonists on capsaicin-induced cellular protection

The pretreatment with capsaicin (10^{-8} to 10^{-6} M) prevented the decrease in the cell viability induced by 10% ethanol, in a concentration-dependent manner, the inhibition by capsaicin being 39.7, 65.3, and 84.7%, respectively (Fig. 5). The protective effect of capsaicin was concentration-dependently abolished by capsazepine (10^{-6} and 10^{-5} M), and the apparent antagonism was observed on

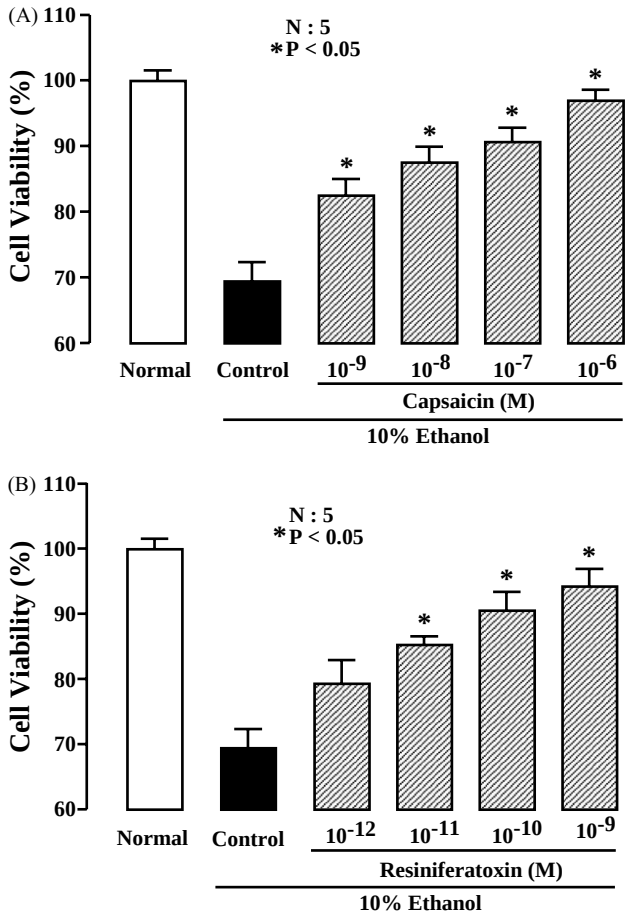

Fig. 3. Effect of capsaicin (A) and resiniferatoxin (B) on cellular damage induced by ethanol in RGM-1. The cells were treated with culture medium including either capsaicin (10^{-9} to 10^{-6} M) or resiniferatoxin (10^{-12} to 10^{-9} M) for 30 min, and then exposed to the medium containing 10% ethanol for 30 min. The cell viability was determined by MTT assay. Data are presented as the mean ± SE for five cultures per group. Statistically significant difference from control (ethanol alone) at $^{*}P < 0.05$.

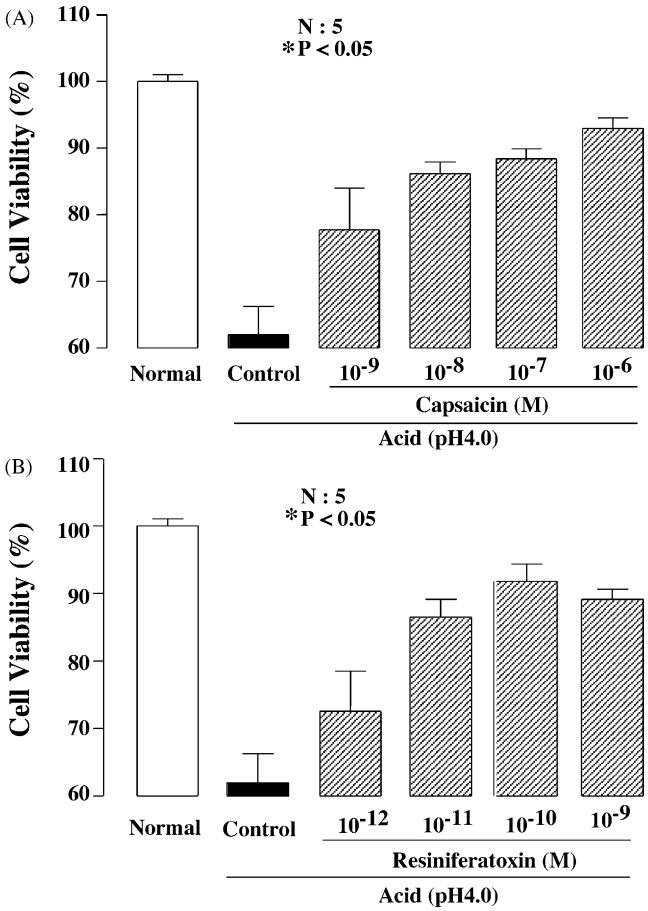

Fig. 4. Effect of capsaicin (A) and resiniferatoxin (B) on cellular damage induced by acid (pH 4.0) in RGM-1. The cells were treated with culture medium including either capsaicin (10^{-9} to 10^{-6} M) or resiniferatoxin (10^{-12} to 10^{-9} M) for 30 min, and then exposed to the medium containing acid (pH 4.0) for 30 min. The cell viability was determined by MTT assay. Data are presented as the mean ± SE for five cultures per group. Statistically significant difference from control (acid alone) at $^{*}P < 0.05$.

co-treatment with 10^{-7} or 10^{-6} M of capsaicin, the inhibition by capsaicin decreasing to 49.0 and 66.0%, respectively. Similarly, ruthenium red (10^{-6} and 10^{-5} M) significantly attenuated the protective effect of capsaicin (10^{-6} M) against 10% ethanol-induced cell damage, the inhibition by capsaicin being 71.2 and 109.8%, respectively (Fig. 6). Ruthenium red alone showed a slight antagonism of the decrease in cell viability in response to 10% ethanol, although the effect at neither concentration was significant.

4. Discussion

In the present study, we demonstrated the expression of VR1 in cultured rat gastric mucosal cell line, RGM-1, at both protein and mRNA levels using Western blotting and RT–PCR, respectively, and confirmed that the sequence of the PCR product was almost identical to that of the authentic rat VR1. We further showed that pretreatment with capsaicin and resiniferatoxin prevented damage induced by ethanol and acid in RGM-1, and this effect of capsaicin was attenuated by either capsazepine or ruthenium red, the VR1 antagonist. These findings suggest that VR1 is expressed not only in the neural system but in gastric mucosal epithelial cells as well and plays a protective role at the cellular level against noxious stimuli.

The presence of VR1 has been established in the dorsal root, trigeminal, nodose ganglia [21], and brain [22,23] in addition to the originally reported dorsal root ganglia [3]. Subsequently, several vanilloid receptor variants and homologues have been recognized in not only neural but also various non-neural cells, such as bronchial epithelial cells [17], keratinocytes in epidermis [18,19], and urinary bladder epithelial cells [20]. These observations suggest that VR1 and its homologues are more broadly distributed than previously thought. In the present study, we provided for the first time direct evidence for the existence of VR1 in RGM-1, the rat gastric epithelial cell line. In Western blot analysis, the immune complex with the specific anti-VR1

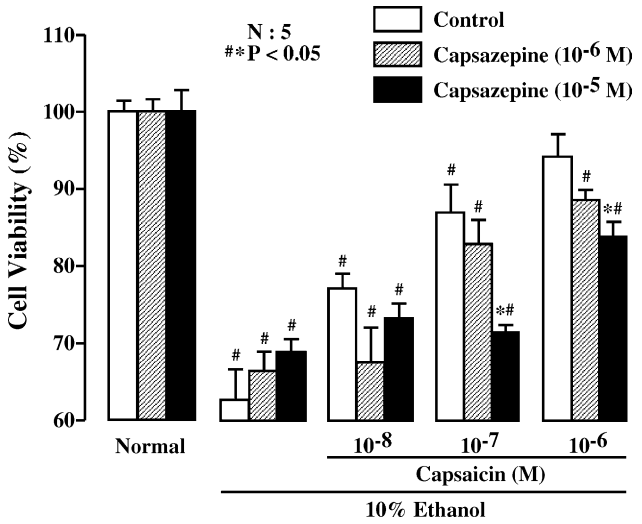

Fig. 5. Effect of capsazepine on the capsaicin-induced cellular protection in RGM-1. The cells were treated with capsaicin (10^{-8} to 10^{-6} M) with or without capsazepine (10^{-6} or 10^{-5} M) for 30 min, and then exposed to culture medium containing 10% ethanol for 30 min. The cell viability was determined by MTT assay. Data are presented as the mean ± SE for five cultures per group. Statistically significant difference from the corresponding control at each concentration of capsaicin (without capsazepine) from control (ethanol alone) at $^{*}P < 0.05$. Statistically significant difference from the corresponding values for ethanol alone at each concentration of capsaicin at $^{\#}P < 0.05$.

antibody was detected in the membrane fraction of RGM-1 as well as the rat stomach and spinal cord, although the amount expressed in RGM-1 cells appeared to be lower than that in the latter tissues. We also showed by RT–PCR

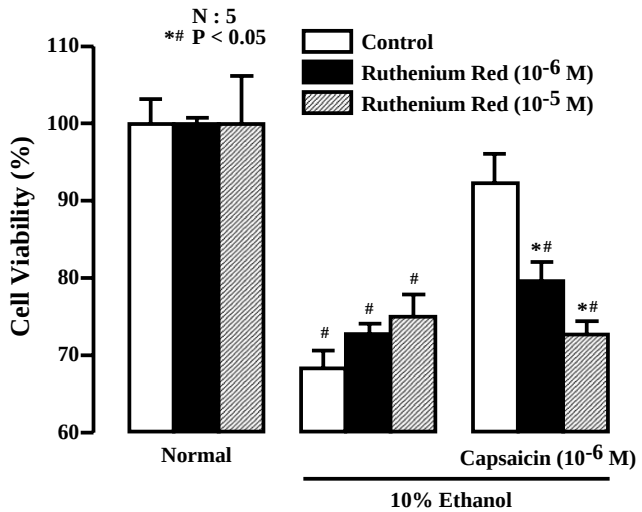

Fig. 6. Effect of ruthenium red on the capsaicin-induced cellular protection in RGM-1. The cells were treated with capsaicin (10^{-6} M) with or without ruthenium red (10^{-6} or 10^{-5} M) for 30 min, and then exposed to the culture medium containing 10% ethanol for 30 min. The cell viability was determined by MTT assay. Data are presented as the mean ± SE for five cultures per group. Statistically significant difference from the corresponding control at each concentration of capsaicin (without ruthenium red) from control (ethanol alone) at $^{*}P < 0.05$. Statistically significant difference from the corresponding values for ethanol alone at each concentration of capsaicin at $^{\#}P < 0.05$.

using specific primers for rat VR1 that a distinct VR1 mRNA expression was observed in RGM-1 as well as the stomach and spinal cord. Furthermore, as the result of subcloning for the PCR product, we found that the sequence was almost totally identical to that of rat VR1 mRNA (99.8%), which has been reported by Caterina *et al.* [3]. These results strongly suggest that VR1 is expressed in gastric mucosal epithelial cells, in addition to neural tissues. It should be noted in Western blotting that the amount of VR1 expressed in RGM-1 cells was less than that of the stomach as well as spinal cord. Since the stomach sample used in this study contains various cell populations; smooth muscle, connective tissues and neurons in addition to epithelial cells, the real amount of VR1 expressed in the epithelial cells of the rat stomach might be less than that of neuronal tissues.

Capsaicin, the main ingredient of hot chili peppers, activates a subpopulation of primary sensory neurons, generally referred to as polymodal nociceptors [1,2]. These nociceptive nerves, so-called capsaicin-sensitive afferent neurons, participate in afferent signaling and local responses to a potentially harmful stimulation. In the gastrointestinal tract, these neurons play an important role in maintaining mucosal integrity, and activation of the neurons by capsaicin, indeed, provides gastric protection against various injuries [7,8]. Since VR1, originally cloned as a capsaicin receptor from rat dorsal root ganglia, has been shown to be a receptor responsive to proton (acid) and noxious heat (>43°), in addition to capsaicin [3], it has been assumed that the gastroprotective action of capsaicin is mediated by the activation of VR1 located on capsaicin-sensitive sensory neurons. In the present study, we observed that capsaicin significantly prevented the cell damage induced by 10% ethanol and acid (pH 4.0) in RGM-1. This effect of capsaicin was mimicked by an ultrapotent capsaicin analogue, resiniferatoxin, though the action of the latter was more potent on a concentration basis. In addition, the VR1 antagonists, capsazepine and ruthenium red, significantly attenuated the capsaicin-induced cellular protection against 10% ethanol. Because RGM-1, established from the normal rat stomach, differs from transferred or cancer cell lines, it is considered to be a potentially important model of gastric mucosal cells for the evaluation of various functions, without any intervention by nerves and blood circulation. Based on these results, it is possible that capsaicin protects the gastric mucosa at the cellular level, via a process probably mediated by VR1.

Trevisani *et al.* [24] have recently reported that ethanol directly activated VR1 and potentiated the VR1 response induced by capsaicin, similar to acid [4]. Thus, it is possible that ethanol and acid used in this study for induction of cell damage also directly activate VR1 expressed in RGM-1. In general, high concentration of ethanol and acid produces gastric damage, while the pre-treatment with low concentration of ethanol and acid

affords adaptive cytoprotection against various noxious stimuli [25]. The latter phenomenon is accompanied by activation of the mucosal defensive pathways such as mucosal blood flow and bicarbonate secretion, mediated partly by capsaicin-sensitive afferent neurons *in vivo* [26,27]. In the present study, we used injurious concentration of ethanol (10%) and acid (pH 4.0) to induce damage in RGM-1 cells *in vitro*. The concentrations of ethanol and acid used in this study may be too high to show the adaptive protection *in vitro*. Even if these agents have both direct injurious and VR1-mediated protective actions at the cellular level, the latter would be totally masked by the injurious action, resulting in damage. Since capsaicin prevents gastric mucosal damage induced by high concentration of ethanol and acid *in vivo*, through stimulation of afferent neurons mediated by VR1 [28] and since RGM-1 cells express VR1 as demonstrated in this study, it would be reasonable that this agent exerts protection action against ethanol- and acid-induced cell damage in RGM-1 cells. On the other hand, it is possible that the VR1 expressed in RGM-1 might be different from the authentic cloned VR1, transfected in HEK293 cell used in the study of Trevisani *et al*. [24]. Moreover, the conditions or the amounts of VR1 expressed in RGM-1 cells might be different from those of the cloned VR1 transfected in HEK293 cells. Further study should certainly be required for clarifying these points.

It is generally considered that the protective effect of capsaicin results from the activation of sensory afferent neurons in the stomach. This concept has been confirmed by several investigators who showed that the gastroprotective action of capsaicin was almost totally abolished by chemical ablation of capsaicin-sensitive afferent neurons following pretreatment with a neurotoxic dose of capsaicin. Thus, the capsaicin-induced cellular protection observed in cultured RGM-1 cells might not be so important in *in vivo* conditions. Nozawa *et al*. [16] also showed that a neurotoxic dose of capsaicin markedly reduced the immunoreactivity of VR1 as well as calcitonin-gene related peptide in the rat stomach. Thus, it is possible that a large dose of capsaicin might cause down-regulation of VR1 expression in gastric epithelial cells as well as dysfunction of the primary afferent neurons. Further study is required for clarifying the physiological role of VR1 expressed in gastric epithelial cells.

The mechanism underlying the capsaicin-induced gastric protection is associated with an increase in mucosal blood flow [10–13], the secretion of mucus [14], and bicarbonate [15] mediated by the activation of sensory neurons. However, it is unclear how capsaicin prevents the decrease in viability induced by ethanol or acid in cultured RGM-1 cells, devoid of a neural system or blood circulation. RGM-1 has been shown to secrete mucus in response to prostaglandin E_2 and some mild irritants [29,30]. In addition, several reports showed that Ca^{2+} as well as cAMP is an intracellular mediator for agonist-stimulated the secretion of mucus in cultured gastric mucosal cells [31,32]. Since VR1 acts as a nonselective cation channel with high permeability for divalent cations, especially Ca^{2+}, and since VR1 responds to capsaicin, proton, and noxious heat by increasing the Ca^{2+} concentration intracellularly [3], it is possible that capsaicin affords cellular protection in RGM-1 through an increase in mucus secretion mediated by Ca^{2+}. Certainly, further study should be conducted to verify this point.

In conclusion, the present findings together suggested that VR1 is expressed in gastric mucosal epithelial cells and plays a protective role at the cellular level against damage in response to ethanol. Thus, it is assumed that the gastroprotective action of capsaicin observed under *in vivo* conditions is, at least partly, accounted for by peripherally expressed VR1 in gastric epithelial cells.

Acknowledgments

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