Effects of capsaicin on human intestinal cell line Caco-2

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Abstract

The influence of capsaicin processing on human intestinal cell line Caco-2 was examined by measuring transepithelial electrical resistance (TER). There was an increase in permeability at high concentration (200 to 500 μ M) of capsaicin, and the effect was inhibited by pretreatment of capsazepine, which is a competitive antagonist of the vanilloid receptor 1 (VR1). LDH-activity as well as changes in intracellular Ca^{2+} were determined to know whether or not capsaicin affected TER activity through its influence on the tight junction. We also determined the expression of the VR1-like protein on Caco-2 cells in time-dependent manner by western blotting using vanilloid receptor (VR1) antiserum. Our results showed that the permeability increase by capsaicin was through binding to VR1-like protein of Caco-2 cells.

Abbreviations: DMEM, Dulbecco's modified eagle's medium; FCS, fetal calf serum; TER, transepithelial electrical resistance; LDH, lactic dehydrogenase; VR-1, vanilloid receptor 1.

Introduction

Capsaicin, a homovanillic acid derivatives (8-methyl-N-vanillyl-6-nonenamide) (Figure 1), which is the main pungent ingredient in 'hot' chili peppers, is used widely due to its taste and digestive properties associated with its pungent principles. Capsaicin is an active component of the red pepper of the genus *Cap-sicum* and has a variety of physiological actions. The digestive properties of capsaicin may be attributed to enhancement of the activity of digestive enzymes (Platel et al., 1996) or to indirect effects on vascular endothelia, smooth muscles and mast cells, resulting in increase of vascular permeability and of mucosal blood flow (Gronbech et al., 1994; Reynier et al., 1994). Besides these neuronal and vascular effects, direct interaction of capsaicin with gastrointestinal epithelia is likely to occur, but little is known about such direct interactions.

The capsaicin receptor has recently been reported as a receptor for pain, while its role in the nervous system has recently become a focus of attention (Caterina

et al., 1997). Capsaicin is known to bind to the capsaicin receptor, also known as the vanilloid receptor subtype1 (VR1) containing an open reading frame of 2514 nucleotides. This VR1 encodes a protein of 838 amino acids with a molecular mass of 95 kDa. At the amino terminus, VR1 has three ankyrin repeat domains. The carboxy terminus has no recognizable motifs. Predicted membrane topology of VR1 features six transmembrane domains and a possible pore-loop between the fifth and sixth membrane-spanning regions.

We now focus on the human intestinal cell line Caco-2 from a human colon carcinoma, which express brush-border enzymes, nutrient transporters, and intercellular tight junction (Arpad et al., 1999) as a model to examine the existence of VR1 and the TER-decreasing activity of capsaicin. This paper intends to clarify the relationship between capsaicin and its receptor and the role they play in the human intestinal tract. The mechanism of permeability in Caco-2 cells by capsaicin was also investigated.

Materials and methods

Materials

Capsaicin, Dulbecco's modified Eagle's medium (DMEM), and fetal calf serum (FCS) were obtained from Sigma (U.S.A.). Non-essential amino acid was purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan). Millicell-ERS instrument with Ag AgCl⁻¹ electrodes was purchased from Nihon Millipore (Tokyo, Japan). Lactic acid dehydrogenase (LDH) Test and Fluo-3/AM were purchased from Wako Pure Chemical Industries (Osaka, Japan). VR1 antiserum, capsazepine, and ruthinium red were gifts from by Dr. David Julius of department of cellular and molecular pharmacology, University of California.

Cell culture

Caco-2 cells were maintained in DMEM supplemented with 10% FCS, 1% penicillin-streptomycin, and 1% non-essential amino acids. They were incubated at 37 ° in humidified atmosphere of 5% CO₂ in air. The cells were passaged at a split ratio of 4 to 8 every 3 or 4 days. For TER measurement, cells were seeded on Transwell (Coastar, U.S.A.) coated with collagen at a density of 2×10^5 cells cm⁻². For the LDH activity and [Ca²⁺]_i assays, cells were seeded on 96-well plate at density of 2×10^5 cells cm⁻².

Western blotting

Caco-2 cells (2×10^6 cells) were incubated with 100 μM of capsaicin for 24 hr. Lysates were prepared from cells growing as monolayers or in suspension. Cells were washed with cold phosphate-buffered saline (PBS) and lysed in RIPA lyses buffer (1% Nonidet P-40, 20 mM Tris pH 8.0, 137 mM NaCl, 10% glycerol, and 1 mM Na₃VO₄) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 0.1 trypsin inhibitory units ml⁻¹ aprotinin and 20 μg ml⁻¹ leupeptin). The lysates were centrifuged for 10 min at 4 °C to remove debris, and the protein concentrations were determined using the Bio-Rad DC Protein Assay (Hercules, CA, U.S.A.). The proteins were resolved by SDS-PAGE and PVDF (BIO RAD). The immunoblots were incubated for 90 min with the VR-1 antiserum (diluted 2000-fold), washed three times with TBS-tween buffer. It was then incubated for 1 hr with goat anti-rabbit IgG (diluted 2500-fold), and washed three times with TBS-tween buffer. Immunodetection was performed using the enhanced chemi-

luminescence (ECL) Western blotting kit (Amersham Pharmacia Biotech).

TER measurement

The permeability of Caco-2 cell monolayer was evaluated by measuring the transepithelial electrical resistance (TER) value. TER is believed to be correlated with the change in paracellular permeability of the cell monolayer (Hashimoto et al., 1997). The cell monolayers on Millicell-HA were rinsed with DMEM and then set in a 24-well plate containing DMEM. DMEM was added to the apical side, and the TER value was measured with a Millicell-ERS instrument. The TER value of the Caco-2 monolayers was measured before and after adding assay sample, and the effect of each assay sample is expressed as the relative TER value to the value at zero time.

[Ca²⁺]_i measurement

[Ca²⁺]_i measurement was performed by modified method of Aase et al. (1991). Cells in 96-well plate were washed with 200 μl of HEPES-buffered saline (120 mM NaCl, 5 mM KCl, 0.62 mM MgSO₄, 1.8 mM CaCl₂, 10 mM HEPES, 6.0 mM glucose, pH 7.4) and incubated with 50 μl of Fluo-3/AM at 37 °C for 60 min. Loading was terminated by removing the media, and cells were washed with 200 μl of HEPES-buffered saline. The cells were then treated with capsaicin. Fluorescence was recorded with a Fluoroscan ascent FL (Labsystems) microtiter fluorometer using the fluorescent filter pairs (excitation wavelength, 485 ± 2 nm; emission wavelength, 538 ± 3 nm). Calibration was done with cells incubated with the ionophore A-23187 (10 μM in HEPES-buffered saline), which allowed sufficient influx of Ca²⁺ to attain the saturation level of binding with the intracellularly trapped Fluo-3 ligand (F_{max}). The fluorescence was quenched with 2 mM CuCl₂ dissolved in 0.9% NaCl plus 10 μM A-23187 to obtain the minimal fluorescence level (F_{min}). The observed relative fluorescence values for the cells were used with the following well-known equation to calculate the free cytosolic Ca²⁺ concentration [Ca²⁺]_i:

$$[\text{Ca}^{2+}]_i = K_D (F - F_{\min}) / (F_{\max} - F)$$

Where F is the observed fluorescence, which increases on binding of Ca²⁺ without shifts in excitation or emission wavelengths.

LDH activity measurement

LDH activity was determined using LDH-Cytotoxic Test kit. The 96-well plates were inoculated with 2×10^5 cells/well in 50 μ l of medium and then incubated for 2 days in a 5% CO₂ incubator at 37 °C. The medium was then carefully removed, and the cells washed twice with PBS (–). After washing, 50 μ l of PBS (–) was dispensed into each well, followed by treatment of the cells with 50 μ l of varying concentrations of capsaicin. PBS (–) and 0.2% Tween 20 dissolved in PBS were used as negative and positive controls, respectively. After a treatment for 15 min at room temperature, 50 μ l of coloring solution [Nitro-tetrazolium blue (3.7 mg/vial), diaphorase, and NADH dissolved in buffer solution (50 g l^{–1} DL-Lithium lactate)] were added into each well and allowed to stand for 60 min at room temperature. The reaction was terminated by the addition of 100 μ l of reaction terminator (0.5 N HCl) into each well. The absorbance of the reaction mixture was then measured at 570 nm using a microplate reader (BIO RAD, Model 550).

Results and discussion

Western blotting

Western blotting was carried out to check whether the capsaicin receptor exists in the Caco-2 cells. The Caco-2 cells was processed with capsaicin concentration of 10 μ M and 100 μ M. The former is the capsaicin concentration (the 10 μ M following) at which the capsaicin receptor expresses in the nerve cell (Caterina et al., 1997), and the latter is the capsaicin concentration (100 μ M over) at which the TER of the human intestinal epithelial monolayers is reduced (Erika et al., 1997). However, in the study, there was no expression of the capsaicin receptor at 10 μ M concentration (data not shown).

Results of the Western blotting showed the expression of the capsaicin receptor VR1 or VR1-like protein in Caco-2 cells after 8 to 24h incubation in 100 μ M capsaicin (Figure 2).

A 95 kDa protein was cross-linked with VR1 anti-serum, and the expression of the protein was enhanced in time-dependent manner after treatment with 100 μ M of capsaicin.

Effect of capsaicin on TER of Caco-2 monolayers

The permeability of the Caco-2 monolayers was increased by capsaicin (Figure 3). The permeability of the Caco-2 monolayers was increased significantly after 45 min of treatment with capsaicin (from 300 to 500 μ M). HEK293 cells responded to low-concentration of capsaicin (0.6–3 μ M) (Caterina et al., 1997). In contrast, the Caco-2 monolayers responded to high-concentration of capsaicin (from 100 to 500 μ M). This is because the cells differ in their sensitivity to capsaicin.

On the other hand, the pretreatment of the Caco-2 monolayers with 10 μ M capsazepine, which is a competitive antagonist of the vanilloid receptor, showed almost no change in the permeability of the Caco-2 monolayers. This result suggests that capsazepine prevented the interaction between capsaicin and capsaicin receptor (Figure 4).

As shown in Figure 5, the pretreatment of the Caco-2 monolayers with 10 μ M ruthenium red, which is a noncompetitive antagonist of the vanilloid receptor, resulted in increased permeability only at capsaicin concentration of 200 μ M. In the case of the HEK293 cells, capsazepine and ruthenium red inhibited the interaction between capsaicin and capsaicin receptor (Caterina et al., 1997). Therefore, this result suggests that the vanilloid receptor of HEK293 and the receptor of the Caco-2 cells are different.

Measure of capsaicin-induced increase in intracellular Ca²⁺ and LDH activity in Caco-2 cells

VR1 ion channel has high Ca²⁺ permeability (Arpad et al., 1999). We observed the capsaicin-induced increase in intracellular Ca²⁺, and this result shows that VR1 (or VR1-like protein) exists in the Caco-2 cells (Figure 6). The increased in the intracellular Ca²⁺ was very high at capsaicin concentration of 200 μ M.

Moreover, LDH activity of the Caco-2 cells was high at 200 μ M of capsaicin (Figure 6). This is the same as in the case of intracellular Ca²⁺. However the high LDH activity of the Caco-2 cells was not seen at 300 and 400 μ M of capsaicin. These results suggest that the interaction between capsaicin and capsaicin receptor is observed specifically at 200 μ M of capsaicin.

From these results, we proposed the existence of capsaicin receptor-like protein in human intestinal cell line Caco-2.

There are needs for further studies on DNA sequence, searching homology with VR1 of the VR1-

Figure 1. Structure of capsaicin.

Figure 2. Time-dependent expression of the capsaicin receptor VR1 in Caco-2 cells after incubation with 100 μ M capsaicin as determined by immunoblotting using VR1 antiserum.

Figure 3. Effect of capsaicin on the TER. TER values are presented as relative to the values at zero time. (○) 0 μ M; (●) 100 μ M capsaicin; (Δ) 200 μ M capsaicin; (▲) 300 μ M capsaicin; (□) 400 μ M capsaicin; (■) 500 μ M capsaicin.

Figure 4. Effect of capsazepine (10 μ M, 30 min) on the TER-decreasing activity of capsaicin. (○) 0 μ M; (●) 100 μ M capsaicin; (△) 200 μ M capsaicin; (▲) 300 μ M capsaicin; (□) 400 μ M capsaicin; (■) 500 μ M capsaicin. Standard deviation is < 10%.

Figure 5. Effect of ruthenium red (10 μ M, 30 min) on the TER-decreasing activity of capsaicin. (○) 0 μ M; (△) 200 μ M capsaicin; (▲) 300 μ M capsaicin. Standard deviation is < 10%.

Figure 6. Increase in $[Ca^{2+}]_i$ and rate of released LDH activity. Standard deviation is < 10%.

like protein, and the influence of the degree of permeability by capsaicin.

Conclusion

In this study we observed the VR1 (or VR1-like protein) in Caco-2 cells. The permeability of the Caco-2 monolayers increased significantly after 45 min treatment with capsaicin (from 300 to 500 μ M). The permeability of the Caco-2 monolayers was not observed in the presence of the competitive antagonist of the vanilloid receptor capsazepine. These results show that the rise in the permeability of the Caco-2 monolayers is due to the binding of capsaicin and capsaicin receptor.

Furthermore, as shown in Figure 6, the numerical value of LDH and intracellular Ca^{2+} concentration were high at 200 μ M of capsaicin. Thus it could be argued that the binding of receptor and ligand was remarkably inhibited by about 200 μ M of capsaicin. Moreover, there were no remarkable changes in the intracellular Ca^{2+} concentration and LDH activity at

concentrations above 300 μ M of capsaicin (300 ~ 500 μ M capsaicin). These experimental results suggest that the decrease in TER value by the capsaicin treatment was not due to any damage to the cell monolayer, but rather due to the reversible opening of a paracellular route.

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