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Effect of Sodium Azulene Sulfonate on Capsaicin-Induced Pharyngitis in Rats

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Abstract: Sodium azulene sulfonate is a water-soluble derivative of azulene which is an antiinflammatory component of chamomile of the family of *Asteraceae*. Sodium azulene sulfonate is clinically used as a therapeutic agent in the treatment of pharyngitis as well as other inflammatory diseases such as tonsillitis, stomatitis and conjunctivitis. There has been no documentation on the effect of sodium azulene sulfonate on pharyngitis in laboratory models, probably because of no availability of such models. We recently established a pharyngitis model using capsaicin application on pharyngeal mucosa in rats. The present study investigated the antipharyngitis activity of sodium azulene sulfonate comparing with those of ruthenium red (vanilloid receptor antagonist, 8.5 and 85 mg/ml), ascorbic acid (antioxidative compound, 100 μg/ml), povidone iodine (gargle as disinfectant, oxidative compound, 5 and 20 mg/ml) and diclofenac sodium (cyclooxygenase inhibitor, 0.1 and 1 mg/ml). As an antipharyngeal effect, the capsaicin-induced plasma exudation in the pharyngeal mucosa of the rat was evaluated. The capsaicin-induced plasma exudation in the pharyngeal mucosa was inhibited by sodium azulene sulfonate (100 and 200 μg/ml) as well as ruthenium red and ascorbic acid, but not by povidone iodine and dicrofenac sodium; povidone iodine rather promoted the plasma exudation. In conclusion, the antipharyngitis effect of sodium azulene sulfonate was demonstrated for the first time in a laboratory model. Although the mechanism by which sodium azulene sulfonate inhibited the capsaicin-induced pharyngitis is not yet unraveled, antioxidative effect, but not inhibitory effect on cyclooxygenase pathway, might be involved.

The pharyngitis is a common inflammatory disease of the oropharynx presenting symptoms of sore throat, erythema and chapping of throat, which is attributed predominantly to infections of viruses and bacteria. Irritation and sore of throat accompanied by mild oedema and erythema of the pharynx are present in about 80% of patients with the common cold syndrome due to viruses such as rhinoviruses, coronaviruses, influenza viruses, parainfluenza viruses and adenoviruses, and due to bacteria including *Streptococcus pyogenes* (Lang & Singh 1990; Peter 1992). Cigarette smoking also becomes a cause of sore throat. The complaint of pharyngitis is responsible for an estimated 40 million outpatient visits in the United States in a year (Vukmir 1992).

Sodium azulene sulfonate is a water-soluble derivative of azulene that is an anti-inflammatory component of chamomile, *Matricaria recutita, Asteraceae.* Azulene and sodium azulene sulfonate are clinically used for the medical treatment of pharyngitis as well as gastric ulcer, gastritis, conjunctivitis, aenoiditis and stomatitis. It has been reported that guaiazulene, a lipophilic azulene derivative, shows not only an antiinflammatory effect (Yanagisawa *et al*. 1990) but also an antioxidative effect (Kourounakis *et al*. 1997). Thus, a possibility that sodium azulene sulfonate, which is a guaiazulene relative compound, also has an antioxididative effect can be presumed. An antioxidative effect may inhibit the tissue damage induced by the nimious oxygen radicals which leukocytes and macrophages produce in inflammatory process, and may bring about antiinflammatory effect (Nowak *et al*. 1991). Recently, the microbes might be killed by proteases, activated by oxidase through the generation of a hypertonic, K^+-rich and alkaline environment in the phagocytic vacuole (Reeves *et al*. 2002). Furthermore it was showed that K^+ crosses the membrane through large-conductance Ca^{2+} activated K^+ channel in neutrophil (Ahluwalia *et al*. 2004).

Capsaicin is known to be the prototype of neurogenic irritants. Topical application of capsaicin to rat skin leads to excitation of afferent neurons (Kinins 1982), increase in skin blood flow (Inoue *et al*. 1993), and vasodilation (Lynn *et al*. 1992). Neuropeptides such as substance P and neurokinin A, which are released by capsaicin from peripheral endings of afferent neurones via vanilloid receptor (Caterina *et al*. 1997), have been considered as chemical mediators of skin inflammation (Holzer 1988 & 1991; Maggi & Meli 1988; Saria *et al*. 1988). Substance P which is a tachykinin found in the C-fiber nerve endings of the airways of a variety of species including man, influences several airway functions; it increases mucus secretion, epithelial chloride secretion, and vascular permeability and stimulates airway smooth muscle contraction (Martling 1987). Though suitable animal models for studying pharyngitis and for development of effective drugs for the disease have not hitherto been reported, we lately established a pharyngitis model

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using capsaicin in rats. In the capsaicin-induced pharyngitis, tachykinins were mainly involved and a participation of NK_1 receptor was suggested (Yamabe *et al.* 1998).

Prostaglandin E_2, a prostanoid derived from arachidonic acid metabolism through the enzymatic action of cyclooxygenase and prostaglandin E_2 synthase, is released from a number of cells in the respiratory organs during various airway inflammatory reactions (Holtzman 1991). The airway epithelium, which is the primary target of initial assault by the inhaled irritants, is also the major cellular source of prostaglandin E_2 (Holtzman 1991). The prostaglandin E_2 activates sensory ending in the lungs; for example, inhalation of aerosolized prostaglandin E_2 elicits coughs and retrosternal soreness (Costello 1985; Taguchi 1992). Furthermore, inhaled prostaglandin E_2 enhances the sensitivity of the cough reflex elicited by capsaicin in man (Choudry *et al.* 1989), suggesting a prostaglandin E_2-induced sensitization of pulmonary C-fiber afferents. Prostaglandin E_2 generated by cyclooxygenase may therefore participate in the capsaicin-induced pharyngitis.

In the present study, we investigated the effects of sodium azulene sulfonate on the capsaicin-induced pharyngeal plasma exudation in rats. Furthermore, in order to investigate the mechanism(s) of the capsaicin-induced plasma exudation, the effects of ruthenium red (vanilloid receptor antagonist), ascorbic acid (antioxidative compound), povidon iodine (oxidative compound) and diclofenac sodium (cyclooxygenase inhibitor) were also examined.

Materials and Methods

Animals. Animals were housed for appropriate time intervals in the animal center of Hoshi University after their arrival. Constant temperature and humidity (22±1°, 55±10%) were maintained with a fixed 12 hr light-dark cycle and free access to food and water. Experiments were performed under the guiding principles for the care and use of laboratory animals approved by the Animal Care Committee of Hoshi University (Tokyo, Japan).

Effects of sodium azulene sulfonate and several drugs on pharyngeal plasma exudation induced by capsaicin. Male Wistar rats, weighing 280–490 g (Tokyo Laboratory Animal Co., Japan), were used. Animals were anaesthetized with urethane (2 g/kg, intraperitoneally), placed in the supine position and given spontaneous respiration through a tracheal cannula after treatment with atropine sulfate (0.2 mg/kg, intraperitoneally). To study the effects of the drugs on the capsaicin-induced pharyngeal plasma exudation, rats were administered with a 0.5 ml drug solution into the oral cavity 30 min. prior to capsaicin treatment, after binding the upper part of the oesophagus and the trachea with thread. The treatment drugs were as follows: Azunol® (1:800, 1:400 and 1:200 dilute distilled water; 50, 100 and 200 µg/ml as sodium azulene sulfonate), vehicle of Azunol®, ruthenium red (8.5 and 85 mg/ml, vanilloid receptor antagonist), ascorbic acid (100 µg/ml), povidone iodine (5 and 20 mg/ml), diclofenac sodium (0.1 and 1 mg/ml) and saline as a control. Experimental pharyngitis was induced by application of capsaicin solution with a cotton-tripped applicator on the surface of pharyngeal mucosa. Before capsaicin application, oral cavity was washed two times with 0.5 ml saline. When the capsaicin solution was applied, the tongue was slightly pulled out with a foreceps and the pharynx area was opened deep in the oral cavity with a small rib spreader. A 0.3 mM (0.25 ml) capsaicin-soaked cotton was swabbed each for about 3 sec. gently totally three times. Because capsaicin was dissolved in a mixture of 10% ethanol-10% Tween 80–80% distilled water, the rats in the control group were given vehicle alone. After capsaicin solution was applied, a period of 60 min. was allowed before evaluation of plasma exudation. For a quantitative evaluation of the capsaicin-induced plasma exudation in the rat pharyngeal mucosa, extravasation of Evans blue dye into the pharyngeal tissue was determined. Evans blue dye (30 mg/kg, intravenously) was injected into the femoral vein 10 min. prior to the application of capsaicin. Sixty min. after the capsaicin application, exsanguination was done from the abdominal aorta. Each animal's head portion was perfused with 180 ml of citric acid buffer (5% of paraformaldehyde in 0.05 M sodium citrate solution adjusted to the pH 3.5 with 0.05 M citric acid solution) at a rate of 15 ml/min.

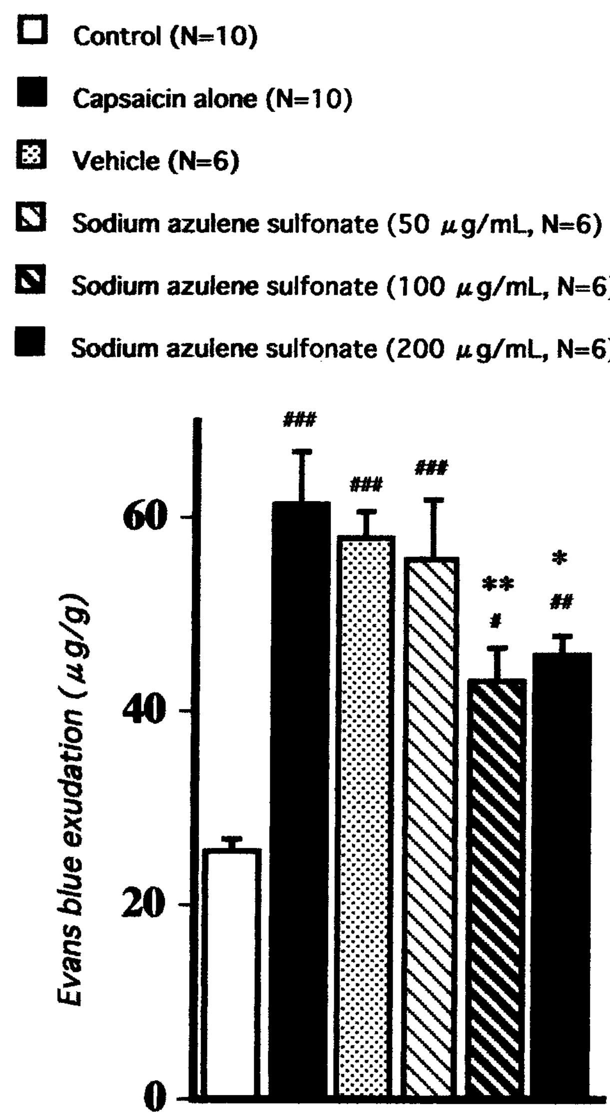

Fig. 1. Effect of sodium azulene sulfonate on capsaicin-induced Evans blue exudation in the rat pharynx. Treatment with 0.3 mM capsaicin significantly increased Evans blue exudation ($P<0.001$). Sodium azulene sulfonate was administered into the oral cavity 30 min. before capsaicin application. Pretreatment with sodium azulene sulfonate (100 and 200 µg/ml) significantly inhibited the Evans blue exudation induced by capsaicin. Values are means±S.E. from 6–10 experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ versus control. $^{*}P<0.05$ and $^{**}P<0.01$ versus capsaicin.

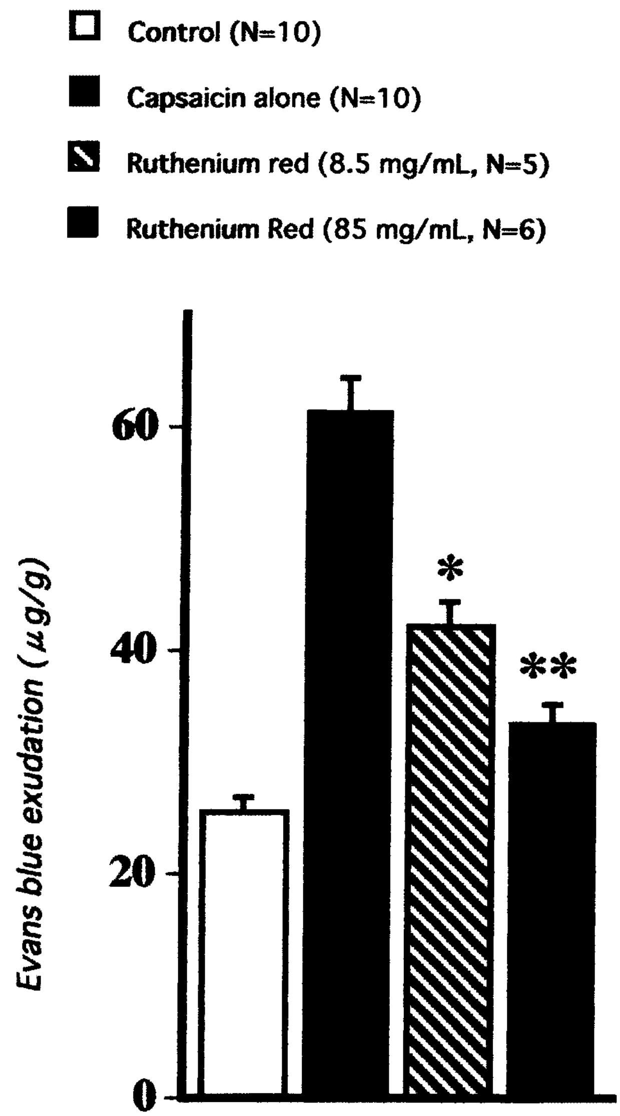

Fig. 2. Effect of ruthenium red on capsaicin-induced Evans blue exudation in the rat pharynx. Ruthenium red was administered into the oral cavity 30 min. before capsaicin application. Pretreatment with ruthenium red (8.5 and 85 mg/ml) significantly inhibited the Evans blue exudation induced by capsaicin ($P<0.01$). Values are means±S.E. from 5–10 experiments. *$P<0.05$ and **$P<0.01$ versus capsaicin.

via the bilateral carotid arteries to expel the intravascular dye; the perfused buffer being eliminated from an incision of the right atrium. Then, the bilateral musculus masseter of the rat was incised and the lower jaw was removed to enable extirpation of the pharynx. The pharyngeal mucosa was isolated by separation from the oesophagus and trachea; the soft palate, tongue, larynx and nasal tissues were removed. The isolated pharynx contained the portion ranging from the caudal end of the soft palate to the epiglottis just at the beginning of the larynx, and weighed 60–90 mg. Evans blue dye in the tissue was extracted in formamide at 60° for 24 hr and determined spectrophotometrically at 620 nm. The tissue dye content was expressed as micrograms of dye per gram of wet weight of tissue.

Reagents. Azunol® gargleliquid was gifted from Nippon Shinyaku Co. Ltd. (Kyoto, Japan). Urethane (ethyl carbamate) was obtained from Sigma (St. Louis, MO, USA). The other reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Statistical analyses. All data are expressed as mean±S.E. Statistical significance was determined by one-way analysis of variance (ANOVA). ANOVA was carried out with Bonferroni/Dunn's test.

Results

Effects of sodium azulene sulfonate and several drugs on pharyngeal plasma exudation induced by capsaicin.

The amount of Evans blue leakage increased significantly in the capsaicin-treated group (61.2±5.4 μg/g) compared with that in the control group (vehicle of capsaisin, 25.4±1.2 μg/g, $P<0.001$) (fig. 1). Sodium azulene sulfonate

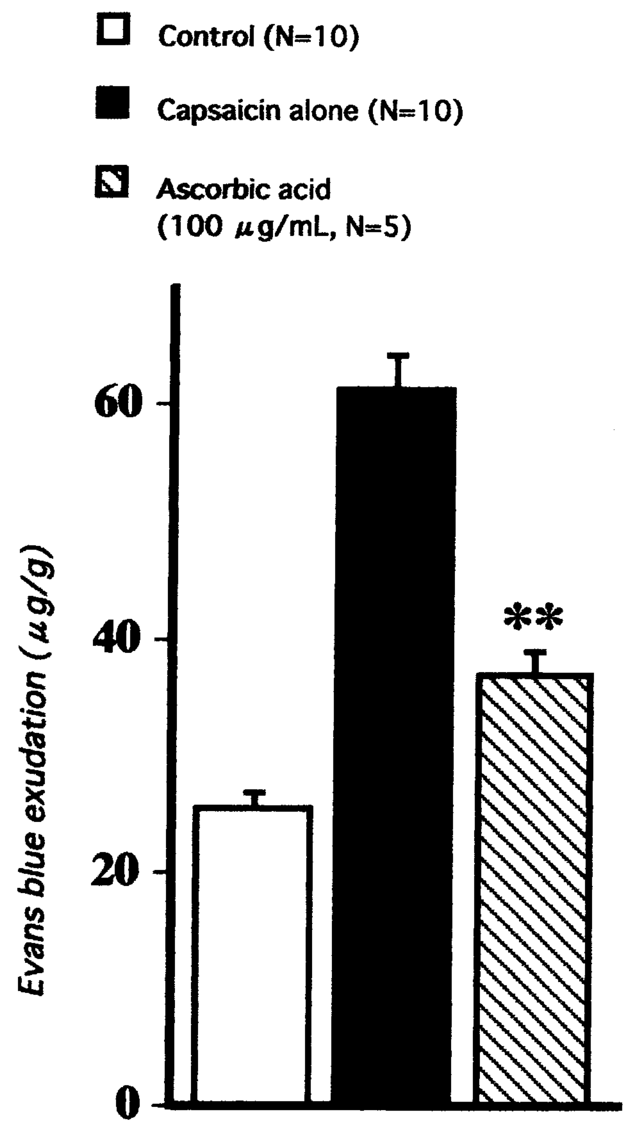

Fig. 3. Effect of ascorbic acid on capsaicin-induced Evans blue exudation in the rat pharynx. Ascorbic acid was administered into the oral cavity 30 min. before capsaicin application. Pretreatment with ascorbic acid (100 μg/ml) significantly inhibited the Evans blue exudation induced by capsaicin ($P<0.01$). Values are means±S.E. from 5–10 experiments. **$P<0.01$ versus capsaicin.

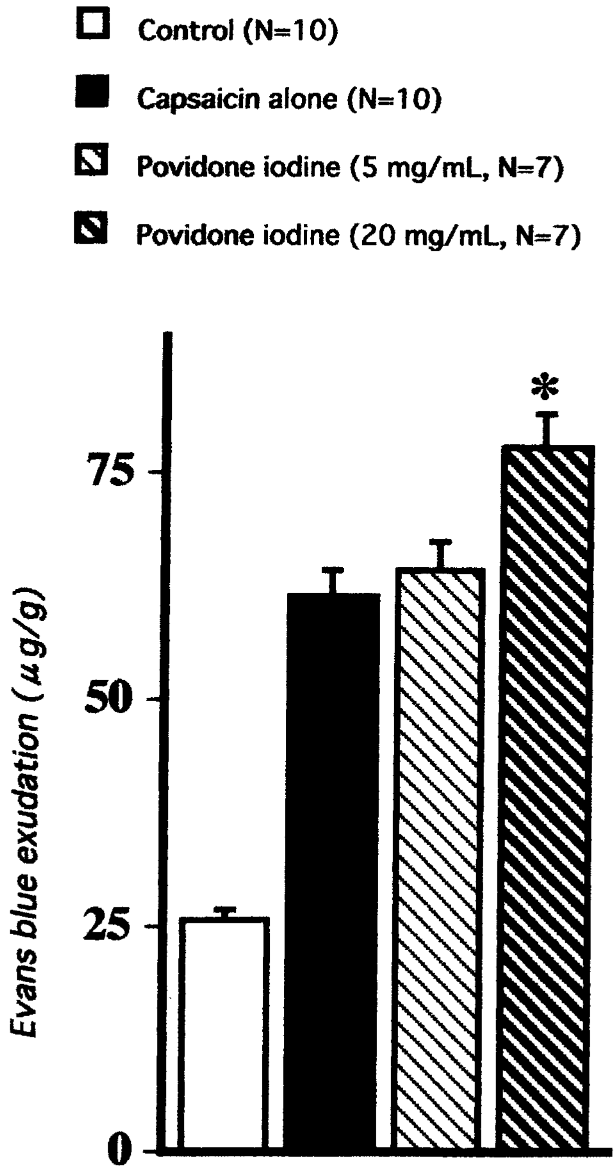

Fig. 4. Effect of povidone iodine on capsaicin-induced Evans blue exudation in the rat pharynx. Povidone iodine (5 and 20 mg/ml) was administered into the oral cavity 30 min. before capsaicin application. Pretreatment with povidone iodine (20 mg/ml) significantly increased the Evans blue exudation induced by capsaicin ($P<0.05$). Values are means±S.E. from 7–10 experiments. *$P<0.05$ versus capsaicin.

in concentrations of 100 and 200 μg/ml significantly inhibited the capsaicin-induced pharyngeal plasma exudation (43.1±3.3 μg/g and 45.7±2.1 μg/g, ratio of inhibition: 50.6±9.3 and 43.4±6.0%, IC50=64.1 μg/ml, $P<0.01$ and $P<0.05$, respectively). Amount of the capsaicin-induced Evans blue leakage was significantly decreased by ruthenium red (antagonist of vanilloid receptor) in concentrations of 8.5 mg/ml and 85 mg/ml (42.1±6.8 and 33.3±3.4 μg/g, ratio of inhibition: 53.4±19.1 and 77.9±23.0%, $P<0.05$ and $P<0.01$, respectively) in a concentration-dependent manner (fig. 2). The finding suggestes that the increase in Evans blue leakage by capsaicin was attributed to the effects mostly via vanilloid receptors. Pretreatment with 100 μg/ml of ascorbic acid (antioxidative compound) significantly decreased the capsaicin-induced Evans blue extravasation to 36.9±1.8 μg/g (ratio of inhibition: 67.9±5.0%, $P<0.01$) (fig. 3). Fig. 4 shows the effect of povidon idoine (oxidative compound) on the pharyngeal plasma exudation. Povidon iodine (20 mg/ml) significantly increased the amount of Evans blue leakage induced by capsaicin to 77.6±8.7 μg/g (ratio of inhibition: −45.8±24.3, $P<0.05$). Diclofenac sodium (cyclooxygenase inhibitor) (0.1 and 1 mg/ml) was without effect

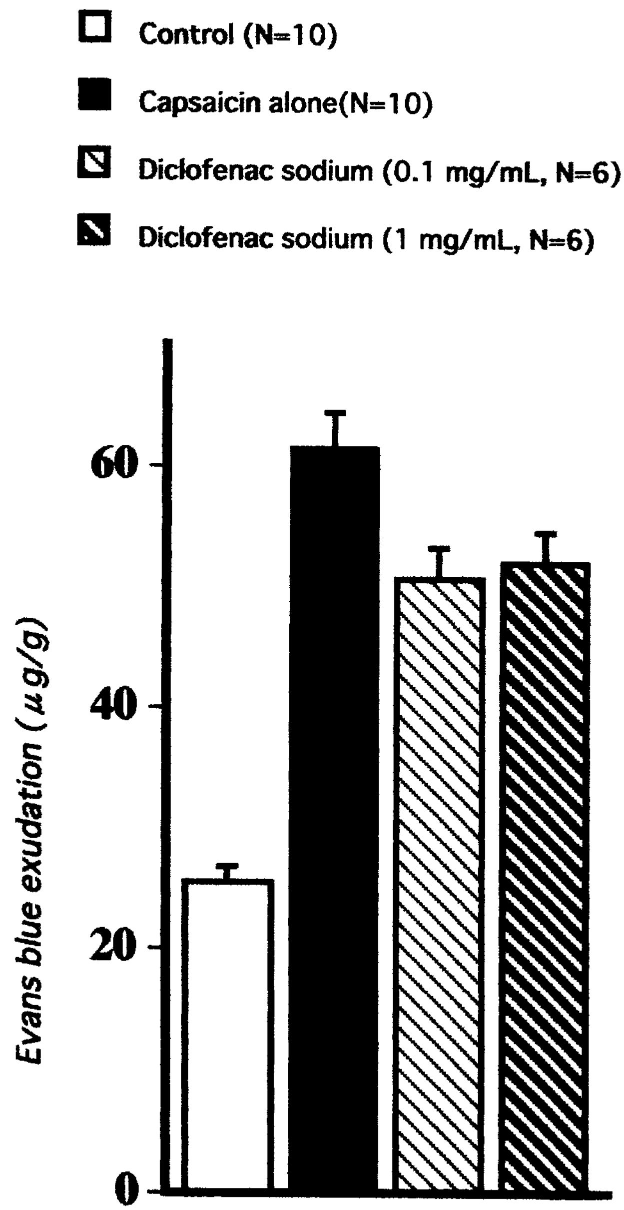

Fig. 5. Effect of diclofenac sodium on capsaicin-induced Evans blue exudation in the rat pharynx. Diclofenac sodium (0.1 and 1 mg/ml) was administered into the oral cavity 30 min. before capsaicin application. Pretreatment with dicrofenac sodium (20 mg/ml) was without effect on the capsaicin-induced the Evans blue exudation. Values are means±S.E. from 6–10 experiments. Figure 1

on the capsaicin-induced Evans blue leakage (ratio of inhibition: 29.4±11.8 and 26.0±18.8%) (fig. 5).

Discussion

Capsaicin is known to cause excessive activation of primary afferent sensory neurones and to cause a release of neuropeptides including substance P and neurokinin A from nerve endings (Buck & Burks 1986). Substance P and neurokinin A have potent proinflammatory effects in the airway tissues. Substance P is localized in afferent nerve terminals and sensory neuronal cell bodies of the trigeminal ganglion (Saria *et al.* 1988; Baraniuk & Kaliner 1991; Baraniuk *et al.* 1991; Barnes 1987; Barnes *et al.* 1991 & 1998). After released from the afferent nerve terminals, substance P acts on NK_1-receptors and mediates neurogenic inflammation. Neurogenic inflammation can be described as increased vascular permeability, plasma extravasation, glandular secretion and pro-inflammatory cell influx which are mediated by substance P. Recently, it has been proposed that the phenomena found in neurogenic inflammation may be partly mediated via activation of capsaicin vanilloid receptor 1 known as a polymodal receptor (Caterina *et al.* 1997; Tominaga *et al.* 1998; Trevisani *et al.* 2002). Ruthenium red, a functional vanilloid receptor antagonist, inhibits the capsaicin effect on sensory neurons by an action on the plasma membrane to prevent opening of capsaicin-coupled ion channels (Dray *et al.* 1990; Amann & Maggi 1991). In the present study, the capsaicin-induced pharyngitis was inhibited by ruthenium red, suggesting that capsaicin caused pharyngeal plasma exudation via vanilloid receptors. Indeed, we priviously reported that the capsaicin-induced pharyngitis was abolished by tachykinins antagonists (Yamabe *et al.* 1998). In a preliminary study, we evaluated the binding activity of sodium azulene sulfonate to NK_1 and NK_2 receptor using radioligand binding assay. Sodium azulene sulfonate did not significantly bind to NK_1 and NK_2 receptors. It is thus suggested that sodium azulene sulfonate did not inhibit the capsaicin-induced pharyngitis by directly blocking NK_1 and NK_2 receptors. Diclofenac sodium which is one of the non-steroidal antiinflammatory drugs (NSAIDs) did not inhibit the capsaicin-induced pharyngitis. The result shows that cyclooxygenase products such as prostaglandin E_2 are not participating in the pharyngitis that capsaicin induces. This finding was in agreement with our previous report using indomethacin (Yamabe *et al.* 1998).

Preliminarily we demonstrated that sodium azulene sulfonate inhibited lipid peroxidation in rat hepatic microsome study. The finding suggests that sodium azulene sulfonate has an anti-oxidative activity. Radical mediated impairment of cellular functions has been known to associate with a number of disorders. Since biological membranes contain highly oxidizable polyunsaturated fatty acids, they are particularly vulnerable to radical attack, and the oxidation of biomolecules such as lipids, proteins and DNA is considered to be critical in the development of inflammation.

It was observed that povidon iodine (20 mg/ml) rather worsened the capsaicin-induced pharyngitis. Povidon iodine which is widely used for the purpose of the disinfection by oxidative action to various types of pharyngitis is therefore considered to have a paradoxical aspect: disinfectant effect as a beneficial one, and enhancing effect on microvascular exudation as a disadvantageous one. The capsaicin-induced pharyngitis was inhibited by ascorbic acid, antioxidative compound. Thus, oxidative radicals such as superoxide may be mediated in the capsaicin-induced pharyngitis in rats. Although mechanism of the therapeutic effect of sodium azulene sulfonate on the capsaicin-induced pharyngitis in the present study is not enough explainable, the antioxidative effect might be involve in mechanism(s) of inhibition of capsaicin-induced pharyngitis by sodium azulene sulfonate.

In conclusion, using the capsaicin-induced pharyngeal plasma exudation model in rats, the beneficial effect of sodium azulene sulfonate on pharyngitis was for the first time demonstrated experimentally. Moreover, our present pharyngitis model was shown to be useful to screen effective drugs.

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