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INDIAN RED SCORPION VENOM-INDUCED AUGMENTATION OF CARDIO-RESPIRATORY REFLEXES ELICITED BY TRPV1 AGONIST IS MEDIATED THROUGH GENERATIVE REGION OF PULMONARY C FIBRE

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Abstract

Intravenous injection of phenyldiguanide (PDG), phenylbiguanide (PBG) or capsaicin produces cardio-respiratory reflexes. It is shown that PBG (5-HT_3) and capsaicin ($TRPV_1$) involve different afferent pathways to evoke cardio-respiratory reflexes. It is known that *Mesobuthus tamulus* (MBT) venom augments PDG/PBG reflex responses. Therefore, this study was undertaken to evaluate whether MBT venom also augments capsaicin-induced reflex responses and the underlying mechanisms. Blood pressure, respiratory excursions and ECG were recorded in urethane anaesthetized adult rats. capsaicin was injected intravenously before and after MBT venom (100 µg/kg). At the end, pulmonary water content was determined. Injection of capsaicin (0.1-10 µg/kg) produced apnoea, hypotension and bradycardia in a concentration-dependent manner. Ondansetron (5-HT3 receptor antagonist) did not block capsaicin induced reflex response. After exposure to venom (100 µg/kg) capsaicin-induced responses were augmented and there was increased pulmonary water content. Methylene blue (guanylyl-cyclase inhibitor, 5mg/kg) did not block the venom–induced augmentation of capsaicin-response. However, after antagonist pretreatment there was no increase in pulmonary water content. In another series, sildenafil (phosphodiesterase V inhibitor, 100 µg/kg) did not augment the capsaicin-induced reflexes even though there was increased pulmonary water content. These observations indicate that venom-induced augmentation of cardio-respiratory reflex responses by capsaicin does not involve serotonin (5-HT_3) receptor mediated G-cyclase-cGMP pathway.

Keywords: guanylyl-cyclase inhibitor; Indian red scorpion venom; methylene blue; pulmonary edema; pulmonary C reflexes; sildenafil.

Introduction

Intravenous injection of phenyldiguanide (PDG), phenylbiguanide (PBG), 5-HT, capsaicin, acetylcholine or lobeline produces cardio-respiratory reflex responses by stimulating J receptors/pulmonary C fibre receptors located in the lung interstitium (4, 14, 16). PDG has been used extensively to evoke J-reflex which manifests as bradycardia, hypotension and apnoea/tachypnoea (5, 14). Pulmonary edema and pulmonary congestion are the natural stimuli for J-receptors (14). Indian red scorpion (*Mesobuthus tumulus*; MBT) venom produced pulmonary edema and augmented cardiorespiratory reflexes evoked by PDG by various inflammatory mediators such as prostaglandin bradykinin, histamine, kinins etc (2, 5, 12, 13). The augmentation is attributed to the increased vagal discharges evoked by PDG (3). It is further demonstrated that augmentation of PDG reflex and pulmonary edema by venom involve B_2 kinin receptors activating NO-cGMP pathway (11). The cGMP in turn increases the capillary permeability so as to sensitize the J receptors (11, 14). Recently, we have shown that PBG and capsaicin produce cardio-respiratory reflexes but the PBG-sensitive vagal afferents are not responsive to capsaicin (8, 9). Thus, involvement of separate afferent pathways for PBG and capsaicin has been implicated (8). Therefore, the present study was undertaken to examine whether venom also augments the reflex responses elicited by capsaicin involving similar mechanisms.

Materials and methods

Animals, anesthesia and recording procedure

Experiments were performed according to the guidelines of the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India for conducting animal experiments. Adult female rats of Charles Foster strain weighing 190 ± 25 g were used. The animals were anaesthetized with urethane (1.5 g/kg i.p). An additional dose of urethane (0.1-0.15 g/kg i.p) was injected whenever required. Trachea, jugular vein and femoral artery were cannulated. Tracheal cannulation was used to keep the respiratory tract patent; jugular venous cannulation for drug administration; and femoral artery cannulation for recording blood pressure via pressure transducer. Eletrocardiographic potentials were recorded by connecting the needle electrodes in standard limb lead-II configuration. Respiratory movements were recorded by securing a thread to the skin over the xiphisternum to a force-displacement transducer. All the recordings were taken on a chart recorder.

Drugs and Solutions

Capsaicin was obtained from Sigma Chemical Company St. Louis, MO, USA. MBT venom was procured from Haffkine Institute, Mumbai, India and methylene blue was from qualigens fine chemicals, Mumbai, India. Sildenafil citrate was from Mankind Pharma (New Delhi, India). Ondansetron was purchased from CIPLA Ltd, Mumbai. Stock solution of capsaicin (1 mg/ml) was prepared in ethanol. Scorpion venom, sildenafil (1 mg/ml), methylene blue (10 mg/ml) and ondansetron (1mg/ml) were prepared in distilled

water. Subsequent dilutions of all the drugs were made with normal saline at the time of administration. The volume of the injections was kept at 0.1 ml.

Experimental protocol

The animals were divided into 5 groups. In group-I (n=8), concentration (0.1-10 µg/kg)-response relation of capsaicin was determined before and after MBT venom. After obtaining the initial recordings (respiration, ECG, blood pressure), capsaicin (0.1 µg/kg) was injected in the jugular vein and the reflex changes in respiration, heart rate and arterial pressure were recorded for 60 s. Subsequently, the responses to next higher concentrations (0.3, 1, 3 and 10 µg/kg) of capsaicin were recorded, allowing 5 min between two successive concentrations. After 10 min, MBT venom (100 µg/kg) was injected and 30 min later capsaicin concentration-response was repeated as earlier.

In group-II (n=4), capsaicin (1 µg/kg) was injected initially and 10 min after ondansetron (10 µg/kg) pretreatment.

In group-III (n=10), animals were exposed to a concentration (10 µg/kg) of capsaicin and 10 min later venom was injected. After 30 min, responses to capsaicin were obtained again.

In group-IV (n=9), the effect of methylene blue for venom-induced changes in capsaicin response was ascertained. After obtaining the initial capsaicin (10 µg/kg) reflex response, methylene blue (5 mg/kg) was injected and 10 min later, capsaicin reflex response was obtained again. Subsequently, MBT venom was injected and 30 min later, capsaicin responses were recorded.

In group-V (n=3), after obtaining the initial capsaicin (10 µg/kg) response, sildenafil citrate (100 µg/kg) was injected and 30 min later capsaicin response was obtained again.

Determination of pulmonary water content

The pulmonary water content was determined by the method described earlier (5). Briefly, at the end of each experiment, the lungs were excised, weighed and dried to a constant weight in an oven (at 90º C for 48 h). The difference between wet weight and dry weight was calculated to determine the water content.

Analysis of data

Maximum fall in mean arterial pressure after capsaicin were computed and were normalized to the initial. In case of HR/RF, the decrease in rate in the first 15 s after capsaicin administration was computed and was normalized to the initial (before capsaicin) rate. The data were pooled to obtain mean ± SEM values. The concentration-response of capsaicin was tested by using one-way ANOVA and the effect of venom was compared by using two-way ANOVA. The multiple comparisons at various concentrations were

performed by using Student Newman-Keuls test. Student's *t*-test for paired/unpaired observations was also performed as required. A $P < 0.05$ was considered significant.

Results

Augmentation of capsaicin-induced reflex responses by MBT venom

The values of HR, RF and MAP at the beginning of the experiments (before) were 298 ± 17.4 bpm, 83 ± 6.0 per min and 101 ± 7.1 mmHg, respectively. Jugular venous injection of capsaicin (0.1-10 µg/kg; n=8) produced hypotension, bradycardia and apnoea in a concentration-dependent manner (Fig. 1).

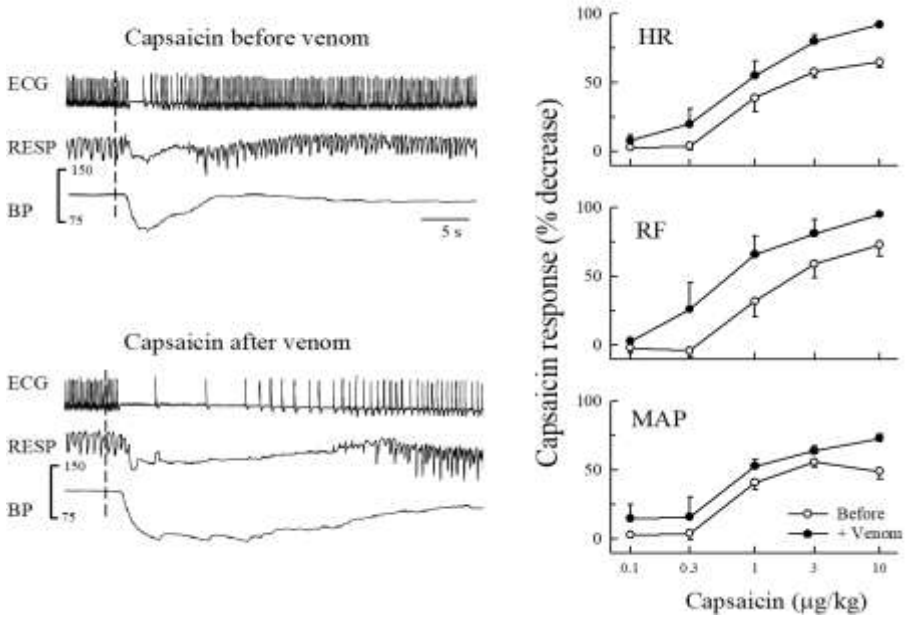

Fig. 1. *MBT* venom augmented the apnoeic, bradycadiac and hypotensive responses evoked by capsaicin. The original tracings of an experiment showing the capsaicin (10 µg/kg)-induced responses (respiration, RESP; electrocardiogram, ECG; and blood pressure, BP) before and after venom are given in the left. Vertical dashed line indicates the point of capsaicin administration. Horizontal line = 5 s for both. Line graphs to the right, show the concentration-response of capsaicin (0.1-10 µg/kg) before and after venom. The mean ± SEM values of mean arterial pressure (MAP), heart rate (HR) and respiratory frequency (RF) are obtained from 8 different experiments. The responses after venom are significantly different from the before values ($P < 0.05$, two-way ANOVA).

The threshold concentration of capsaicin to produce the responses was 1 µg/kg and the maximal concentration was 10 µg/kg. After injecting MBT venom (30 min), the HR, RF and MAP values were not different from before values. However, the reflex responses induced by capsaicin were augmented at all the concentrations and there was a shift of the concentration-response curve to the left ($P < 0.05$, two-way ANOVA followed by Student-Newman-Keuls test; Fig. 1). After venom, the threshold concentration of capsaicin was 0.3 µg/kg and was lesser than before value. In this group, the pulmonary water content was

79.9 ± 0.44% and was significantly greater than the saline treated group ($P < 0.05$, Student's *t* test for unpaired observations).

Ondansetron failed to block the capsaicin-induced reflex response

In this series of experiments (n=4), the resting (before) HR, RF and MAP were 282±24.2 bpm, 108±12.0 per min and 82.2± 22.1 mmHg, respectively. Capsaicin (1 µg/kg) produced hypotension, bradycardia and apnoea. Ondansetron (10 µg/kg) per se did not produce any alteration in the resting RF, MAP and HR. Further, ondansetron did not block the capsaicin (1 µg/kg)-induced reflex response (Fig. 2).

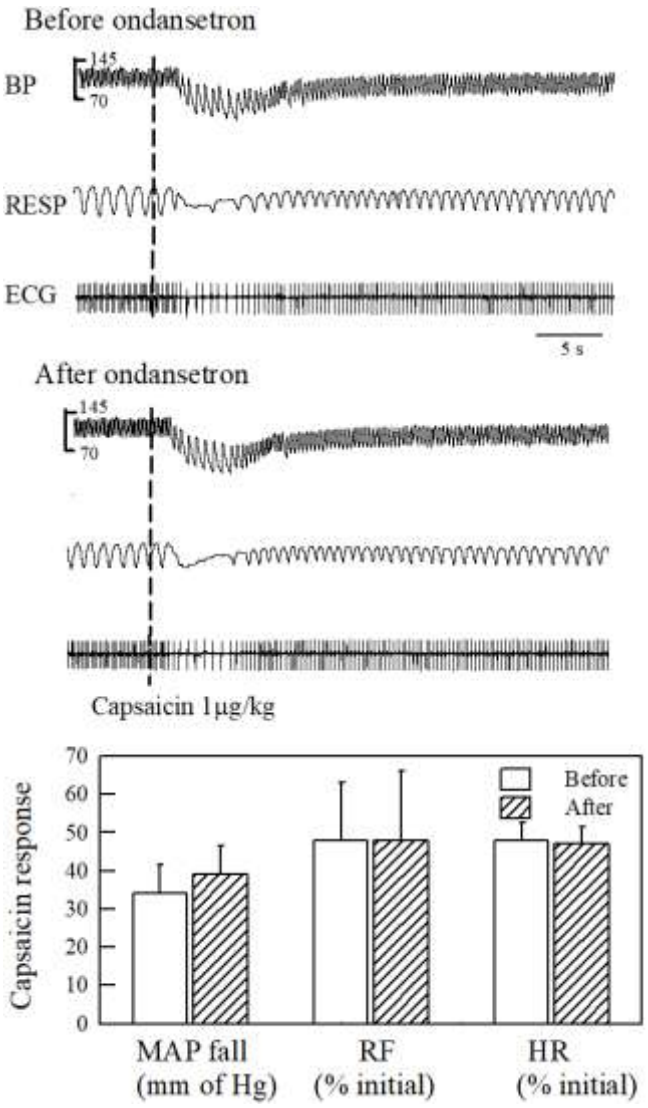

Fig. 2. Ondansetron did not block the capsaicin induced bradycardiac, apnoeic and hypotensive responses. The original tracings of respiration (Resp), electrocardiogram (ECG) and blood pressure (BP) before and after ondansetron at 1 µg/kg of capsaicin concentration are presented in upper panel. Vertical dashed line indicates the point of capsaicin administration in each record. Histograms showing the mean ± SEM values ΔMAP in mm of Hg and heart rate (HR) and respiratory frequency (RF), as % of initial at 1 µg/kg of capsaicin concentration before and after ondansetron.

G-cyclase inhibitor did not block venom-induced augmentation of capsaicin response but blocked the pulmonary edema

These experiments were performed with 10 µg/kg of capsaicin to have a comparable control data for evaluating the effect of G-cyclase inhibitor, methylene blue, on venom-induced augmentation of capsaicin response.

Venom only group

In this set of experiments (n=4), 10 µg/kg of capsaicin produced hypotension, bradycardia and apnoea. Time-matched responses (after 10 min exposure to saline) were not different from the initial capsaicin responses. After venom, the capsaicin responses were augmented significantly (Fig. 3C; $P < 0.05$, Student's *t* test for paired observations).

Methylene blue+venom group

In this series (n=5), the resting (before) HR, RF and MAP were 300 ± 14.7 bpm, 111 ± 4.7 per min and 89 ± 6.2 mmHg, respectively. Capsaicin (10 µg/kg) produced hypotension, bradycardia and apnoea as seen in the venom only group. After exposure to methylene blue (5 mg/kg; 10 min) the resting HR, RF and MAP were not different from the before values. The capsaicin reflex responses after methylene blue were also not different from the initial capsaicin responses (Fig. 3B). In methylene blue pretreated animals, exposure to venom did not alter the resting HR, RF and MAP. However, there was augmentation of capsaicin responses after venom as seen in venom only group (Fig. 3B).

Effect on pulmonary water content

The pulmonary water content in venom only group was 80% and in methylene blue treated group was 78.3%. The value was significantly lower than the venom only group (Fig. 3C; $P < 0.05$, Student's *t* test for unpaired observations) and was similar to saline treated group.

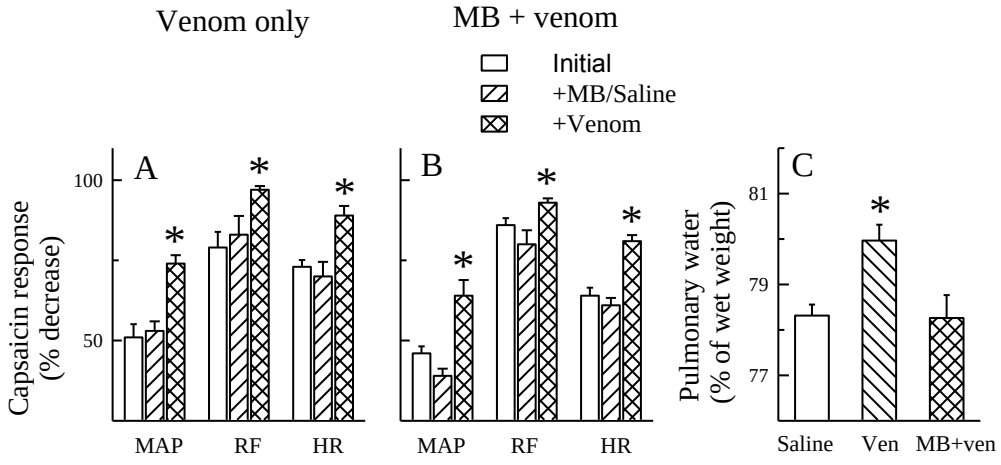

Fig. 3. Methylene blue failed to block the venom-induced augmentation of capsaicin response. Histograms in (A) and (B), capsaicin response in venom only and methylene blue pretreated group are shown. Histograms in C show the mean ± SEM values of pulmonary water content from venom treated and methylene blue pretreated experiments and compared with saline treated group (n=6). An asterisk '*' indicates P < 0.05 as compared to saline group (Student's *t*–test for unpaired observations). Ven=Venom only; MB+ven=Methylene blue+venom.

Phosphodiesterase V (PDE-V) inhibitor did not augment capsaicin-induced reflex responses but produced pulmonary edema

In this series of experiments (n=3), the resting (before) HR, RF and MAP were 292 ± 38.2 bpm, 96 ± 20.8 per min and 74 ± 8.1 mmHg, respectively. In these animals, capsaicin produced hypotension, bradycardia and apnoea as seen in the earlier groups. Phosphodiesterase V inhibitor, sildenafil, per se did not alter the values. After sildenafil, the capsaicin-induced responses were similar to the before responses ($P < 0.05$, two-way ANOVA followed by Student-Newman-Keuls test; Fig. 4). However, there was significant increase in pulmonary water content in this group ($P < 0.05$, Student's *t* test for unpaired observations; Fig. 4).

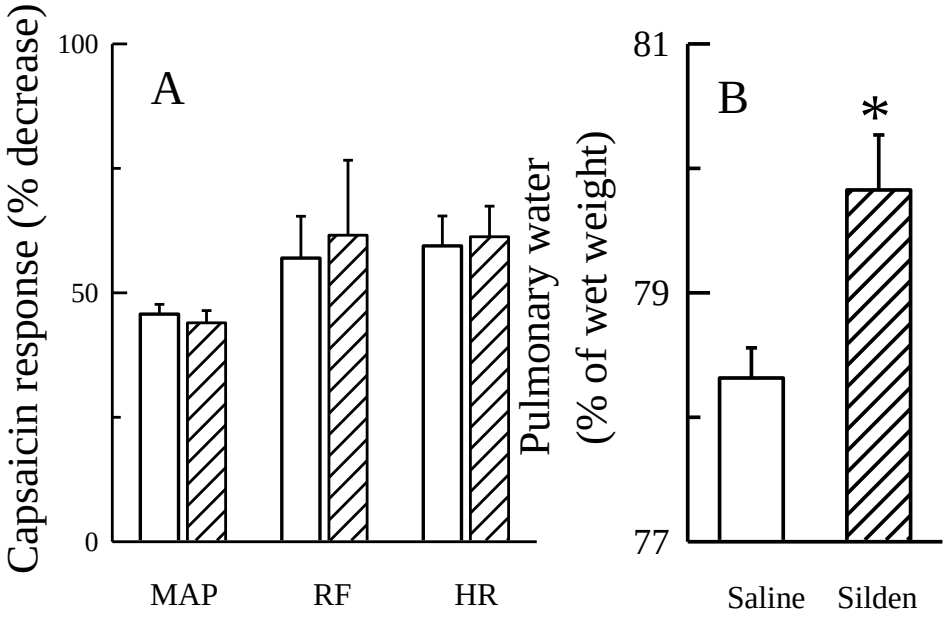

Fig. 4. Sildenafil did not augment the capsaicin-induced reflex response but produced pulmonary edema. Histograms in A depict capsaicin-induced reflex responses (respiratory frequency, RF; heart rate, HR; and mean arterial pressure, MAP) before and after sildenafil. In B, pulmonary water content in these animals are shown and compared with saline treated group (data taken from Fig. 3; * P < 0.05; Student's *t*–test for unpaired observations).

Discussion

Present observations demonstrate that MBT venom augments the capsaicin-induced cardio-respiratory reflexes similar to PDG responses as shown elsewhere (3, 5, 6, 10, 11). Augmentation of capsaicin responses does not involve G-cyclase mechanisms. Further, the venom-induced augmentation of capsaicin response is independent of pulmonary edema. It has been shown that capsaicin does not involve 5-HT_3 receptors to mediate the cardio-respiratory responses.

In our earlier studies, it has been shown that capsaicin does not involve PBG sensitive vagal afferents or 5-HT_3 receptors to mediate the cardio-respiratory response (8). Existence of two different afferent pathways for PBG and capsaicin has been suggested. Even though the responses induced capsaicin involve different afferents than PBG, venom augmented capsaicin responses in same magnitude. The data showing the left shift after venom support the sensitization of vagal nerve terminals. Hence, the capsaicin responses possibly utilize the mechanisms by sensitizing the nerve terminals.

In the earlier study it has been shown that ondansetron (5-HT3 receptor antagonist) completely blocked PDG induced reflex response and after venom the reflex response reappeared. Thus, there was sensitization of 5-HT3 receptors after envenomation by pulmonary edema induced by several inflammatory mediators. However, in this study, it has been observed that capsaicin induced reflex response through TRPV1 receptors are independent of 5-HT3 receptor mediated sensitization mechanisms.

In the earlier study, it is shown that venom-induced augmentation of PDG response and pulmonary edema involves NO-G-cyclase-cGMP pathways (11). Since G-cyclase is an important signaling molecule in this pathway, we tested the involvement of G-cyclase by using methylene blue. However, methylene blue did not block the augmentation of capsaicin response (Fig 3). Even the PDE-V inhibitor (sildenafil) that is known to increase cGMP level (17) also did not augment the capsaicin responses (Fig 4). These observations indicate the non-involvement of G-cyclase-cGMP pathway in mediating the augmentation of capsaicin response. However, in the earlier report, PDE-V inhibitor has augmented the PDG reflex response and also produced pulmonary edema (11).

Pulmonary edema is an important factor for the sensitization of J receptor/pulmonary C fibre receptor (1, 11, 14, 16). Previously, we have shown that MBT venom or its toxin augmented PDG response by producing pulmonary edema (3, 5, 6, 10, 11). However, in this study there was no pulmonary edema but the augmentation of capsaicin response persisted in methylene blue pretreated group. Further, in sildenafil pretreated group, pulmonary edema was observed but capsaicin response was not augmented. These observations suggest that pulmonary edema dependent factors are not involved for the augmentation of capsaicin response. Thus, factors other than pulmonary edema may be responsible for venom-induced augmentation of capsaicin response. Bradykinin can be one of such factor as bradykinin is shown to sensitize vagal nerve terminals especially that are sensitive to capsaicin (7).

Kinin mechanism has been implicated in the MBT venom-induced augmentation of PDG reflexes and pulmonary edema (3, 5). Further, aprotinin, kinin synthase inhibitor, protected the animals against toxicity and also reversed the pulmonary edema (15). Thus, kinins play a vital role in scorpion toxicity. The kinins are inflammatory mediators, trigger the signaling pathways via B_2 kinin receptor involving NO, G-cyclase, cGMP mechanisms (11). Thus, blocking of G-cyclase does not alter the kinin levels. Hence, it is likely that the kinins generated after venom in turn sensitizes the capsaicin-sensitive nerve terminals to augment capsaicin response. In a study elsewhere it has been shown that there are generative region and regenerative region in medullated as well as non-medullated vagal C fibre (18). TRPV1 receptors are located in the generative region and there are several other receptors responsive to inflammatory mediators located in the same region. Thus, sensitization of TRPV1 receptors by adjacent inflammatory mediator receptor may be a possibility (Fig 5).

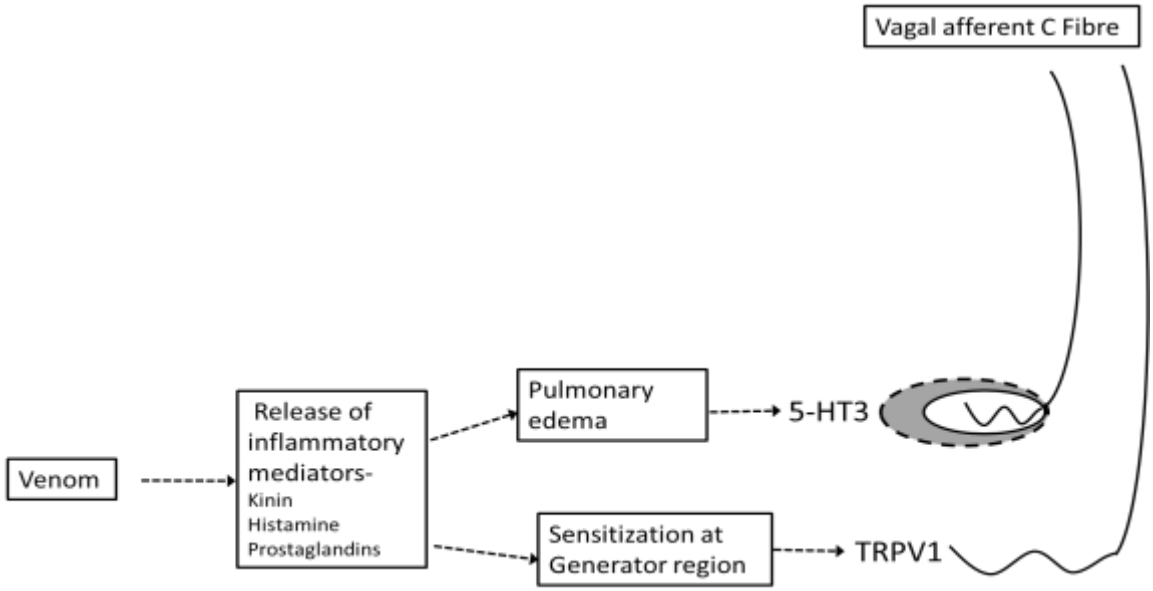

Figure 5. Schematic showing the action of venom on 5-HT3 and TRPV1 sensitive vagal afferent C fibre. Swelling of the connective tissue surrounding 5-HT3 receptors in vagal C fibre in response to pulmonary edema is represented by shaded area.

In conclusion, MBT venom augments the capsaicin induced reflex response however, the mechanisms underlying the augmentation of capsaicin response is different from those operating for PBG. Pulmonary edema is not involved in venom-induced augmentation of capsaicin response as shown for PDG earlier. Sensitization of capsaicin-sensitive neurons by cell injury products/inflammatory mediators after envenomation may be a possibility

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