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Bradykinin-evoked sensitization of neuropeptide release from afferent neurons in the guinea-pig lung

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1 It has been shown that bradykinin (BK) causes sensitization of airway sensory neurons and an enhancement of the cough reflex in guinea-pigs. In the present study, the guineapig isolated perfused lung was used to investigate the possible enhancement by BK of histamine-evoked neuropeptide release from peripheral terminals of primary afferent neurons, and to determine the contribution of cyclooxygenase products of arachidonate metabolism to this effect.

2 The lung was perfused with oxygenated physiological salt solution containing peptidase inhibitors (thiorphan, bestatin and captopril, 1 μM each). BK and histamine were added to the perfusate for 10 and 5 min, respectively.

3 BK alone (0.1 μM) evoked the release of 10.35 ± 2.4 fmol immunoreactive calcitonin gene-related peptide (CGRP), histamine alone (100 μM) evoked the release of 12.7 ± 1.6 fmol CGRP. Stimulation with 100 μM histamine in the presence of 0.1 μM BK (added 5 min before histamine and present during histamine) evoked the release of 67.1 ± 5.3 fmol CGRP.

4 Prostaglandin (PG) release was stimulated by BK (418 ± 71 pmol 15-keto-13,14-dihydro-$PGF_{2\alpha}$ and 345 ± 59 pmol 6-keto-$PGF_{1\alpha}$), and, to a lesser extent, by histamine (36.1 ± 7.4 pmol 15-keto-13,14-dihydro-$PGF_{2\alpha}$, and 24.6 ± 3.9 pmol 6-keto-$PGF_{1\alpha}$). Prostaglandin release induced by histamine in the presence of BK was not significantly higher than with BK alone.

5 Indomethacin (5 μM) as well as the bradykinin B_2 receptor antagonist HOE140 (icatibant, 1 μM) inhibited prostaglandin release following stimulation with histamine in combination with BK. CGRP release evoked by histamine in combination with BK was attenuated by indomethacin and HOE140 to 22.1 ± 7.8 fmol and 16.4 ± 3.8 fmol, respectively, significantly less than the value obtained in control experiments (67.1 ± 5.3 fmol).

6 The results suggest that BK-induced stimulation of prostaglandin synthesis results in facilitation of histamine–evoked release of pro-inflammatory neuropeptides from afferent neurons, a mechanism that probably becomes relevant during inflammation, and that can be blocked by a bradykinin B_2 receptor antagonist.

Keywords: Guinea-pig lung; bradykinin; histamine; prostaglandins; neuropeptides; release

Introduction

Peripheral sensitization of afferent neuron terminals is known to be an important factor in the development of inflammatory hyperalgesia. Bradykinin, in particular, has been shown to be very effective in sensitizing afferent neurons (*c.f.* Dray & Perkins, 1993). Its mode of action seems to be mainly indirect, secondary to stimulation of the cyclooxygenase pathway of arachidonic acid metabolism and resulting prostaglandin (PG) formation (*c.f.* Levine *et al.*, 1993).

Fox *et al.* (1996) have shown that in guinea-pigs, inhibition of the breakdown of endogenous BK by an angiotensin converting enzyme (ACE) inhibitor causes sensitization of airway afferent neurons, and an enhancement of the cough reflex in response to an inhaled irritant that can be inhibited by a bradykinin B_2 receptor antagonist. This observation suggests that an increase of the BK concentration in the pulmonary circulation can increase neuronal excitability leading to enhanced reflex responses. It seems reasonable to assume, therefore, that in this case the excitatory effect of histamine will exceed that found under normal conditions. Apart from sensitization of the afferent pathway and the resulting facilitation of autonomic reflexes, there is another intriguing aspect concerning the 'local effector function' (Holzer, 1988) of afferent C-fibres: Sensitization of C-fibre afferent neurons may induce a corresponding facilitation of the local release of pro-inflammatory neuropeptides (e.g. tachykinins, calcitonin gene-related peptide). In the respiratory system, this may contribute to vasodilation and bronchial edema formation, and in case of tachykinin release, bronchoconstriction (Barnes *et al.*, 1991; Lundberg *et al.*, 1991).

The present study was, therefore, aimed at investigating the possible influence of BK on histamine-evoked neuropeptide release in the guinea-pig isolated perfused lung.

Methods

Guinea-pig isolated lung perfusion

Experiments were conducted as described previously (Amann *et al.*, 1989). Guinea-pigs (300–450 g) of either sex were killed by an overdose of pentobarbital, and the pulmonary artery was cannulated through the right ventricle. The left atrium was opened to collect the outflow. The lung was perfused (6 ml/min) with oxygenated (95% O_2 5% CO_2) physiological salt solution (NaCl 118, KCl 4.6, $MgSO_4$ 1.17, $CaCl_2$ 2.5, NaH_2PO_4 1.17, $NaHCO_3$ 25, glucose 10 mM) at 37°C. All experiments were performed in the presence of thiorphan, bestatin and captopril (1 μM each). After an equilibration period of 20 min the outflow was collected (5 min fractions,

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for 30 min) on ice in glass vials containing acetic acid to give a final concentration of 0.5 M. An aliquot was immediately neutralized for determination of prostaglandin content, the remaining sample was desalted and concentrated on SepPak C_{18} cartridges (Waters). The first two fractions were collected to determine the basal concentrations of mediators. In fraction three, BK, [des-Arg^9]-BK, or PGE_1 were added to the perfusate for 10 min. Histamine was added in fraction 4 for 5 min, either in the presence, or in the absence of the other drugs.

Radioimmunoassay

After lyophilization, samples were used for radioimmunoassay (RIA). CGRP was determined using antiserum RAS 6009 N (Peninsula) raised against human CGRP. (2-[I^{125}]iodohistidyl^{10}) CGRP, human (Amersham) was used as radioligand and synthetic human alpha-CGRP (Peninsula) as standard. Substance P (SP) was determined using antiserum RD2 (gift of Dr. S. E. Leeman, Boston University School of Medicine, Dept. of Pharmacol., Boston MA 02118, U.S.A.), [I^{125}]Boston-Hunter-SP (Amersham) as radioligand and synthetic SP (Sigma) as standard.

Immunoreactive 15-keto-13,14-dihydro-$PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were determined as described previously (Liebig *et al*., 1974; Peskar *et al*., 1979) using 15-keto-13,14-dihydro[5,6,8,9,11,12,14(n)-^{3}H]$PGF_{2\alpha}$and 6-keto[5,8,9,11,12,13,15 (n)-^{3}H] $PGF_{1\alpha}$(Amersham) as radioligands and synthetic 15-keto-13, 14-dihydro-$PGF_{2\alpha}$ (Pesel & Lorai; Frankfurt/Main) and 6-keto-$PGF_{1\alpha}$ (Cayman) as standards, respectively.

15-keto-13, 14-dihydro-$PGF_{2\alpha}$ ($KH_2PGF_{2\alpha}$) was determined because in the guinea-pig it represents a major metabolite of endogenous pulmonary $PGF_{2\alpha}$ (Liebig *et al*., 1974), and, in contrast to PGE_2 and its corresponding metabolite, it is stable in an acidic environment. 6-keto-$PGF_{1\alpha}$ represents a major metabolite of endogenous pulmonary PGI_2 (Wong *et al*., 1978; Robinson *et al*., 1984) which is less extensively metabolized than PGs of the E or F series. In addition, a number of reports have shown that PGI_2 sensitizes afferent neurons similar to PGE_2 (Geppetti *et al*., 1991; Hintgen & Vasko, 1994; Vasko *et al*., 1994).

Determination of histamine

The concentration of histamine was determined after condensation with o-phtaldialdehyde by high pressure liquid chromatograph (HPLC) according to Skofitsch *et al*. (1981).

Materials

Bradykinin acetate, [des-Arg^9] bradykinin acetate, histamine dihydrochloride, bestatin hydrochloride, captopril (all obtained from Sigma), and HOE140 (D-Arg-[Hyp^3,Thi^5,D-Tic^7,Oic^8]-Bradykinin; provided by Hoechst AG) were dissolved in distilled water. A 23 187 (Sigma) was dissolved in dimethyl sulfoxide, PGE_1 and dl-thiorphan (both obtained from Sigma) were dissolved in 70% and 100% ethanol, respectively. Indomethacin (Sigma) was dissolved in 2% sodium bicarbonate and titrated to pH 7.2 with monosodium phosphate. Stock solutions (1 mM PGE_1, 10 mM thiorphan, 1 mM indomethacin) were diluted to final concentrations in physiological salt solution.

Data analysis

Total evoked release (TER) was calculated by subtracting the basal outflow from the total amount of released immunoreactivity in the fractions during and following stimulation. Without stimulation, the spontaneous release of eicosanoid metabolites remained constant throughout the course of the experiment. The amount of 6-keto-$PGF_{1\alpha}$ and of KH_2 $PGF_{2\alpha}$ released during the first 5-min collection period was 25.1 ± 3.8 pmol and 2.4 ± 0.2 pmol, respectively; values that were not significantly different ($P > 0.05$) from those obtained between 30 and 35 min after start of the collection period: 22.86 ± 1.62 pmol 6-keto-$PGF_{1\alpha}$, and 2.29 ± 0.21 pmol $KH_2PGF_{2\alpha}$; ($n = 5$). CGRP release seemed to declined gradually by less than 15% (from 9.23 ± 0.59 fmol to 7.88 ± 1.1 fmol released between 30 and 35 min after start of the collection period; $n = 5$; $P > 0.05$).

Values are expressed as means $\pm$ s.e.mean, statistical analysis was performed by the Kruskal Wallis Test and Dunn's Multiple Comparison Test. (SigmaStat statistical software, Jandel Scientific, Erkrath, Germany).

Results

Effect of bradykinin and of histamine on neuropeptide release

BK (10 min contact time) as well as histamine (5 min contact time) caused a concentration-dependent release of CGRP (Figure 1). When histamine (30 – 300 μM) was added to the perfusate in the presence of 0.1 μM BK, the total evoked release (TER) of CGRP was considerably higher than that produced by either compound alone (Figure 1). [des-Arg^9]-BK (1 μM, 10 min) did not evoke the release of CGRP (TER: -6.6 ± 5.2 fmol, $n = 4$), nor did the presence of 1 μM [des Arg^9]-BK significantly influence histamine (100 μM)-evoked release of CGRP (18.7 ± 5.5 fmol, $n = 4$).

In one group of experiments, substance P (SP) and CGRP were determined in parallel. The ratio SP/CGRP was 0.75 ± 0.09 ($n = 5$) when release was stimulated by histamine (100 μM) in the presence of BK (0.1 μM). This value was not significantly different from the SP/C GRP ratios obtained after

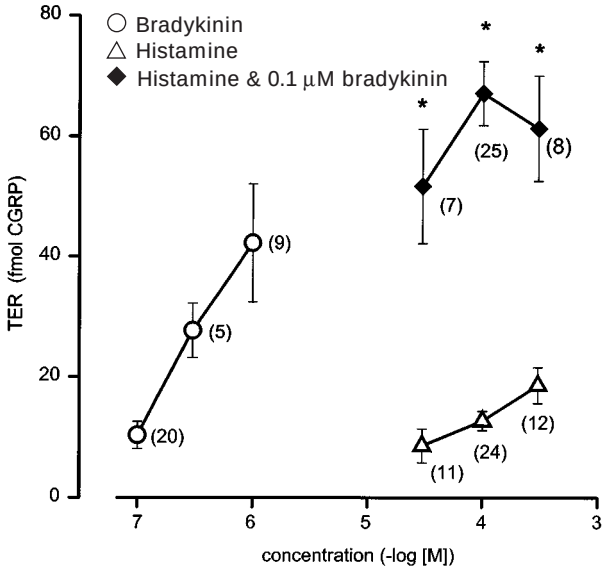

Figure 1 Total evoked release (TER) of CGRP by bradykinin, histamine and by histamine in the presence of bradykinin (0.1 μM). Values are means $\pm$ s.e.mean; n in parenthesis; $^*P < 0.05$ as compared to the corresponding concentration of histamine alone.

stimulation with histamine or bradykinin alone ($n=5$ each, data not shown).

In another group of experiments ($n=4$) we have determined whether or not 0.1 μM bradykinin stimulates histamine release from the isolated perfused lung. Without stimulation, the histamine concentration was 1.5–1.8 ng/ml, and remained constant for the duration of the experiment. Addition of 0.1 μM bradykinin for 10 min did not increase the concentration of histamine. The calcium ionophore A 23187 (30 μM, 5 min) was used as a positive control 30 min after the end of bradykinin exposure, and increased the concentration of histamine to 30–40 ng/ml.

Effect of bradykinin and of histamine on prostaglandin release

The BK (0.1 μM)-induced total evoked release of 15-keto-13,14-dihydro- $PGF_{2\alpha}$ (KH_2 $PGF_{2\alpha}$) and 6-keto-$PGF_{1\alpha}$ was 418 ± 71 pmol and 345 ± 59 pmol ($n=8$ each), respectively. Although histamine (100 μM) also caused a significant increase of these metabolites, its effect was moderate as compared to that of BK (Figure 2). The total evoked release of KH_2 $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ induced by histamine in the presence of BK was not significantly different from that induced by BK alone (Figure 2).

Influence of HOE140 and of indomethacin on prostaglandin and CGRP release

HOE140 (1 μM) prevented the BK (0.1 μM)-induced release of KH_2 $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ and the BK (0.1 μM)-induced release of CGRP ($n=9$, data not shown). In the presence of HOE140 prostaglandin release following stimulation with histamine (100 μM) in combination with BK (0.1 μM) was inhibited (Figure 2), and the evoked release of CGRP was 16.4 ± 3.8 fmol ($n=5$), a value that was not significantly different from that obtained with histamine alone (12.7 ± 1.6 fmol, $n=24$).

Indomethacin (5 μM) nearly abolished prostaglandin release induced by BK (0.1 μM), histamine (100 μM) ($n=4$ each, data not shown), or the combined administration of BK and histamine (Figure 2). In the presence of indomethacin, BK (0.1 μM) as well as histamine (100 μM) still evoked a detectable release of CGRP (Figure 3). The CGRP release following the combined administration of BK and histamine was significantly lower (22.1 ± 7.8 fmol, $n=8$) than in the absence of indomethacin (67.1 ± 5.3 fmol, $n=25$, see Figure 1), but higher than that produced by histamine alone (Figure 3).

In the presence of indomethacin, PGE_1 (1 μM, 10 min contact time) evoked a small but significant release of CGRP (11.2 ± 2.7 fmol, $n=4$). Addition of histamine (100 μM, 5 min) after a 5 min pre-exposure to PGE_1 resulted in the release of 38.8 ± 4.9 fmol CGRP ($n=7$), significantly higher than the effect produced by histamine alone (12.7 ± 1.6 fmol, $n=24$).

Discussion

The present results demonstrate enhancement by BK of histamine-induced release of CGRP in the guinea-pig isolated perfused lung. There is good reason to assume that the source of CGRP are capsaicin-sensitive neurons (Martling, 1987), belonging to the group of afferent C-fibres that are sensitive to mechanical and chemical stimuli (Fox *et al.*, 1993). It is known that cutaneous C-polymodal nociceptors respond to a wide range of chemical stimuli, including several autacoids that are formed during inflammation (*c.f.* Perl, 1996). It has been reported that about 50% of C-polymodal nociceptors in the rat skin respond to BK, while only 14% are responsive to histamine (Handwerker, 1992). The small number of histamine-sensitive neurons may explain that, in the guinea-pig respiratory tract, Fox *et al.* (1993) found no histamine-sensitive units at all in a relatively small sample of C-fibres investigated. It may also explain that high concentrations of histamine (10^{-4} M) are needed to observe an only moderate release of neuropeptides in the guinea-pig vascularly perfused lung (Saria *et al.*, 1988).

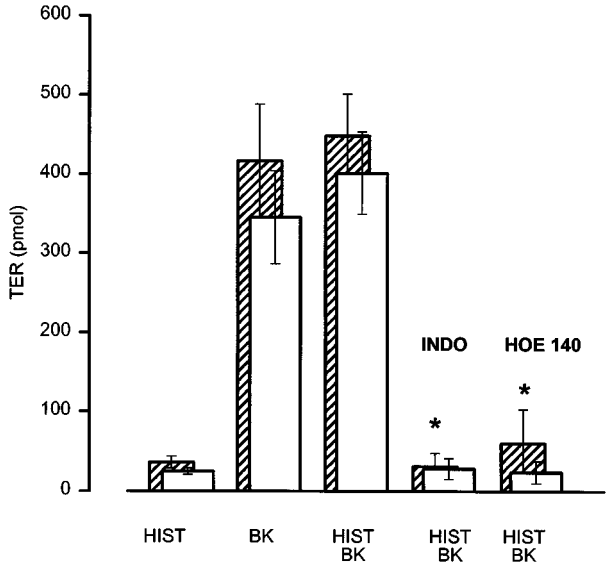

Figure 2 Total evoked release (TER) of $KH_2PGF_{2\alpha}$ (hatched columns) and 6-keto $PGF_{1\alpha}$ (open columns) by histamine (HIST; 100 μM), bradykinin (BK; 0.1 μM), and histamine in the presence of bradykinin (HIST-BK). Indomethacin (INDO; 5 μM) or HOE 140 (1 μM) significantly attenuated prostaglandin release. Values are means$\pm$s.e.mean; $n=4$-8; $^*P<0.05$ as compared to values obtained under control condition.

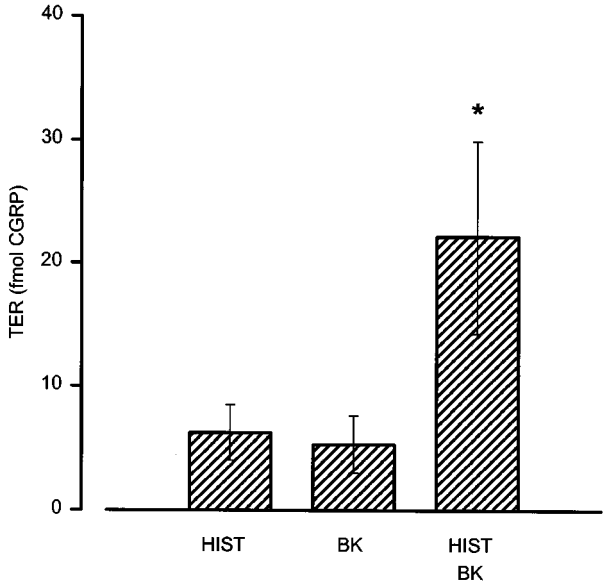

Figure 3 Total evoked release (TER) of CGRP in the presence of 5 μM indomethacin by histamine (HIST; 100 μM), bradykinin (BK; 0.1 μM) and histamine in the presence of bradykinin (HIST-BK). Values are means$\pm$s.e.mean; $n=8$-10; $^*P<0.05$ as compared to histamine alone.

One of the reasons to conduct our study were observations obtained by electrophysiological studies of C-polymodal nociceptors, showing that different autacoids can mutually potentiate their excitatory effect (*c.f.* Handwerker & Reeh, 1991). With regard to the present study it seems particularly interesting that Koppert *et al.* (1993) have shown that application of BK to rat afferent nerve endings increased the response, and the number of units that responded, to a subsequent histamine application. Because capsaicin-sensitive fibres are known to serve a dual function, providing afferent input to the CNS and releasing bioactive neuropeptides at the stimulated site (*c.f.* Holzer, 1988; Maggi, 1995), our present results seem to be in close correlation to those mentioned above. The observation by Koppert *et al.* (1993) that BK exposure induces histamine sensitivity in normally non-responsive afferents led us to look at the possibility that neuropeptide release from additional fibres may have detectable consequences on the relative amount of SP/CGRP that is released. However, we could not detect an effect of BK sensitization on the ratio of released SP/CGRP, suggesting therefore that, if BK-induced recruitment of afferents was involved in our experiments, it did affect fibres that contain CGRP as well as SP.

Biochemical evidence for facilitation of neuropeptide release by mediators of inflammation has been obtained in several preparations (Hua & Yaksh, 1993; Hintgen & Vasko, 1994; Vasko *et al.*, 1994). The effect of BK has been analysed in detail in the rat spinal cord by Andreeva & Rang (1993). They found that BK augmented the evoked release of CGRP, an effect that was inhibited by indomethacin or by $1-10$ μM HOE140, and mimicked by several prostaglandins, indicating that BK facilitates release via stimulation of the cyclooxygenase pathway of arachidonic acid metabolism. In our experiments, indomethacin significantly reduced CGRP release that was evoked by histamine in the presence of BK. However, also with indomethacin added, the release of CGRP evoked by histamine in the presence of BK was higher than in that evoked by either compound alone, the results being compatible with an additive effect of histamine and BK on CGRP release. It seems unlikely that the concentration of indomethacin (5 μM) was not sufficient to block prostaglandin synthesis because at this concentration indomethacin nearly prevented the BK and histamine-induced increase of immunoreactive KH_2 $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ in the outflow. In addition, using 1 μM indomethacin, we observed similar inhibition of CGRP release evoked by histamine in the presence of bradykinin (TER:25.1 ± 5.3 fmol, $n=4$; results not shown) as with 5 μM indomethacin. Therefore, the indomethacin-resistant evoked release of CGRP may be caused by a prostaglandin-independent, direct effect of histamine and BK on primary afferent neurons. This view is supported by a number of studies showing that, although usually attenuated, BK can excite and/or evoke neuropeptide release from primary afferent neurons also when endogenous prostaglandin synthesis is prevented (Franco-Cereceda, 1989; Geppetti *et al.*, 1991; Fox *et al.*, 1993; Vasko *et al.*, 1994).

A role of prostaglandins in the interaction of BK and histamine in the present experiments is also suggested by the observation that, in the presence of indomethacin, PGE_1 (which has been shown to facilitate evoked release of CGRP in the rat spinal cord (Andreeva & Rang, 1993)) enhanced histamine-evoked CGRP release, the effect being, however, considerably smaller than that observed with BK. In view of the fact that prostaglandins usually do not produce overt pain or cause excitation of afferent neurons, the present observation that 1 μM PGE_1 produced a detectable release of CGRP seems surprising. However, previous reports have shown that high concentrations of PGE_1 (100 μM: Franco-Cereceda, 1989), PGE_2 and PGI_2 (10-100 μM: Geppetti *et al.*, 1991) can evoke CGRP release from capsaicin-sensitive neurons. Geppetti *et al.* (1991) have discussed the problems that arise from the apparent absence of algesic effects of these substances and point to the possibility that prostaglandins might evoke local neuropeptide release without generating afferent impulse conduction. With regard to BK-evoked neuropeptide release, Geppetti *et al.* (1991) discuss the view that a large part of the BK effect is mediated by prostaglandins, i.e. that prostaglandins serve as final mediators of release.

Therefore, there are at least two alternative mechanisms that could lead to the present results showing enhancement by BK of histamine-induced CGRP release: (A) BK-induced prostaglandins sensitize afferent neurons for subsequent histamine exposure or (B) the combined administration of BK and histamine act together to stimulate the cyclooxygenase, resulting in a higher concentration of endogenous prostaglandins acting on afferent terminals. The observation that the amount the prostaglandin metabolites, KH_2 $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, that was released by histamine in the presence of BK, and by BK alone was not significantly different while the evoked CGRP release was at least five-fold higher, favours alternative (A). The observation that exogenous PGE_1-evoked detectable CGRP release on its own could be explained by assuming PG-induced sensitization of afferent neurons for other endogenous excitants that may be present in the perfused tissue preparation (it seems interesting in this respect that Lang *et al.* (1990) report that 1 μM PGE_2 has no clear excitatory effect on C-polymodal nociceptors in the rat skin, but may increase pre-existing ongoing activity).

Taken together, the present results show that exposure of the guinea-pig lung to a concentration of BK that has only a moderate effect on neuropeptide release stimulates the formation of prostaglandins, which, in turn, condition afferent fibres for the subsequent histamine stimulation. In various guinea-pig tissues, it has been shown that 1 μM HOE140 has neither agonist activity, nor does it antagonize histamine effects (Pruneau *et al.*, 1995). Therefore, the ineffectiveness of [desArg^9]-BK to mimic and the effectiveness of HOE140 (Lembeck *et al.*, 1991; Hock *et al.*, 1991) to inhibit BK evoked release and sensitization suggests the involvement of bradykinin B_2 receptors prior to BK-induced stimulation of prostaglandin synthesis.

Increased concentrations of BK can also be expected to be present in asthmatic disease (Christiansen *et al.*, 1992). Under these conditions, another afferent neuron excitant, e.g. histamine, which is found in lung perfusates of guinea-pigs during anaphylaxis in high concentrations (Piper & Vane, 1969; Gryglewski & Korbut, 1976), may act on BK-sensitized afferent neuron endings. Therefore, it can be assumed that in asthmatic disease, BK-induced facilitation of local neuropeptide release from C-fibre afferents in the respiratory tract may be important for the development of neurogenic edema and bronchoconstriction.

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