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Effects of bradykinin and bradykinin analogues on spleen cells of mice

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Abstract

The present study was undertaken to examine the effects of bradykinin and selected bradykinin analogues on mononuclear cells derived from mouse spleen. Bradykinin as well as des-Arg^9-bradykinin, a bradykinin B_1 receptor agonist, were able to induce the release of so-called charge-changing lymphokines, which could be identified as interleukin-1, interleukin-6, interleukin-2 and as interleukin-2 receptor. The cytokine release evoked by bradykinin and all analogues showed a bell-shaped dose dependence in a range of 10^{-8} M to 10^{-6} M and could be inhibited by the specific bradykinin receptor antagonist, D-Arg^0[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin (HOE140), and by bradykinin analogues with *N*-methyl-phenylalanine at position 2 in concentrations as low as 10^{-12} M and 10^{-13} M, respectively. Obviously the *N*-terminus of bradykinin seems to be responsible for the interaction with the mononuclear cells concerning all peptides investigated.

Keywords: Bradykinin; Bradykinin analog; Bradykinin receptor antagonist; HOE 140; Mononuclear cell; Cytokine; Interleukine

1. Introduction

Bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) is an endogenous peptide that is involved as a regulator molecule in various physiological and pathophysiological processes. Receptors for bradykinin, particularly the bradykinin B_2 receptor, are widely distributed throughout mammalian tissues and have been shown to mediate a number of specific responses including neurotransmitter release, vasodilation, smooth muscle contraction, exocrine secretion and pain (for review see Hall, 1992; Sharma, 1993). The bradykinin B_1 receptor was especially characterized in isolated vascular smooth muscles, for which des-Arg^9-bradykinin and des-Arg^{10}-kallidin are potent spasmogens (Farmer and Burch, 1991). Bradykinin B_1 receptors are usually not apparent in freshly isolated preparations; they are induced as a result of products of tissue damage or by noxious agents (Regoli et al., 1977; Bouthillier et al., 1987). Therefore it has been suggested that bradykinin receptors play a role in inflammatory processes. For instance, cells in inflamed tissues are more responsive to bradykinin than those in normal tissues (Kirchhoff et al., 1990; Perkins et al., 1993). This increased sensitivity to bradykinin could be due also to the effect of inflammation-associated cytokines (Ferreira et al., 1993). Along these lines, it has been shown that bradykinin induces interleukin-6, synergizes with interleukin-1 (Vandekerckhove et al., 1991) and stimulates tumor necrosis factor and interleukin-1 release from macrophages (Tiffany and Burch, 1989).

In 1985 we reported the release of so-called charge-changing lymphokines from mononuclear cells after stimulation with bradykinin and other related peptides (Werner and Paegelow, 1985; Paegelow and Werner, 1986). Moreover, an internal alteration in the bradykinin sequence was obviously consistent with a change in the potency to induce cytokine secretion.

The present report describes the effects of bradykinin and of selected analogues on mononuclear cells derived from spleens of mice. The cytokines released could be characterized as interleukin-1, interleukin-6, interleukin-2 and soluble interleukin-2 receptor. We show furthermore that the cytokine release can be blocked by specific bradykinin receptor antagonists.

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2. Materials and methods

2.1. Cell cultures

Spleens were prepared from male AB mice 6–8 weeks of age. After washing, a total of 1.5×10^6 spleen cells was incubated with the given concentration of the peptides in 2 ml serum-free Eagle's minimal essential medium at 37° C for 5 h. The cell-free supernatants were obtained by centrifugation. In order to test the effect of antagonistic activities of selected bradykinin analogues, cells were preincubated for 30 min at 37° C before the addition of the optimal dosage of bradykinin. In some cases an incubation of 6×10^7 cells (whole spleen of a mouse) per 15 ml in a culture flask was done followed by concentration (20–30-fold) of the supernatants by Amicon filtration (PM 10 filter/cut off 10000 MW); no cytokine activity was found in the ultrafiltrate. The supernatants derived from both culture conditions were fractionated on ULTROGEL AcA54 (column 0.9×60 cm; flow 1.25 ml fractions/5 min). The column was calibrated with proteins of known molecular weight as well as with reference cytokines derived from the mouse system. After gel separation of interleukin-1 or interleukin-2 standards under the experimental conditions used, double peaks were observed, which may correspond to the monomer and dimer molecular forms of these interleukins. The supernatants and the fractions were measured in the tanned erythrocyte electrophoretic mobility test as described below or using enzyme immunoassays.

In some cases single fractions were preincubated for 2 h at 23° C with the corresponding monoclonal antibody (0.4 μg antibody/sample) against the cytokine before the measurement in the tanned erythrocyte electrophoretic mobility test was done. Using this equipment only identification, no quantification, of the cytokines released by the peptides was possible.

2.2. Tanned erythrocyte electrophoretic mobility test

The test system is based on the determination of so-called charge-changing lymphokines as products of activated mononuclear cells by means of sulfosalicylic acid-stabilized tanned sheep red blood cells as target cells (Shenton et al., 1977; Werner, 1986). Cell electrophoresis measurement of the indicator cells was performed using a commercial cytopherometer (ZEISS, Oberkochen, FRG). The velocity of the stabilized sheep red blood cells was recorded by microscopic observation using an electronic timer and plotter. The difference in the migration time is defined as slowing effect (%):

$$\%slowing = \frac{mobility\ time_{specimen} - mobility\ time_{control}}{mobility\ time_{control}} \times 100.$$

Controls were pure target cells and/or target cells plus peptides to exclude artefacts. None of the peptides investigated had any effect on the mobility of the stabilized red blood cells in the concentrations used (10^{-15} M to 10^{-5} M).

2.3. Mouse enzyme immunoassays

The measurements of interleukin-2, interleukin-3 and interleukin-6 were performed using highly specific enzyme immunoassays. The immunoassays for interleukin-2, interleukin-3 and interleukin-6 detected concentrations as low as 10–50 pg/ml without any cross-reactivity for other interleukins. The activity in all supernatants was obtained by comparing the concentrated supernatants with standard concentrations of recombinant interleukins.

2.4. Data analysis

Each value represents the mean ± S.D. taken from n experiments. Statistical analysis was performed with Student's t-test for unpaired samples.

2.5. Materials

Bradykinin and the bradykinin analogues were synthesized by S. Reißmann, Jena; HOE 140 was a kind gift from B. Schölkens (Hoechst AG, FRG).

The following reagents were purchased from Genzyme, IC, Cambridge, MA, USA: the recombinant cytokines interleukin-1, interleukin-2, interleukin-3, interleukin-6, tumor necrosis factor α, interferon, the monoclonal antibodies rat anti-mouse interleukin-6, rat anti-mouse interleukin-3, hamster anti-mouse interleukin-1α, rat anti-mouse interleukin-2, hamster anti-mouse tumor necrosis factor and the Mouse Interleukin 2 Elisa. Other materials were obtained from the following sources: Endogen Murine Interleukin-3 ELISA and Endogen Murine Interleukin-6 ELISA (Edogenous, MA, USA), anti-interleukin-2 receptor, interleukin-1 receptor antagonist and human Interleukin-2-Receptor-ELISA (Boehringer, Mannheim, Germany), des-Arg^9-bradykinin, substance P (Serva, Heidelberg, Germany), histamine (Jenapharm, Jena, Germany); concanavalin A (Sigma-Aldrigde, Deisenhofen, Germany).

3. Results

3.1. Characterization of the bradykinin-induced cytokine release

The dose-response curves for cytokine release by bradykinin and by various analogues were analysed in

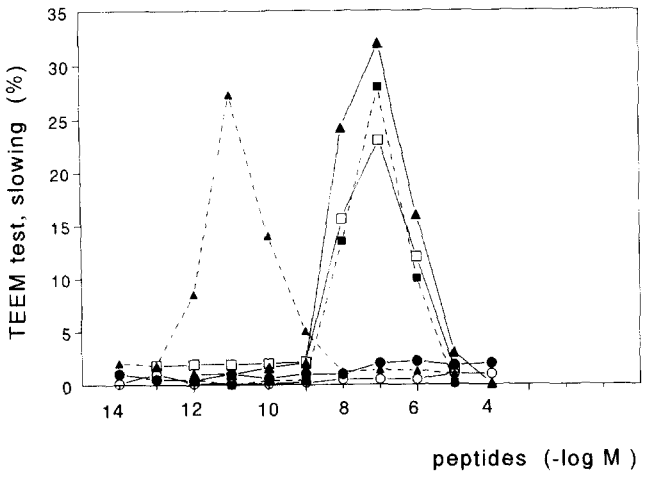

Fig. 1. Concentration-response curves to bradykinin (▲), des-Arg^9-bradykinin (■) and [D-NMe-Phe^7]bradykinin (□) as well as to HOE 140 (●), [L-NMe-Phe^2]bradykinin (○) and to substance P (- - ▲ - -) on spleen cells of mice. The cytokine release induced was measured after a short-time culture (5 h, 37° C) of mouse spleen cells. The activity in the supernatants is expressed as slowing per cent measured by the tanned erythrocyte electrophoretic mobility (TEEM) test. The values represent the means taken from three animals in duplicate cultures. The mean of all S.D. is 3.4 in the range from 0.5 to 5.5. Differences > 3% are significant ($P < 0.05$).

spleen cells of mice, using the measurement as so-called charge-changing lymphokines, which were first unfractionated. The bell-shaped dose-response curves of bradykinin, des-Arg^9-bradykinin and some analogues peaked mostly at 10^{-8} M to 10^{-7} M (Fig. 1/Table 2). For comparison, the dose-response evoked by substance P is shown in Fig. 1. To characterize the cytokines released by bradykinin the supernatants were fractionated, activities were measured in the tanned erythrocyte electrophoretic mobility test and compared with those of the reference cytokines, which were treated and measured by the same procedure. As shown in Fig. 2 bradykinin is able to induce the release of

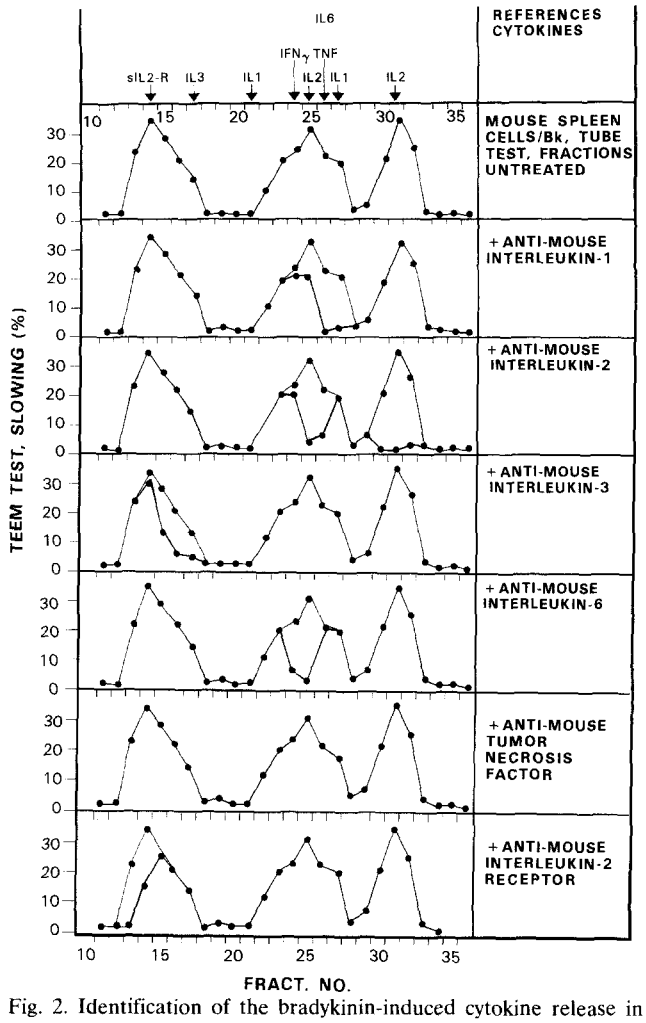

Fig. 2. Identification of the bradykinin-induced cytokine release in cultures of mouse spleen cells after separation on ultrogel AcA54 by different monoclonal antibodies. The activity of the samples is expressed as slowing per cent measured by the tanned erythrocyte electrophoretic mobility (TEEM) test and was estimated without and after the pretreatment (2 h) with the monoclonal antibodies (0.4 μg/sample) (dashed part of the curves). Data points are shown for one representative experiment which was repeated three times. Differences > 3% are significant ($P < 0.05$).

Table 1
Cytokine release from mouse spleen cells cultured with bradykinin (BK; 10^{-7} M) in comparison with controls and after pre-exposure to HOE140 (10^{-7} M) as well as to [L-NMe-Phe^2]bradykinin

Sample mouse spleen cells	Cytokines (pg/ml)					
	Interleukin-2	(n)	Interleukin-6 [a]	(n)	Interleukin-2 receptor [b]	(n)
Control	< 0.5	(7)	90 ± 30	(4)	0.5 ± 0.2	(4)
Bradykinin	5.6 ± 2	(9)	180 ± 50	(5)	2.2 ± 0.3	(4)
Hoe 140	< 0.1	(5)	70 ± 14	(3)	n.d.	
Bradykinin + HOE 140	0.6 ± 0.8	(5)	57 ± 6	(3)	n.d.	
[L-NMePhe^2]BK	0.9 ± 0.8	(5)	63 ± 6	(3)	n.d.	
Bradykinin + [L-NMePhe^2]BK	0.9 ± 0.4	(5)	67 ± 20	(3)	n.d.	
Concanavalin A	15 ± 3	(3)	60 ± 6	(3)	n.d.	

Amicon (PM 10 filter)-concentrated supernatants were used in the enzyme immunoassays. The values were calculated as concentrations of the original non-concentrated culture supernatant. Mean values ± S.D. derived from (n) experiments. [a] From non-concentrated cultures. [b] Interleukin-2 receptor derived from a human peripheral mononuclear blood cell culture was measured in a human enzyme immunoassay. [c] n.d., not done.

interleukin-1 and interleukin-6 whose peaks correspond in both cases with the peaks of the reference interleukins. The specificity of the cytokines was assessed by means of the monoclonal antibodies against interleukin-1 and interleukin-6. Additionally, the release of interleukin-2, soluble interleukin-2 receptor and interleukin-3 was demonstrated by means of the pattern of molecular weights, reference cytokines, and monoclonal antibodies. Tumor necrosis factor could not be detected.

After amicon concentration of the supernatants derived from flask cultures of isolated spleen cells that were incubated with the peptides, cytokine release could also be evidenced using enzyme immunoassays. Table 1 demonstrates the activities of interleukin-2 and interleukin-6 calculated as pg/ml of the original supernatant: 5.6 ± 2 pg/ml interleukin-2 were secreted from a whole spleen after the incubation with 10^{-7} M bradykinin. The T-cell mitogen, concanavalin A, evoked interleukin-2 secretion in an amount of 15 ± 3 pg/ml under the same conditions.

The interleukin-3 production was at the detection limit of the immunoassay test system (not shown). Interleukin-6 was found to be secreted by bradykinin from the spleen cells in an amount of 180 ± 50 pg/ml. The soluble interleukin-2 receptor could also be detected in an enzyme immunoassay. However, in this case a culture from human mononuclear cells derived from peripheral blood and an enzyme immunoassay for the human system had to be used (see Table 1); the amount was about 2.2 pg soluble interleukin-2 receptor/ml supernatant.

3.2. Structure-activity relationship of bradykinin analogues

Using the measurement of the non-fractionated cytokines in the tanned erythrocyte electrophoretic mobility test, the influence of various changes in the bradykinin sequence was evaluated (Table 2). Bradykinin analogues with a maximal agonistic effect at 10^{-7} M showed the same pattern of activity after gel separation of the supernatants as bradykinin itself (not shown). Because the concentration of 10^{-7} M bradykinin was generally able to elicit the maximal releasing activity, this concentration was used to investigate inhibitory effects of selected analogues. The pre-exposure of the cells to bradykinin analogues with a potential antagonistic activity reduced the capacity to induce the cytokines by bradykinin as shown in Fig. 3 and Tables 1 and 2. A potent inhibitory effect was demonstrated for [L-NMePhe^2]bradykinin and [D-NMePhe^7]bradykinin as well as for D-Arg^0-[Hyp^3,Thi^5,D-Tic^7,Oic^8]bradykinin (HOE 140) in a concentration of 10^{-13} M and 10^{-12} M. With histamine (10^{-5} M) or substance P (10^{-11} M) as stimulants for

Table 2
Secretion of cytokines, measured by the tanned erythrocyte electrophoretic mobility (TEEM) test, induced from spleen cells of mice by bradykinin (BK) and bradykinin analogues and the inhibition by bradykinin receptor antagonists

Peptide	TEEM test	
	ED_{max} (M)	Inhibition (M)
Bradykinin	10^{-7}	no effect
Des-Arg^9-bradykinin	10^{-7}	10^{-11}
[D-Phe^7]BK	10^{-7}	10^{-10}
[D-NMe-Phe^7]BK	10^{-7}	10^{-11}
[L-NMe-Phe^7]BK	10^{-7}	10^{-12}
[Hyp^3,Thi^5,D-NMe-Phe^7]BK	10^{-7}	10^{-12}
[D-NMe-Phe^7,Oic^8]BK	10^{-7}	10^{-5}
[L-NMe-Phe^3]BK	10^{-7}	10^{-9}
[D-NMe-Phe^3]BK	10^{-7}	10^{-9}
D-Arg^0[L-NMe-Phe^7]BK	no effect	10^{-11}
D-Arg^0[Hyp^3,Thi^5,D-NMe-Phe^7]BK	no effect	10^{-6}
D-Arg^0[Hyp^3,D-NMe-Phe^7,L-NMe-Phe^8]BK	no effect	10^{-6}
[D-NMe-Phe^2]BK	no effect	10^{-12}
[L-NMe-Phe^2]BK	no effect	10^{-13}
D-Arg^0[L-NMe-Phe^2]BK	no effect	10^{-13}
D-Arg^0[Hyp^3,Thi^5,D-Tic^7,Oic^8]BK	no effect	10^{-12}

Concentrations indicated demonstrate the ED_{max} of the appropriate dose-response curves and the lowest concentration which antagonized the bradykinin-induced effect.

cytokine secretion from spleen cells, the bradykinin receptor antagonists were not able to inhibit this effect (not shown). As described previously (Paegelow and Werner, 1987), histamine induced preferably interleukin-1 release; the pattern of activities after incuba-

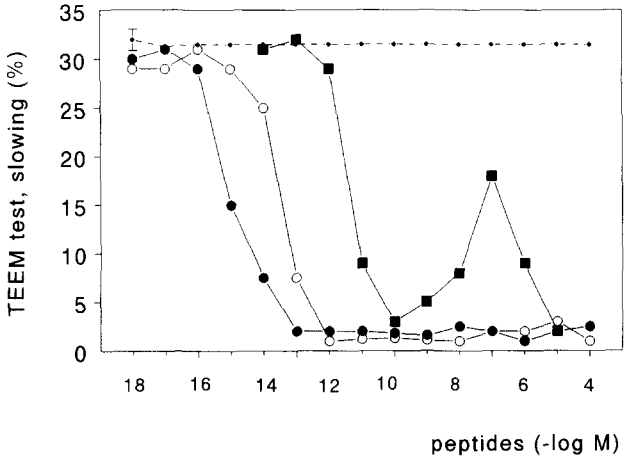

Fig. 3. Effect of increasing concentrations of bradykinin receptor antagonists on the bradykinin-induced secretion of cytokines expressed as charge-changing lymphokines in slowing per cent measured by the tanned erythrocyte electrophoretic mobility (TEEM) test. Bradykinin (10^{-7} M) (♦) was used to induce a maximal cytokine release in the spleen cell cultures. The cells were preincubated (30 min, 37° C) with increasing concentrations of the bradykinin antagonists: HOE 140 (○), [L-NMe-Phe^2]bradykinin (●) and [D-NMe-Phe^7]bradykinin (■). Data points are means of all S.D. is 3.4 in the range from 0.5 to 5.5. Differences > 3% are significant ($P < 0.05$).

tion with substance P was comparable with that of bradykinin (Werner et al., 1987).

In some cases – for instance [D-Phe^7]bradykinin and analogues having D- or L-NMePhe at position 3 or 7 – the compounds have their own intrinsic agonistic as well as antagonistic activity (see Table 2).

4. Discussion

Bradykinin and some of its analogues were found to induce the secretion of several cytokines in spleen cells of mice. Using the fractionation of the supernatants and the inhibition by monoclonal antibodies, the interleukins could be characterized as interleukin-1, interleukin-6, interleukin-2, interleukin-3 and as soluble interleukin-2 receptor, measuring the activities by means of the tanned erythrocyte electrophoretic mobility test. Experiments with enzyme immunoassays for selected cytokines – especially for interleukin-2, soluble interleukin-2 receptor and interleukin-6 – confirmed these results. For this purpose the supernatants were concentrated after the incubation with bradykinin. As expected, interleukin-6 was found in the culture supernatants under these conditions. The sensitivity of the test system for interleukin-3 was limited, but that for interleukin-2 was reproducible, measured in the range of 120–200 pg/ml in the concentrated cultures. This means a content of about 5 pg interleukin-2/ml original supernatant. In comparison, concanavalin A was capable of inducing a 3-fold higher secretion of interleukin-2.

Bradykinin is known to stimulate the release of interleukin-1, interleukin-6 and tumor necrosis factor α from macrophages and to act synergistically with interleukin-1 (Tiffany and Burch, 1989; Vandekerckhove et al., 1991). We suggest that, in the present experiments using spleen cells of mice, bradykinin stimulates also preferably the macrophages/monocytes. Interleukin-1 and interleukin-6, mainly produced by these cells, were found in the supernatants after the incubation with bradykinin. However, interleukin-2, interleukin-3 in traces and interleukin-2 receptor as T-cell products were also detected in the supernatants. These cytokines induced by bradykinin could be secreted, possibly by T-cells directly. But, more probably they are expressed by an indirect mechanism, in which bradykinin for instance first activates phospholipase A_2 as the source of the arachidonic acid metabolism (Cisa et al., 1993). It is known that bradykinin can rapidly enhance prostaglandin synthesis not only in fibroblasts (Lerner and Modeer, 1991) but also in monocytes (Lerner et al., 1989). On the other hand, interleukin-2 secretion may be induced by the interleukin-1 released in the same culture. In preliminary experiments it was found that the peaks, which characterize T-cell activities in the fractionation pattern of supernatants cultured with bradykinin, could be inhibited after preincubation with the interleukin-1 receptor antagonist, IL-1ra. Therefore, it might be speculated that an indirect release of T-cell-derived cytokines after the stimulation with bradykinin is more probable than a direct event.

It became evident from the present experiments that several bradykinin analogues were also able to induce cytokine secretion. Bell-shaped dose-response curves could be recorded after incubation both with the bradykinin B_1 receptor agonist, des-Arg^9-bradykinin, as well as with analogues having D- or L-NMePhe at position 3 or 7 (see Table 2). Using the tanned erythrocyte electrophoretic mobility test as titration method, a ranking order of the potency of agonists with the ED_{max} at the concentration of 10^{-7} M cannot be given, because the effect of the analogues at the concentration of 10^{-7} M was not quantifiable exactly.

The agonistic activity of the analogues disappeared when the *N*-terminus of the substance was changed: first, the replacement of the amino acid, proline, at position 2 by the more hydrophobic and conformationally restricted imino acid, *N*-methyl-phenylalanine, caused a loss of the agonistic function, but enhanced the antagonistic activity considerably (Table 1; Fig. 3): [L-NMePhe^2]bradykinin was about 10 times more active than HOE 140, known as a bradykinin B_2 receptor antagonist, on several target tissues (Lembeck et al., 1991).

Second, elongation of the bradykinin analogues with D-arginine at position 0 generally led to a loss of the agonistic but not the antagonistic activity. The inhibitory effect of these analogues on mononuclear cells is obviously not only restricted to this change in the molecule, as can be seen for the replacement of the proline at position 2 of bradykinin without any other substitutions.

In previous experiments it had been shown that an exchange of the arginine residue at position 1 as in [Lys^{1,9}]bradykinin or omission of the arginine as in des-Arg^1-bradykinin caused a loss of the cytokine release measured in the tanned erythrocyte electrophoretic mobility test (Paegelow and Werner, 1986). According to these results the *N*-terminus seems to be responsible for the interaction with the monocytes.

Most surprisingly, some agonists themselves were observed to inhibit the bradykinin-induced cytokine release in lower concentrations and to initiate release in the concentration range of 10^{-7} M to 10^{-6} M. These dualistic effects of some bradykinin analogues are still unclear. It is known, however, that drugs with antagonistic properties may have intrinsic activity too. Recently, bradykinin analogues were developed with dehydrophenyl-alanine at position 5. They inhibit the bradykinin action at the guinea pig ileum and have moreover agonistic activities (Reißmann et al., 1995).

However, we suggest that in the present experiments various cell populations present in the spleen cell culture are stimulated directly or indirectly and that receptor subtypes do exist.

The cytokine release from mononuclear cells which was induced by substance P or by the biogenic amine, histamine, could not be inhibited by the bradykinin antagonists. Taken together, our data suggest that the inhibition of the bradykinin effects by specific antagonists and the absence of influence of these substances on the histamine- and substance P-evoked cytokine secretion is due to a bradykinin receptor-mediated cytokine release.

The expression of a bradykinin B_1 receptor by a macrophage cell line, RAW264.7, was already assumed (Burch and Kyle, 1992). This assumption is acceptable because the bradykinin B_1 receptor agonist stimulated cytokine release and the bradykinin B_1-selective antagonist, des-Arg^9-[Leu^8]bradykinin, blocked the kinin-induced cytokine release. But at the present stage of knowledge it is not certain which cytokines are expressed via receptor-mediated events and which kind of bradykinin receptor does exist on macrophages/monocytes. Binding experiments with bradykinin on peritoneal macrophages in our laboratory show mostly bradykinin B_2 receptors on these cells (unpublished observations). The existence of bradykinin receptors on lymphocytes had so far not been described.

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