British Journal of Pharmacology (1997) 122, 1585–1592

Synthesis of kininogen and degradation of bradykinin by PC12 cells

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1 In this study, the abilities of PC12 cells to synthesize and degrade kinins were investigated. Kinin formation was assessed as kinin and kininogen content of cells and supernatants in serum-free incubations by use of a bradykinin-specific radioimmunoassay. Expression of kininogen mRNA was demonstrated by reverse-transcriptase PCR. Kinin degradation pathways of intact PC12 cells were characterized by identification of the kinin fragments generated from tritiated bradykinin either in the absence or presence of the angiotensin I-converting enzyme inhibitor ramiprilat.

2 Kinin immunoreactivity in the supernatant of PC12 cell cultures accumulated in a time-dependent fashion during incubations in serum-free media. This effect was solely due to *de novo* synthesis and release of kininogen (35 pg bradykinin h^{-1} mg^{-1} protein) since it could be suppressed by cycloheximide. Continuous synthesis of kininogen was a specific property of PC12 cells, as it was not observed in cultured macro- or microvascular endothelial cells. PC12 cells contained only minor amounts of stored kininogen. The rate of kininogen synthesis was not affected by ramiprilat, bacterial lipopolysaccharide, nerve growth factor or dexamethasone, but was stimulated 1.4 fold when cells were pretreated for 1 day with 1 μM desoxycorticosterone.

3 By use of cDNA probes specific for kininogen subtype mRNAs, expression of low-molecular-weight kininogen and T-kininogen in PC12 cells was confirmed. Expression of high molecular weight kininogen mRNA was also shown, though only at the lowest limit of detection of the assay.

4 Degradation of tritiated bradykinin by PC12 cells occurred with a half-life of 48 min resulting in the main fragments [1-7]- and [1-5]-bradykinin. The degradation rate of bradykinin decreased to 15% in the presence of ramiprilat (250 nM). Apart from angiotensin I-converting enzyme direct cleavage of bradykinin to [1-7]- and [1-5]-bradykinin still occurred under this condition as a result of additional kininase activities.

5 Along with previous findings of B_2-receptor-mediated catecholamine release, these results now confirm the hypothesis that a cellular kinin system is expressed in PC12 cells. The presence of such a system may reflect a role of kinins as local neuromodulatory mediators in the peripheral sympathetic system.

Keywords: Bradykinin; kininogen; kininogen synthesis; angiotensin I-converting enzyme; PC12 cells

Introduction

While the involvement of the kinin-kallikrein system in acute pathological events such as inflammation or shock is well established, the continuous production of kinins in many tissues also suggests their involvement in physiological mechanisms. Kinins are secretory constituents of exocrine glands, such as the salivary glands, prostate and kidney and may function as local tissue hormones involved in regulating vascular tone, renal function or neuronal processes (reviewed by Bhoola *et al*., 1992).

Under most circumstances, circulating kininogen, supplied abundantly by the liver, is considered as the precursor for kinin production brought about by locally activated kallikreins. Only few organs have been identified where a tissue-specific kininogen synthesis takes place. Kininogen immunoreactivity has been detected in salivary and sweat glands, neutrophils and specific regions of the brain (Chao *et al*., 1988; Poblete *et al*., 1991; Richoux *et al*., 1991; Figueroa *et al*., 1992). However, the only locations where a kininogen mRNA expression has been shown are liver, kidney and the lung (Iwai *et al*., 1988; Chao *et al*., 1993). Whether endothelial cells are able to synthesize kininogens locally is still a subject of discussion (van Iwaarden *et al*., 1988; Schmaier *et al*., 1988). A local generation of kininogen is of particular importance for the proposed physiological roles of kinins in the central nervous system, as these would be totally dependent on local *de novo* synthesis of kinin components which cannot pass the blood brain barrier. Expression of kininogen in neurones, not yet demonstrated by mRNA analysis, would therefore be a prerequisite to propose a role of kinins as neuronal mediators or neuromodulatory hormones. Kinins in the brain could potentially be involved in a variety of regulatory functions (reviewed by Walker *et al*., 1995), and can clearly influence the cardiovascular system via stimulation of sympathetic activity and vasopressin release (Brooks *et al*., 1986; Lopes & Couture, 1992). Thus their participation in the pathogenesis of hypertension is also possible (Martins *et al*., 1991).

In addition, a peripheral adrenergic cell type, the chromaffin cell of the adrenal medulla, appears to be an important target for kinin actions. Bradykinin (BK) activates catecholamine release from adrenal medulla *in vitro* (Staszewska-Barczak & Vane, 1967) and BK infusion potently increases plasma concentrations (particularly adrenaline) in the pithed rat, especially when kinin effects are potentiated by inhibition of angiotensin I-converting enzyme (ACE) (Dominiak *et al*., 1992). Even endogenous BK may stimulate adrenal catecholamine release. The presence of kallikrein and ACE has been demonstrated in this organ, pointing to the existence of a local kinin-kallikrein system (Wilson *et al*., 1987; Nolly *et al*., 1993). However, the specific involvement of chromaffin cells, which would imply a role of kinins as autocrine neuromodulators or even as cotransmitters, has as yet not been investigated.

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In the present study, the rat phaeochromocytoma cell line PC12 was used as an adrenal chromaffin cell model. PC12 cells are well characterized with respect to stimulus-secretion coupling and to the stimulant actions of BK. They possess B_2-receptors, which activate phospholipase C and D (Fasolato *et al*., 1988; Horwitz, 1991) and increase influx and intracellular release of Ca^{2+} (Fasolato *et al*., 1988; Clementi *et al*., 1992). Stimulation of B_2-receptors, characterized by inhibitor studies, also very potently evokes catecholamine release (Dendorfer & Dominiak, 1995). While PC12 cells effectively respond to exogenous BK, it is not known whether they also can generate and degrade BK. Such functions would indicate the existence and basal activity of a local kinin-kallikrein system in this neuronal cell type.

To investigate this, kinin and kininogen release from PC12 cells were measured and conditions which might modulate the expression and activity of kinin generation were tested. The question of kininogen synthesis being a specific function of PC12 cells, but not a general property of any cell type, was addressed in comparative studies on micro- and macrovascular endothelial cells. BK and its precursors low molecular weight (LMW)- and high molecular weight (HMW)-kininogen (K-kininogens) were considered specifically. T-kininogens and T-kinin, which are rat specific kinin subtypes, act as acute phase proteins *in vivo* (Barlas *et al*., 1985). They should therefore not take part in physiological functions of the kinin-kallikrein system, the main subject of this investigation.

Methods

Cell culture

PC12 cells were provided by Professor R. Heumann (Bochum, F.R.G.). Cell cultures were grown in 75 cm^2 flasks (Falcon, Becton Dickinson, Lincoln Park, U.S.A.) in Dulbecco's modified Eagle's medium (1 g l^{-1} glucose) supplemented with 5% foetal calf serum, 10% horse serum, 100 u ml^{-1} penicillin and 0.1 μg ml^{-1} streptomycin. For BK degradation studies, cells were seeded into 24-well tissue culture plates (Falcon, Becton Dickinson, Lincoln Park, U.S.A.) at least 4 days before the experiments.

Macrovascular endothelial cells were isolated from the bovine aorta by superficial collagenase treatment as described by Macarak *et al*. (1977). Microvascular endothelial cells were obtained from rat myocardium according to the procedure of Dendorfer *et al*. (1986). In brief, isolated hearts from male Wistar rats (250–300 g) were completely dissociated by protease perfusion. Capillary fragments were purified from the cell suspension by centrifugation on a density gradient (Percoll, Sigma, Deisenhofen, FRG). Endothelial cells were cultured in Medium 199 containing 10% foetal calf serum and antibiotics (100 u ml^{-1} penicillin and 0.1 μg ml^{-1} streptomycin). Cultures were grown to confluence in 75 cm^2 flasks (Falcon, Becton Dickinson, Linoln Park, U.S.A.) and were used after 3–5 passages.

Kinin and kininogen release studies

Cell cultures, grown in 75 cm^2 culture flasks, were pretreated for up to three days as indicated. For kinin release studies, they were washed three times with incubation medium (DMEM/F12 containing 1 mg ml^{-1} albumin) and then kept in incubation medium at 37°C and 5% CO_2 for 1 h. Subsequently the cells were subjected to consecutive incubation periods of 1 to 6 h duration, with a complete exchange of the incubation medium after each period. Cell cultures being pretreated with ramiprilat, dexamethasone or desoxycorticosterone, were also exposed to these substances throughout the incubation procedures. In some experiments, the incubations were completed by a 15 min treatment of the cells with cycloheximide (100 μg l^{-1}) before the final 1 h incubation period. Supernatants and cell homogenates obtained from these incubations were either immediately processed (for kinin analysis) or inactivated by boiling for 10 min in 32 mM acetic acid (for kininogen analysis).

Kinin and kininogen analysis

Kinins were extracted from the samples by a solid-phase extraction procedure adopted from Hilgenfeldt *et al*. (1995). For kininogen measurements, the precipitated kininogen was sedimented ($1500 \times g$, 15 min, 4°C) and resuspended in 10 ml DMEM/F12. BK was liberated from kininogen by incubation (30 min, 37°C) with trypsin (200 μg) according to the method of Dinitz & Carvalho (1963). The reaction was stopped by 10 min of boiling and the precipitated protein was removed by centrifugation ($4000 \times g$, 15 min, 4°C). Kinins were extracted from the supernatants as described.

Kinin concentrations were quantified by a radioimmunoassay with a bradykinin-specific rabbit antiserum and [^{125}I-Tyr^8]-bradykinin as tracer. The antiserum had a high affinity for BK and significantly bound the kinin fragment [1-8]-BK (24.3% cross-reactivity), while the cross-reactivities to [2-9]-BK (0.011%), T-kinin (Ile-Ser-bradykinin, 0.068%) and all smaller N-terminal BK fragments were negligible (Hilgenfeldt *et al*., 1995). The cross-reactivity to human intact high molecular weight kininogen was found to be 8.7% on a molar basis. The radioimmunoassays were set up according to an assay for angiotensin I described by Menard & Catt (1972). In brief, lyophilized samples were dissolved in RIA buffer (0.1 M tris(hydroxymethyl)aminomethane, 2 mM $CaCl_2$, 0.22 mM neomycin, 1 mg ml^{-1} bovine serum albumin, pH 7.4) and mixed with 5000 c.p.m. of [^{125}I-Tyr^8]-bradykinin and antiserum at a dilution of 1 : 80 000 to a final volume of 500 μl. After 24 h incubation at 4°C, free tracer was adsorbed to charcoal (5 mg in 150 μl RIA buffer containing 33% bovine serum) and sedimented by centrifugation ($3500 \times g$, 10 min, 4°C). The radioactivity of the pellet was measured and related to a standard curve by a γ-counter (LKB Wallac, Turku, Finland). A sensitivity of 1 pg BK per test tube was achieved. Due to the BK-selectivity of the antiserum used, T-kinin was not assayed. Measurements of kininogen contents therefore selectively represent the amounts of BK released by trypsin from K-kininogens (high- and low-molecular weight kininogen), but not from T-kininogen which would be a source of T-kinin (Okamoto & Greenbaum, 1986).

Kininogen mRNA

PC12 cultures (5–9 mg protein) were washed three times with phosphate buffered saline. The cells were removed from the flasks and sedimented ($1200 \times g$, 5 min, 4°C). mRNA was prepared by phenolic extraction by use of RNAzol B according to the suggestions of the manufacturer (WAK-Chemie, Bad Homburg, F.R.G.). Isolated mRNA was dissolved in RNAse-free H_2O and quantified by photometry at 260 nm. As a positive control, pieces of rat liver (200–300 mg wet weight) were processed in the same way.

The genomic structures of the three rat kininogen genes and the mRNA sequences of the four rat kininogen subtypes were taken from Furuto-Kato *et al*. (1985) and from Kitagawa *et al*. (1987). Two forward primers were used to differentiate T-kininogens (T_1- and T_2-kininogen) from K-kininogens (T-F: 5′-CCAAGCACTAGACATGATG-3′ and K-F: 5′-ACTAGACATGACATCAGTG-3′, respectively), while one reverse primer was specific for high molecular weight kininogen (H-R: 5′-TGCATGCAACCAGCCATGC-3′), and a second for both, T- and low-molecular weight kininogens (TL-R: 5′-GCCCTTGTACTCACATGAG-3′). The two existing T-kininogen genes (T_1- and T_2-kininogen) are highly homologous, hence a differentiation of their expression was not attempted. The transcribed sequences spanned intron I of all kininogen genes (Kitagawa *et al*., 1987), so that the detection of cDNA could not be interfered with by the presence of genomic DNA (refer to Figure 1). First strand cDNA was synthesized by use

of a commercial kit (Pharmacia LKB, Woerden, The Netherlands) starting with 5 μg isolated RNA and one of the primers TL-R (5 ng) or H-R (25 ng). The cDNA produced was amplified by polymerase chain reaction (PCR) employing a nucleotide-mixture (Promega, Heidelberg, F.R.G.) supplemented with 2 mM $MgCl_2$, Taq DNA-polymerase (0.5 u, Boehringer, Mannheim, F.R.G.) and any combination of one reverse-primer (H-R or TL-R, 50 ng) and one forward-primer (T-F or K-F, 50 ng). PCR was performed under standard conditions (53°C hybridization temperature) for 40 (PC12 cells) or 35 (liver) cycles. For verification of the PCR products, the presence of specific endonuclease cleavage sites was tested. PCR products were incubated for 60 min with Taq I (10 u, 65°C), Msp I (10 u, 37°C) and Sty I (10 u, 37°C) in incubation buffers supplied by the manufacturer (Boehringer, Mannheim, F.R.G.). The sequences of primer hybridization and the endonuclease cleavage sites for all 4 rat kininogen cDNAs are depicted in Figure 1.

Bradykinin degradation studies

The kinetics of BK degradation were established by incubating PC12 cells in multiwell culture plates (2 cm^2 surface) with tritiated BK. Cell cultures were washed three times with HEPES-DMEM (DMEM without $NaHCO_3$, containing 20 mM N-[2-hydroxyethyl]piperazine-N′-2-ethanesulphonic acid and 0.1% bovine serum albumin, pH 7.4) and incubated in 1 ml of the same buffer supplemented with 0.2 nM [^{3}H-Pro^{2,3}]-bradykinin ([^{3}H]-BK) for up to 2 h at 37°C in a waterbath. At the beginning and at indicated times thereafter, aliquots (70 μl) were sampled, inactivated by addition of 7 μl trifluoroacetic acid (1%) and stored at −20°C until analysis. Experiments were performed with or without inhibition of ACE by ramiprilat (250 nM), which was applied 30 min before and throughout the incubation periods.

The spectrum of BK degradation products was determined as described previously (Dendorfer *et al*., 1997). In brief, peptides were separated on an ET 200 Nucleosil 5 C_{18} column (Macherey-Nagel, Düren, F.R.G) and BK, [1-8]-BK, [2-9]-BK, [1-7]-BK, [1-6]-BK and [1-5]-BK were collected in 1.6 ml fractions at their respective retention times.

Substances

DMEM was obtained as $NaHCO_3$-free salt mixture from Gibco (Eggenstein, F.R.G.). DMEM/F12 (1 : 1), Medium 199, all antibiotics, cell culture supplements and synthetic kinin peptides were obtained either from Sigma (Deisenhofen, F.R.G.) or Bachem (Bubendorf, Switzerland). The bradykinin-specific rabbit antibody was generously donated by Prof. U. Hilgenfeldt (Heidelberg, F.R.G.). [Tyr^8]-bradykinin was labelled with ^{125}I (550 MBq μg^{-1}, Amersham Buchler, Braunschweig, F.R.G.) by the chloramine-T method of Greenwood *et al*. (1963). Oligonucleotide cDNAs were synthesized by MWG-Biotech (Ebersberg, F.R.G.). Chemicals used for mRNA analysis were supplied either by Boehringer (Mannheim, F.R.G.), Pharmacia LKB (Woerden, The Netherlands) or Promega (Heidelberg, F.R.G.), as indicated. [^{3}H-Pro^{2,3}]-bradykinin ([^{3}H]-BK) was obtained from Du Pont (Bad Homburg, F.R.G.) at a specific activity of 2.22 TBq $mmol^{-1}$. It was used in the incubation media at an activity of 20000 c.p.m. ml^{-1}, corresponding to a concentration of 0.2 nM. The ACE inhibitor ramiprilat was provided by Hoechst (Frankfurt, F.R.G.)

Quantification and statistics

Protein contents of cell cultures were measured according to Lowry *et al*. (1951). The rate of kinin-immunoreactivity accumulation was calculated by linear regression. Kininogen is always expressed as the amount of BK released. Peptide concentrations in degradation studies were quantified according to the ^{3}H-activities measured in the specific high performance liquid chromatography (h.p.l.c.) fractions. These values are given as percentages of the total radioactivity of all kinin metabolites detected in the substrate solution. [^{3}H]-BK contributed 96 ± 1% to the total radioactivity of the substrate solution, showing that only minor levels of impurities were

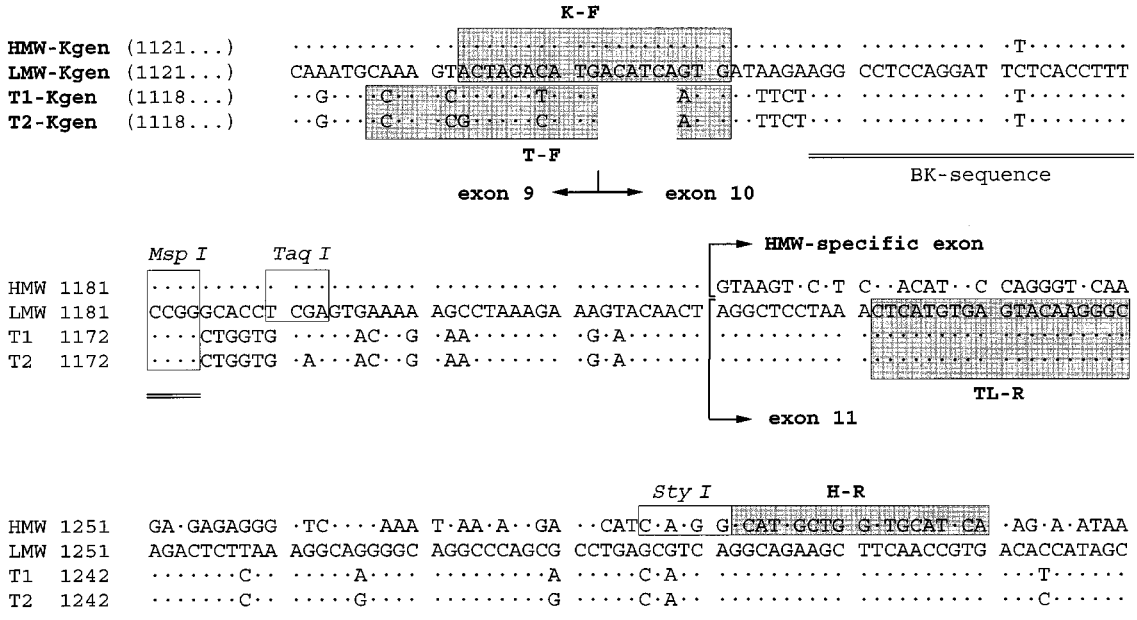

Figure 1 Localizations of PCR primers and restriction enzyme cleavage sites within the rat kininogen cDNA sequences. The structures of all rat kininogen cDNAs around the coding region for kinin peptides were taken from Kitagawa *et al*. (1987). Identity of high-molecular weight kininogen (HMW-Kgen), of T_1-kininogen (T1-Kgen), and T_2-kininogen (T2-Kgen) cDNAs with the sequence of low-molecular weight kininogen (LMW-Kgen) is indicated by dots. The hybridization sites of all PCR-primers (K-F, T-F, H-R, TL-R) are shaded. The amplified sequences spanned intron I (between exons 9 and 10) and intron Ji (between exons 10 and 11 of LMW-, T_1 and T_2-kininogens). This ensures that detection of mRNA is not interfered by genomic DNA. The PCR products contained specific cleavage sites for the restriction endonucleases Msp I, Taq I, and Sty I (boxed areas) which were used for sequence identification.

present. For analysis of degradation kinetics, the complete time course of [^{3}H]-BK degradation was fitted to a function of monoexponential decline ($BK(t) = BK_0 \times e^{-\beta t}$). The velocity of [^{3}H]-BK degradation is always given as the rate constant β or the derived half-life. All data are given as means ± s.e.-mean calculated from 5 independent experiments. The effects of various pretreatments and of cycloheximide on kininogen formation were evaluated by analysis of variance by use of Dunnet's *post-hoc* test. The influence of ramiprilat on [^{3}H]-BK degradation rate was tested with Student's *t* test. Differences were considered as being statistically significant at an error level of $P<0.05$.

Results

Kininogen synthesis by PC12 and endothelial cells

During consecutive incubation intervals under serum-free conditions, kinin-immunoreactivity in the supernatant of PC12 cells increased steadily at a rate of 0.12 ± 0.013 pgBK mg^{-1} protein h^{-1}. However, in experiments aimed at modifying kinin concentrations by ACE-inhibition, ramiprilat failed to enhance kinin-immunoreactivity (rate 0.17 ± 0.019 pgBK mg^{-1} protein h^{-1}, not significantly different vs control, Figure 2). Considering the predominant role of ACE for BK degradation (see below), the increase in kinin-immunoreactivity can therefore not be attributed to an accumulation of authentic BK. It rather must have been brought about by the generation of cross-reactive substances such as kininogens.

Indeed, measurements of trypsin-released BK indicated that K-kininogen (low- and/or high-molecular weight kininogen) was constantly liberated from PC12 cells over a 6 h period at an average rate of 34.7 ± 3.4 pgBK mg^{-1} protein h^{-1}. This process involved *de novo* synthesis of kininogen, since it was nearly eradicated in the presence of 100 μg l^{-1} cycloheximide (6.7 ± 1.1 pgBK mg^{-1} protein h^{-1}, $P<0.05$ vs the preceding incubation, Figure 3). PC12 cells did not contain substantial amounts of stored kininogens. The total cellular content of kininogen (62.9 ± 11.2 pgBK mg^{-1} protein) corresponded to an amount synthesized over approximately 2 h and this was significantly reduced after cycloheximide treatment (23.5 ± 2.9 pgBK mg^{-1} protein, $P<0.05$, Figure 3). When aortic or microvascular endothelial cells were incubated for consecutive 1 h intervals under the conditions used for PC12 cells, no kinin immunoreactivity was found in the supernatants of the cell cultures. Kininogen was released from microvascular endothelial cells only during the first hour of incubation (5.6 ± 1.1 pgBK mg^{-1} protein) and not afterwards. Aortic endothelial cells did not release detectable amounts of kininogen at any time (<1 pgBK mg^{-1} protein).

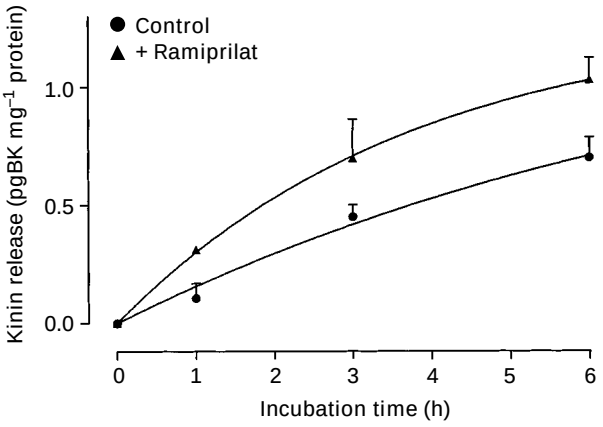

Figure 2 Accumulation of bradykinin (BK) immunoreactivity in the supernatant of PC12 cells. PC12 cell cultures were consecutively incubated under serum-free conditions for the indicated periods. Kinin immunoreactivity in the medium was determined by a bradykinin-specific RIA. The experiments were performed in the absence (control) and presence of ramiprilat (0.25 μM) which effectively inhibited bradykinin breakdown, but did not significantly increase the levels of bradykinin immunoreactivity in the supernatants. Each point represents the mean and vertical lines show s.e.mean of 5 determinations.

In order to investigate whether kininogen synthesis could be modulated, PC12 cells were pretreated under various conditions for up to 3 days. Kininogen release was determined over three 1 h intervals. In these experiments, the rate of kininogen release was about twice as high as that seen in earlier incubations, which might have been due either to a longer cultivation period or other, unidentified alterations of culture conditions. This did not represent a problem for our study, since only data from identical experimental setups were compared. The rate of kininogen release from PC12 cells under control conditions (81.3 ± 8.9 pgBK mg^{-1} protein h^{-1}) was not significantly changed after 1 day treatments with *E. coli* lipopolysaccharides at either 20 μg ml^{-1} (70.2 ± 10.1 pgBK mg^{-1} protein h^{-1}) or 200 μg ml^{-1} (85.7 ± 10.6 pgBK mg^{-1} protein h^{-1}), with 10 μM dexamethasone (86.7 ± 13.2 pgBK mg^{-1} protein h^{-1}), or with 250 nM ramiprilat (78.4 ± 7.7 pgBK mg^{-1} protein h^{-1}). Furthermore, prolonged treatment for 3 days with 30 ng ml^{-1} nerve growth factor (NGF, 85.2 ± 12.1 pgBK mg^{-1} protein h^{-1}), which effectively induced neurone-like cellular differentiation, or with 250 nM ramiprilat (84.3 ± 9.2 pgBK mg^{-1} protein h^{-1}), did not influence the rate of kininogen release. Stimulation of kininogen synthesis was only observed when cells were exposed for 1 day to 1 μM desoxycorticosterone (113.5 ± 10.0 pgBK mg^{-1} protein h^{-1}), ($P<0.05$ vs control).

Expression of kininogen mRNAs

Analysis of rat liver mRNA confirmed that the conditions of RT–PCR were suited to demonstrate specifically the presence of mRNAs for HMW- and LMW-kininogen, and T-kininogen (T_1- and/or T_2-kininogen), since the respective PCR products were of the predicted size (118 bps for LMW- and T-kininogens, 178 bps for HMW-kininogen) and contained specific endonuclease cleavage sites (cDNAs of all kininogen subtypes were cleaved by Msp I, those of both K-kininogens by TaqI, and those of HMW-kininogen by Sty I) (Figure 4). This assay provided sufficient selectivity to allow the differentiation of LMW- from T-kininogen cDNA in the same transcription reaction by modification of one PCR primer (Figure 4).

In PC12 cells, expression of LMW- and T-kininogen mRNA was likewise detected by the appearance of their respective PCR products (Figure 4). However, the level of expression was low compared to the liver. This is delineated from data that smaller signals arose despite of a more extensive PCR amplification, and that a significant production of nonspecific cDNA was observed (Figure 4). A faint band, corresponding to HMW-kininogen mRNA, could also be identified. However, an even stronger amplification of nonspecific sequences superimposed this signal and these were not cleaved by restriction endonucleases (Figure 4). We therefore concluded that HMW-kininogen is expressed only at a very low level.

Degradation of [^{3}H]-BK by PC12 cells

PC12 cell cultures were used at a protein content of 104 ± 3 μg/dish. [^{3}H]-BK was degraded by these cells with a rate constant of 0.014 ± 0.0006 min^{-1} corresponding to a half-life of 49 ± 2 min (Figure 5a). The fragments [1-7]-BK and [1-5]-BK were the predominant degradation products, which comprised 57 ± 5% of the applied radioactivity after 120 min (Figure 5a). 80 ± 5% of the total radioactivity was recovered in the peptide fractions analysed at the end of the incubations. The concentration of [1-7]-BK quickly increased during the initial 5 min, but reached a steady-state thereafter. This indicates the existence of a sequential degradation of BK via [1-7]-BK to [1-5]-BK which is typical for the activity of ACE (Skidgel, 1992). In accordance with this supposition, the rate of

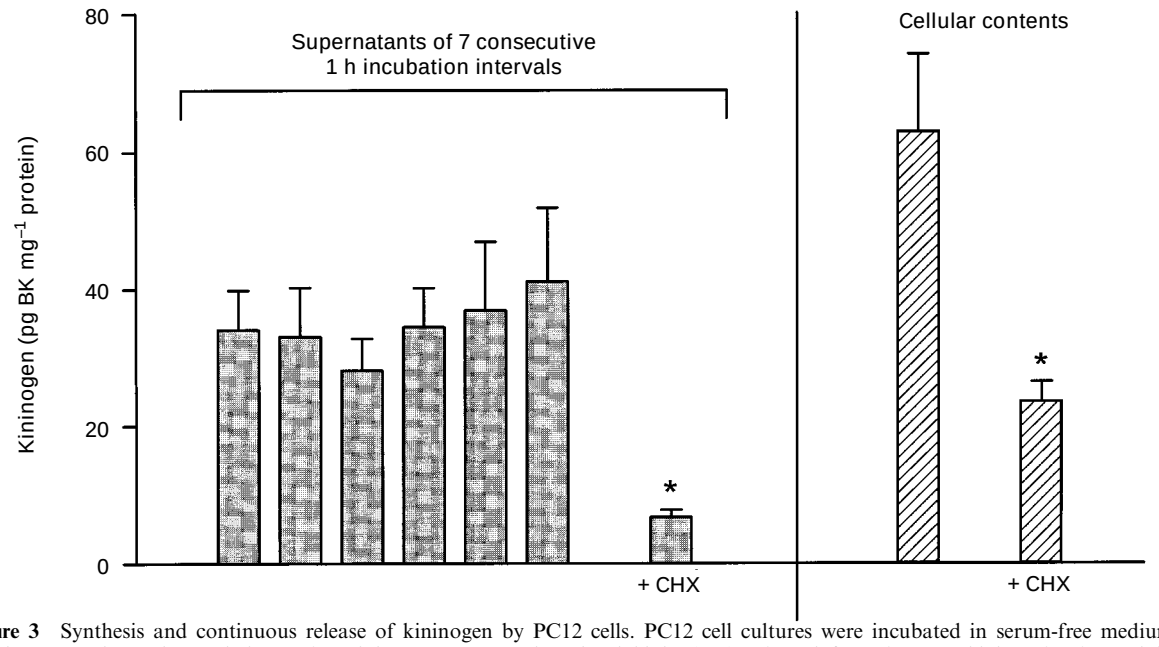

Figure 3 Synthesis and continuous release of kininogen by PC12 cells. PC12 cell cultures were incubated in serum-free medium which was exchanged at 1 h intervals. Kininogen, measured as bradykinin (BK) released from low- or high-molecular weight kininogen by trypsin, was constantly secreted into the medium at an average rate of 34.7 $\pm$ 3.4 pgBK mg^{-1} protein h^{-1} over 6 h. Subsequent application of cycloheximide (CHX; 100 μg l^{-1}) suppressed the release of kininogen (*$P<0.05$). The cellular kininogen contents corresponded to the approximate yield of a 2 h synthesis and were also significantly reduced by cycloheximide (*$P<0.05$). Each column represents the mean $\pm$ s.e.mean of 5 determinations.

[^{3}H]-BK breakdown was 85% lower (rate constant 0.0021 $\pm$ 0.0003 min^{-1}, half-life 366 $\pm$ 45 min) in the presence of ramiprilat (0.25 μM) (Figure 5b). Under these conditions, [1-5]- and [1-7]-BK were still the major metabolites. Their residual generation did not arise from an inadequate inhibition of ACE, but involved additional pathways. This is inferred since direct cleavage at the 5-6 position of BK could be shown by identification of [6-9]-BK, which was produced when unlabelled BK (20 μM) was used as a substrate (data not shown).

Discussion

Generation of kininogens, but not of kinins, by PC12 cells

The data presented demonstrate that PC12 cells continuously synthesize and secrete kininogen(s). At the level of protein expression, this finding applies to intact (kinin-containing) K-kininogen (LMW- and/or HMW-kininogen). Expression of kininogen by PC12 cells was verified by identification of mRNA for both LMW- and T-kininogen. Consequently, the released kinin precursor protein reflected a production of the glandular, but not of the plasma type of kininogen (LMW-, but not HMW-kininogen, respectively). This subtype selectivity corresponds to that of a kininogen produced locally in the kidney (Iwai *et al*., 1988). This first ever demonstration of kininogen expression in a neuronal cell type is also important for the interpretation of earlier histochemical studies, in which kininogens were localized to various regions of rat brain, although their origin could not of course be delineated (Richoux *et al*., 1991).

In contrast to PC12 cells, continuous synthesis of kininogen did not occur in aortic or microvascular endothelial cells. This confirms that kininogen synthesis is a cell-specific function, not shared by other cultured cell types, which also corresponds to the physiological situation where kininogen synthesis has been demonstrated only in specific organs (Iwai *et al*., 1988; Chao *et al*., 1993). The initial release of kininogen observed in microvascular endothelial cells may reflect extracellular binding or even intracellular storage of exogenous kininogen. Such effects have already been demonstrated in human umbilical vein endothelial cells in a study which also confirmed the absence of kininogen synthesis in that model (van Iwaarden *et al*., 1988).

In PC12 cells, the release of kininogen is not accompanied by a continuous generation of BK. The kinin-immunoreactivity accumulating in the medium over 6 h incubations is not degraded by ACE. In view of the predominant role of this enzyme for BK breakdown on PC12 cells (85% of total kininase activity), any contribution of authentic BK to the measured kinin immunoreactivity would have been increased by about a factor of 7 during ACE inhibition. The small and not statistically significant increase of BK immunoreactivity observed under these conditions indicates that the formation of BK from endogenous kininogen was negligible. This interpretation is supported by earlier functional studies in PC12 cells, which demonstrated a potent B_2-mediated stimulation of catecholamine release by exogenous BK, but no effect of Hoe 140 on basal release rates, thereby indicating the absence of endogenously produced kinins in PC12 cell cultures (Dendorfer & Dominiak, 1995).

As such, the functional consequence of kininogen synthesis in PC12 cells is not evident. In adrenal medulla, the presence of glandular kallikrein has been shown and thus local formation of kinins should be possible (Nolly *et al*., 1993). However, the physiological significance of local synthesis of kinin precursors *in vivo* is not really clarified. The situation is analogous to the renin-angiotensin system, where the precursor angiotensinogen is likewise expressed in tissue-specific systems (e.g. in cardiac myocytes) despite its systemic availability (Sawa *et al*., 1992). A favoured hypothesis is that cellular angiotensinogen may be the substrate for specific autocrine or even intracellular mechanisms, a situation which can also be proposed for the kinin-kallikrein system. The best studied example is that of endothelium-derived kinins, which were shown to contribute to the vasodilator action of ACE inhibitors (Wiemer *et al*., 1991), and the generation of which also involves the intracellular occurrence of kininogen. Kinins in adrenal medulla may therefore be assigned to a class of autocrine neuromodulator hormones, such as vasoactive intestinal polypeptide and neuropeptide Y which can be synthesized and released by chromaffin or phaeochromocytoma cells (Eiden *et al*., 1983; Tischler *et al*.,

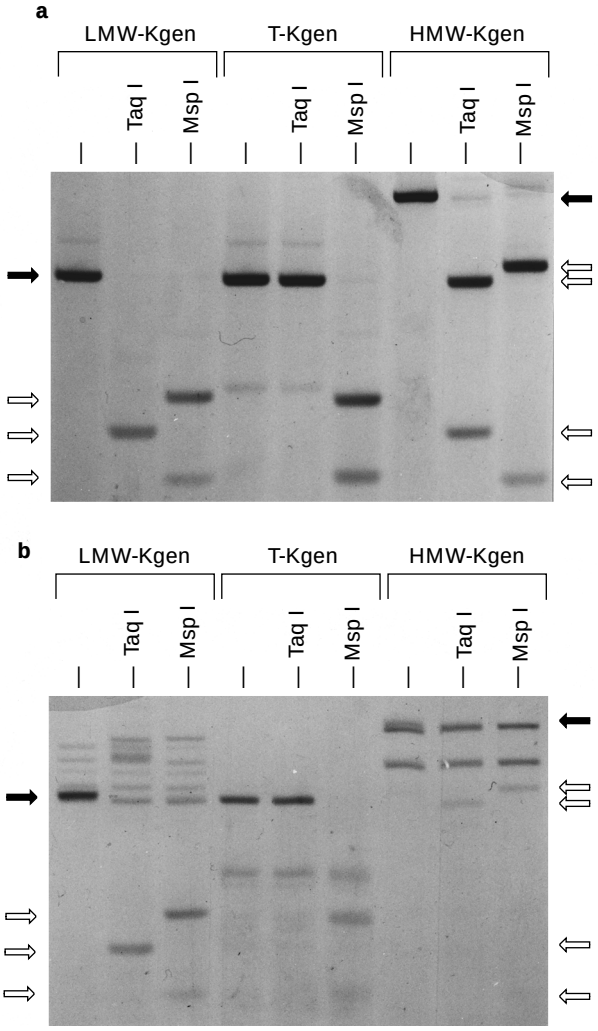

Figure 4 Amplification of kininogen-specific sequences from mRNA of (a) rat liver and (b) PC12 cells. Messenger RNA was transcribed and amplified by PCR by use of oligonucleotide primers in combinations suitable for the specific detection of low-molecular weight kininogen (LMW-Kgen; primers K-F and TL-R), high-molecular weight kininogen (HMW-Kgen; primers K-F and H-R), T_1- and T_2-kininogen (T-Kgen; primers T-F and TL-R). A silver-stained polyacrylamide gel electrophoresis of PCR products with and without restriction endonuclease treatment is presented. PCR products and their cleavage products are indicated by solid and open arrows, respectively. Rightdirected arrows refer to LMW-Kgen and T-Kgen, leftdirected arrows to HMW-Kgen. In the liver, expression of all three types of kininogen was demonstrated by PCR products which had the predicted sizes and endonuclease cleavage sites (all products were cleaved by Msp I, LMW- and HMW- kininogens were cleaved by Taq I). PC12 cells expressed kininogen mRNA at a lower level, as also can be concluded from the occurrence of nonspecific PCR products. In the case of HMW-kininogen, these nonspecific signals even predominated over the faint band of specific mRNA.

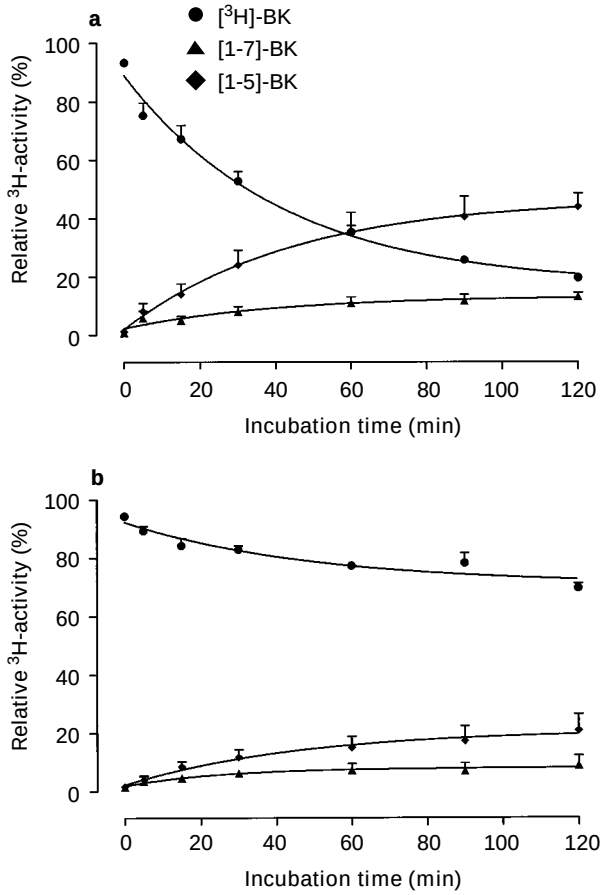

Figure 5 Kinetics of [^{3}H]-bradykinin ([^{3}H]-BK) breakdown by PC12 cells. Time courses of the relative amounts of [^{3}H]-BK and its degradation fragments [1-7]-BK and [1-5]-BK are depicted. The fragments [1-8]-BK and [1-6]-BK contributed less than 3% to the total radioactivity. The experiments were performed either in the absence of peptidase inhibitors (a) or after 15 min preincubation with and in the presence of 0.25 μM ramiprilat (b). The rate constant of [^{3}H]-BK degradation ($0.014 \pm 0.0006\ min^{-1}$ in the control group) was reduced by 85% in the presence of ramiprilat ($P<0.05$). Each point represents the mean and vertical lines show s.e.mean of 5 determinations.

1985). The proposed physiological effect of adrenal kinins on catecholamine secretion can be expected to be as a stimulant, as has been demonstrated repeatedly for exogenous BK (Staszewska-Barczak & Vane, 1967).

Several modes of pretreatment, intended to modify the activity of kininogen generation, failed to exert such effects. Among these were application of nerve growth factor, which was effective in inducing neuronal differentiation and neurite outgrowth, addition of the ACE-inhibitor ramiprilat which was enacted to potentiate the long-term effects of any endogenously formed BK, and administration of *E. coli* lipopolysaccarides, which induces the expression of T-kininogen *in vivo* (Kageyama *et al*., 1985). Only the mineralocorticoid desoxycorticosterone, but not the glucocorticoid dexamethasone, stimulated the rate of K-kininogen release. This appears to be the first demonstration of a regulated generation of K-kininogen, which so far has been found to be constitutively and constantly expressed in the few studies that have addressed this question. Expression of T-kininogen was shown to be induced by urethral occlusion, pulmonary vascular injury, endotoxins, interleukin-6 and oestrogen (Kageyama *et al*., 1985; Bouhnik *et al*., 1989; Chen *et al*., 1991; Chao *et al*., 1993; El-Dahr & Dipp, 1994), whereas a concomitant stimulation of K-kininogens was excluded in some of these studies (Kageyama *et al*., 1985; Bouhnik *et al*., 1989; El-Dahr & Dipp, 1994). However, the influence of mineralocorticoids has not yet been tested. The stimulant effect of a mineralocorticoid on K-kininogen expression which was observed in this study, may be paralleled *in vivo* by a similar effect on glandular kallikrein. A link between renal kallikrein secretion and mineralocorticoid status has been demonstrated in several experimental models and even in human volunteers (Vinci *et al*., 1979).

Kinin degradation by PC12 cells

Degradation of BK on PC12 cells proceeds primarily (by 85%) through the activity of ACE. Minor kininase activities appear

to produce both, [1-7]-BK and [1-5]-BK directly from BK, since no evidence for intermediates such as [1-8]-BK or [1-6]-BK could be found, and the fragment [6-9]-BK was identified as a possible metabolite. These degradation pathways must involve metallo-endopeptidase (EC 3.4.24.15) which is the only enzyme known to cleave the 5-6 bond of BK, as well as neutral endopeptidase (E.C. 3.4.24.11) and/or post-proline cleaving enzyme (EC 3.4.21.26) which both have the ability to produce the metabolite [1-7]-BK (Skidgel, 1992). However, none of these alternative kininases have been identified by inhibitor studies because of their minor significance to BK degradation in PC12 cells.

ACE in its membrane-bound form is found primarily in vascular endothelium and at resorptive epithelium, e.g. in renal tubular or intestinal epithelial cells. Regarding its presence in the nervous system, ACE has been localized to neuroepithelial cells in some brain regions and to the brush border of the choroid plexus (Erdös & Skidgel, 1986). Binding studies have also revealed the presence of ACE in adrenal medulla (Wilson *et al*., 1987), where its cellular distribution is as yet unclear. The preferential expression of ACE by PC12 cells indicates that neuronal cells can express this enzyme constitutively and it may, therefore, contribute to the ACE activity detected in adrenal medulla. Interestingly, ACE of adrenal medulla seems to participate in the control of cardiovascular functions, since its activity varies among different models of hypertension and shows significant correlation with plasma renin activity (Wilson *et al*., 1987). At its proposed location on chromaffin cells, ACE would have the potential to influence adrenal renin-angiotensin and kinin-kallikrein systems, providing an explanation for the sensitization of BK-induced adrenaline release, which is observed during ACE inhibition in rats (Dominiak *et al*., 1992).

In conclusion, this study demonstrates the generation and secretion of K-kininogen (low- or high-molecular weight kininogen), and the expression of mRNA for low-molecular weight and T-kininogen in PC12 cells. K-kininogen accumulates in the medium during incubation, but no significant amounts of kinins are formed. This indicates the absence of kallikrein activity in PC12 cells. The rate of K-kininogen formation is stimulated by the mineralocorticoid desoxycorticosterone, thereby identifying kininogen synthesis as a regulated function in PC12 cells. Bradykinin degradation on PC12 cells was brought about predominantly by ACE. Therefore kininogens, B_2-receptors and kininases are present in PC12 cells as constituents of a cellular kinin system. These properties of PC12 cells, when considered with the previously identified activity of adrenal kallikrein, may indicate the existence of a complete local kinin-kallikrein system in the adrenal medulla. Kinins may therefore play a physiological role as local vasodilator, autocrine neuromodulator or neurotransmitter hormones.

The authors want to thank Prof. U. Hilgenfeldt, Heidelberg, F.R.G. for his generous donation of BK-specific antiserum, and Prof. R. Heumann, Bochum, F.R.G. for providing PC12 cells. The expert technical assistance of Mrs K. Zelazko is greatfully acknowledged. We would also like to thank Dr J.P. Keogh for revising the English in the manuscript.

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(Received July 7, 1997
Accepted September 11, 1997)