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KININASES OF HUMAN POLYMORPHONUCLEAR LEUCOCYTES

MASAO NAKAHARA
Department of Orthopaedic Surgery, Sapporo Medical College, Sapporo, Japan

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Abstract—Four kininases were isolated from human polymorphonuclear (PMN) leucocytes by gel filtration. The molecular weights of kininases I, II, III and IV were 270,000, 90,000, 47,000 and 4000, respectively. No appreciable difference among these kininases was observed in the optimum pH between 7.5 and 8.5. The K_m value of the four kininases for bradykinin hydrolysis was 0.20×10^{-6} M. These kininases did not hydrolyse hippuryl-hystidyl-leucine and hippuryl-L-lysine, in sharp contrast to plasma kininase. The kininase activity of the enzymes was completely destroyed by neutral proteases in the PMN cell lysosomal granule extract or after heating at 56° for 30 min. Kininases II, III and IV were released extracellularly during phagocytosis of kaolin pellets (200 μg/ml). The properties of the PMN cell kininases are discussed in relation to their possible role in the kinin system at the imflammatory site.

Polymorphonuclear leucocytes (PMN) are the first cells to appear in the acute inflammatory exudate. Tissue leucocytosis followed by their accumulation is closely related to the delayed response which is considered to be the essential part of the inflammatory reaction [1–4]; the onset of the delayed permeability response coincides with the onset of leucocytosis and the decline of this response with massive leucocytosis. A number of neutral proteases are present in human PMN cells. A kininase was first described in the PMN cells by Schwab [5] and this finding has been confirmed by many colleagues using cell-lysed samples [6–8]. As well as kininase, a kinin-forming enzyme has been demonstrated in the PMN cells [6, 9, 10] and isolated by Movat *et al.* [11]. With regard to the kinin system in the PMN cells, it has been known that the kininase is present in the extra-lysosomal fraction of the PMN cells while the kinin-forming enzyme is mainly present in the lysosomal fraction. The kininase and kinin-forming enzyme in the PMN cells may be able to shift the balance of bradykinin level to reduction or enhancement at an inflammatory site. For these enzyme activities, a shift of pH to the acidic or alkaline range was suggested to be an important factor [6]. However, the question of how the properties of these enzymes in the PMN cells participate in the kinin system during inflammation has remained unanswered. The author found four kininases in human PMN cells. This paper deals with isolation of four kininases, release of kininases during phagocytosis, and an interaction in the kinin system between PMN cells and plasma.

MATERIALS AND METHODS

Isolation of PMN leucocytes. ADC blood was obtained from Green Cross Blood Bank. To isolate PMN cells, dextran sedimentation techniques [12] were used for the initial separation and the further steps were performed by the method of Fallon *et al.* [13]. The PMN cells were counted and adjusted to 2×10^7/ml.

Preparation of extra-lysosomal fraction and lysosomal granule extract. The pooled cells were disrupted in a Potter–Elvehjem homogenizer with a motor-driven Teflon pestle. The suspension was centrifuged at 400 *g* for 10 min to remove unbroken cells and nuclei. The resulting supernatant fraction was centrifuged at 8000 *g* for 15 min to collect the lysosomal granule fraction. The supernatant obtained was used as an extra-lysosomal fraction. The lysosomal granule fraction was homogenized in 0.15 M KCl using a Potter–Elvehjem homogenizer with a motor-driven glass pestle and centrifuged at 25,000 *g* for 30 min. The supernatant was used as a lysosomal granule extract (80 μg protein/ml). The extract possessed kinin-forming activity and generated kinin equivalent to 2100 ng bradykinin/10 min/mg protein.

Gel filtration of PMN cell kininase. The extra-lysosomal fraction, which is a crude PMN cell kininase preparation, was subjected to gel filtration on a 3.5 × 50 cm column of Sephadex G-200 equilibrated with 0.05 M phosphate buffer, pH 7.4, containing 0.1 M NaCl. Some of the active fractions were again gel-filtered on Sephadex G-200 (3.5 × 50 cm) or G-75 (1.5 × 140 cm) column equilibrated with the same buffer as before.

Plasma kallikrein. Plasma kallikrein was prepared as described previously [14].

Plasma kininase. Plasma was treated according to the method of Rinvik *et al.* [15].

Enzyme assays. Kininase activity was estimated by inactivation of bradykinin as described previously [16]. The hydrolysis of hippuryl-histidyl-leucine (HHL) and hippuryl-L-lysine (HLL) was measured according to Folk *et al.* [17] and Erdös *et al.* [18]. The cleavage of benzoyl-prolyl-phenylalanyl-arginine-*p*-nitroanilide (PPAN) for kallikrein activity was measured by the method of Claeson *et al.* [19]. The kininogenase activity was estimated using kininogen by the method previously described [14].

Protein. The protein content of the solutions was determined by the method of Kalckar [20].

Chemicals. Bradykinin, HHL and HLL were obtained from the Protein Research Foundation, Osaka, Japan; soy bean trypsin inhibitor (SBTI) and lima bean trypsin inhibitor (LBTI) from Sigma Chemical Co., St. Louis, MO; *trans*-aminomethylcyclohexene-carboxylic acid (t-AMCHA) from Daiichi Seiyaku, Co., Tokyo, Japan and PPAN from A B Kabi, Sweden.

RESULTS

PMN cell kininase in the extra-lysosomal fraction and their properties. The extra-lysosomal fraction was first subjected to Sephadex G-200 gel filtration. Four peaks having kininase activity were eluted as shown in Fig. 1. The fractions from each of these peaks were pooled and concentrated. The first (I) and second peak (II) were again subjected to gel filtration on a Sephadex G-200 column, and the third (III) and fourth peak (IV) on a Sephadex G-75 column. Each kininase activity appeared as a single peak. These kininases were used as enzyme sources. The molecular weights of these kininases were estimated on Sephadex columns using human kininase I (mol. wt 280,000), human gamma globulin (mol. wt 160,000), human plasma kallikrein (mol. wt 100,000), bovine serum albumin (mol. wt 67,000), cytochrome *c* (mol. wt 12,500) and trasylol (mol. wt 6500) as protein markers. The molecular weights of PMN kininases I, II, III and IV were approximately 270,000, 90,000, 47,000 and 4000, respectively. No appreciable difference among these four kininases was observed in the optimum pH for the kininase activity, showing that their activity with the optimum pH between 7.5 and 8.0 rapidly declined at a pH less than 6.0. K_m values of the enzymes for bradykinin hydrolysis were determined by means of a Lineweaver–Burk plot. The K_m value of the PMN cell kininase I, II, III and IV was 0.20×10^{-6} M. This value is close to 0.17×10^{-6} M, the value obtained for human plasma kininase II and its subunits [14]. But all of these enzymes did not hydrolyse HHL and HLL, in sharp contrast to plasma kininase [14, 21, 22]. The kininase activities of the enzymes were completely destroyed after heating at 56° for 30 min or at 65° for 10 min as well as bringing the pH to 5.0 at 37° for 15 min, while these enzymes were stable for a few days after standing at 4°. The alterations induced by various agents on the hydrolysis of bradykinin of the enzymes are shown in Table 1. Table 1 shows similarities in behaviour among these kininases except for $CuSO_4$. All of the PMN cell kininases were readily inhibited by $HgCl_2$, SBTI and trasylol but not by 1,10-phenanthroline and DFP, indicating that there is some discrepancy between the kininases discussed in this paper and the non-isolated kininase previously described [5–7, 23]. The behaviour of the enzymes toward EDTA and 1,10-phenanthroline is greatly different from plasma kininases [14].

Inactivation of the PMN cell kininases by lysosomal extract. The concentration of each sample of the PMN cell kininases I, II, III and IV in phosphate buffer, pH 7.4, was adjusted to such a level that 50 per cent hydrolysis was not exceeded during a 10-min incubation, and the samples were incubated with 0.1 ml of the lysosomal extract at 37° for 10 min. All kininases were completely inactivated by neutral proteases in the lysosomal extract, indicating that the neutral proteases in the lysosomal extract contain an enzyme able to destroy the PMN cell kininases.

Release of the kininases from the PMN cell suspension incubated with a kaolin pellet. A suspension of isolated PMN cells containing 5×10^7 per ml was prepared in Krebs III buffer containing 50 mg glucose per ml. Ten milliliters of the PMN cell suspension were incubated with a kaolin pellet at a final concentration of 200 μg per ml at 37° for 30 min in siliconized glassware. The supernatant fraction obtained by centrifugation was subjected to Sephadex G-100 gel filtration. Three peaks having kininase activity were eluted as shown in Fig. 2a. The molecular weights of these three kininases were estimated to be 90,000, 47,000 and 4000. All of the three kininases were inhibited by $HgCl_2$, $CuSO_4$, SBTI and trasylol, but not 1,10-phenanthroline and DFP. The K_m value of these three kininases was 0.20×10^{-6} M for bradykinin hydrolysis. These results suggest that these three kininases represent PMN cell kininases II, III and IV in Fig. 1. A small amount of kinin-forming enzyme was found at a molecular weight of 20,000, as shown in Fig. 2a. The kinin-

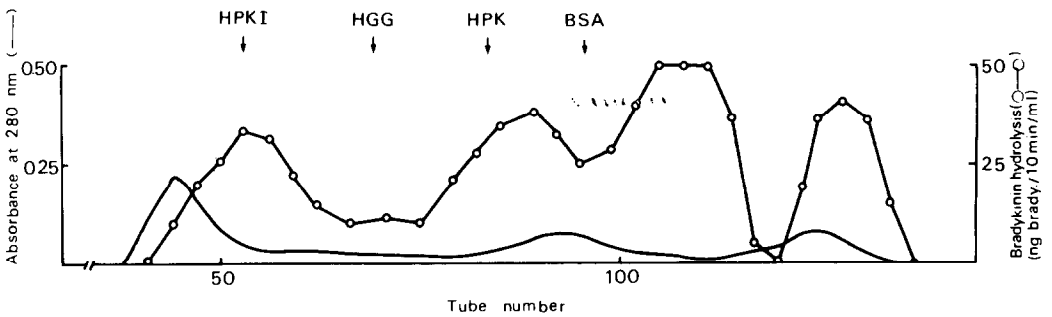

Fig. 1. Gel filtration of PMN cell kininases on a 3.5 × 50 cm column of Sephadex G-200. Ten millilitres of extra-lysosomal fraction (4.2 mg protein/ml) were applied to the column. Three milliliter fractions were collected at a flow rate of 10 ml/hr. The column was calibrated with human plasma kininase I (HPKI), human gamma globulin (HGG), human plasma kallikrein (HPK) and bovine serum albumin (BSA).

Table 1. Inhibition of hydrolysis of bradykinin by PMN kininases*

Agent	Concentration	PMN kininase I	II	III	IV
$HgCl_2$	1×10^{-4} M	++	++	++	++
$ZnCl_2$	1×10^{-4} M	0	0	0	0
$ZnSO_4$	1×10^{-4} M	0	0	0	0
$CuSO_4$	1×10^{-4} M	++	+	+	0
EDTA	1×10^{-4} M	0	0	0	0
1,10-Phenanthroline	1×10^{-4} M	0	0	0	0
SBTI	10 μg	+	+	+	+
LBTI	100 μg	++	+	+	0
Trasylol	10 U	+	+	+	+
	50 U	++	++	++	++
DFP	1×10^{-4} M	0	0	0	0
t-AMCHA	1×10^{-4} M	0	0	0	0

* ++, more than 60 per cent inhibition; +, 20–59 per cent inhibition; 0, 0–19 per cent inhibition. For assay of inhibition, the kininase samples were preincubated with inhibitor at 37° for 10 min.

forming enzyme may be the same as the kininogenase described by Movat *et al.*]11]. When the PMN cell suspension was incubated with kaolin pellet at a final concentration of 1000 μg per ml, a large amount of the kinin-forming enzyme having a molecular weight of 20,000 and four kininases were eluted with a striking contrast to their distribution in Fig. 2a, as shown in Fig. 2b. The findings obtained from molecular weight and behaviour toward kininase inhibitors suggest that these four kininases represent the PMN cell kininases I, II, III and IV in Fig. 1. The result showing that all of the PMN cell kininases were found extracellularly may be due to lysis of the PMN cells. In fact, severe lysis of the PMN cells was observed microscopically after incubation. A negligible amount of kininase and kinin-forming enzyme was eluted in the control experiment carried out without addition of a kaolin pellet.

Effect of neutral proteases in the lysosomal extract on the kinin system in plasma. The experiment was carried out using phosphate buffer, pH 7.4. Kinin-forming activity of plasma kallikrein was adjusted to a concentration of 100 ng bradykinin generation per 10 min per ml, and kininase activity of plasma kininases to a concentration stated in the experiment on the inactivation of the PMN cell kininases. Neither plasma kallikrein nor plasma kininases showed changes in their activities after incubation with 0.1 ml lysosomal extract at 37° for 10 min. Non-contact plasma (0.1 ml), lysosomal extract (0.1 ml) and phosphate buffer (0.8 ml) were mixed and incubated at 37° for 30 min. No evidence of prekallikrein

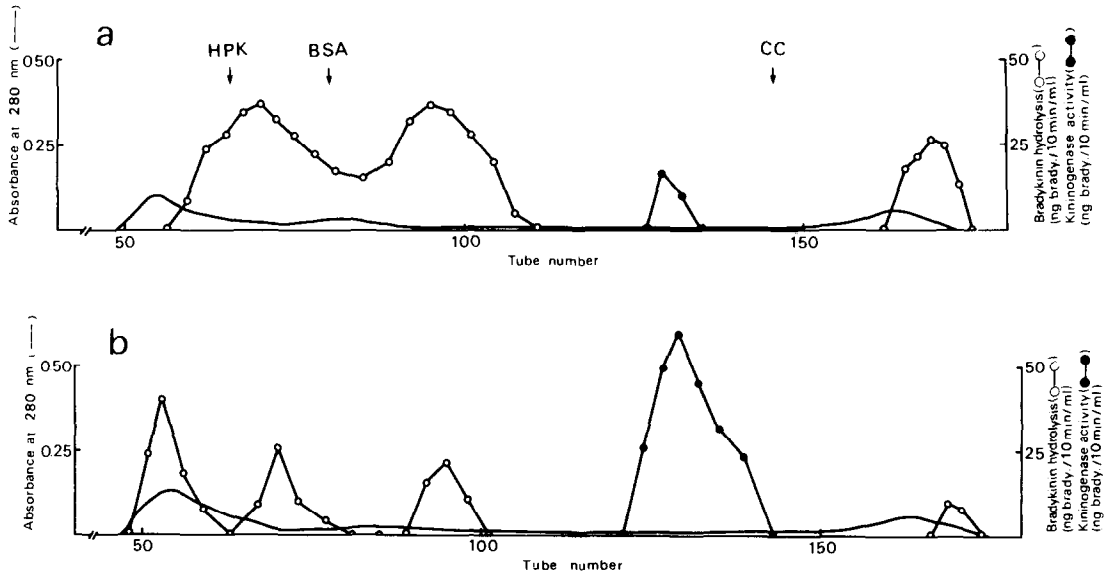

Fig. 2. Gel filtration on a 2.3 × 128 cm column of Sephadex G-100 of the supernatant obtained from the PMN suspension after incubation with a kaolin pellet. Three milliliter fractions were collected at a flow rate of 13 ml/hr. Panel a: the kaolin pellet was suspended at a final concentration of 200 μg/ml. Panel b: the kaolin pellet was suspended at a final concentration of 1000 μg/ml. The columns were calibrated with human plasma kallikrein (HPK), bovine serum albumin (BSA) and cytochrome *c* (CC).

activation was observed in the mixture by PPAN cleavage for kallikrein activity.

DISCUSSION

The release of bradykinin and prostaglandin has been described in exudate produced in various types of experimental inflammation [24–27]. The early inflammatory response yields an extravascular reaction dominated by PMN cells which may be accumulated by bradykinin released in the inflammatory site. Thus, phagocytosis of microorganisms or immune complexes may be commenced by phagocytic function of the accumulated PMN cells. In this study, four kininases were isolated by gel filtration. Three kininases except for the 270,000 mol. wt kininase were released from the PMN cells during phagosytosis of kaolin pellet (200 μg per ml). This finding suggests that the release of kininases observed during phagocytosis is not due to lysis of the PMN cell membrane, although the invagination of cell membrane and formation of a digestive pouch are contained in the phagocytic process [28–30]. Furthermore, it is implied that an extracellular release of the neutral proteases in lysosomal granules was not involved in the process of the kininase release, because a concomitant release of the kininases and neutral proteases would result in inactivation of the kininases by the neutral proteases as described in the results. The finding that the PMN cell kininase activities were reduced with addition of the kaolin pellet as shown in Fig. 2b may be attributed to a concomitant release of the kininases and the neutral proteases induced by cell lysis during phagocytosis.

PMN cell granules contain a number of neutral proteases [31] such as kinin-forming enzyme [6, 11], the PMN cell kininase-inactivating enzyme described in the results, collagenase [31] and elastase [32–34]. It is known that controlled granule lysis occurs for discharge of the neutral proteases into the digestive pouch during phagocytosis [30, 35–37]. The controlled granule lysis induced by phagocytosis may be only responsible for the extracellular release of the kininases from the PMN cells but not for the extracellular release of the neutral proteases. The extracellular release of the whole enzymes in the PMN cells could be induced by lysis of the digestive pouch and cell membrane which may occur during phagocytosis of an excess amount of small particles. Thus, participation of the kininase and kinin-forming enzyme in the kinin system at the inflammatory site might be different whether lysis of the digestive pouch and cell membrane develops or not. In an early inflammatory state in which the controlled granule lysis is in progress, the PMN cell kininases may play a role as an extra-plasma kininase source, resulting in an enhancement of kininase activity in the interstitial fluid which contains plasma kininases. In a state in which the lysis of the digestive pouch and cell membrane is in progress, the plasma kininase resistant to the neutral proteases in the PMN cell granules becomes the main kininase because the PMN cell kininases are inactivated by the neutral proteases. Furthermore, plasma kallikrein resistant to the neutral proteases and the kinin-forming enzyme in the neutral proteases may contribute to shifting the balance of local bradykinin level to enhancement. The perpetual generation of bradykinin induced by lysis of the PMN cells could be responsible for a prolonged reaction such as the delayed response of enhancement of vascular permeability. In summary, it is conceivable that the PMN cells are able to act in inflammation by enhancement of local bradykinin inactivation or formation.

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