Kinin-Forming Enzyme of Rat Cardiac Tissue
Subcellular Distribution and Biochemical Properties
JORGE BRITOS, B.S., AND HÉCTOR NOLLY, M.D.

SUMMARY We have previously reported the presence of a cardiac kinin-generating enzyme (CKGE) in human cardiac tissue. The present study was undertaken to examine the subcellular localization and the biochemical properties of this system in rat cardiac tissue. Cardiac homogenate (20 mg) was incubated with heat-treated plasma substrate and citrate-phosphate buffer (pH 5.3) in the presence of peptidase inhibitors, and the vasoactive substance released was tested on the isolated cat jejunum and the arterial pressure of the rat. Rat cardiac tissue contains an enzyme which releases a kinin from a substrate present in rat plasma. The kinin-releasing substance is nondialyzable and heat labile. The rate at which it releases kinin is similar to the rate of kinin released by kallikrein and trypsin, but it is unaffected by incubation of the enzyme with soybean trypsin-inhibitor or trasylol. The study of its subcellular distribution by differential centrifugation showed a similar pattern to that of acid phosphatase. With sucrose density gradient centrifugation, the equilibrium density for the CKGE-containing particles was 1.164 ± 0.002 g/ml and the distribution of the CKGE was also similar to that of acid phosphatase. The CKGE showed a pi of 4.5 ± 0.2 and an apparent molecular weight of about 30,000 to 35,000 by gel filtration (Aca-54). The kinin released by cardiac homogenate resembles bradykinin in its chemical and pharmacological properties, and, by using antibodies that specifically inhibit kinins, the biological action was completely abolished. These results indicate that the subcellular distribution of the CKGE is similar to that of a lysosomal protease, and that its physicochemical characteristics differ from those of plasmatic kallikrein but are quite similar to those of kallikreins of glandular origin. (Hypertension 3 (suppl II): II-42-II-45, 1981)

KEY WORDS • cardiac kinin-generating enzyme • subcellular fractionation • bradykinin • lysosomal protease

Present studies indicate that kinins have an important influence on coronary vascular resistance by acting directly on resistance vessels or by inducing prostaglandin release. Kinins are appreciably elevated in the coronary sinus blood of dogs after occlusion of the anterior descending coronary artery. Furthermore, an activated kinin-generating system has been observed in the coronary sinus and aortic blood in seven of 11 patients with myocardial ischemia. We previously observed that proteolytic activity capable of producing kinins from kininogen is present in crude extracts of human, bovine, and dog cardiac tissue.

This study describes the subcellular distribution and biochemical properties of the kinin-forming enzyme present in cardiac tissue of the rat.

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Materials and Methods
Cardiac Homogenate
Wistar rats weighing between 200–250 g were sacrificed by decapitation and exsanguination, a polyethylene tube was inserted in the aorta, and the heart was immediately perfused retrogradely with oxygenated Krebs-Henseleit solution (32°C) for 60 minutes to eliminate blood. The tissues were homogenized in distilled water (100 mg/ml), centrifuged (20,000 g) for 10 minutes, and the supernatant dialyzed for 24 hours against a saline solution and stored at −20°C until used.

Kallikrein Substrate
The substrate was prepared from citrated rat plasma incubated for 3 hours at 56°–58°C. Ammonium sulfate was then added and the fraction that precipitated between 34% and 50% saturation was saved. This fraction was dialyzed against running tap water and then lyophilized.
Cardiac Kinin-Generating Enzyme (CKGE)

The CKGE activity was determined by incubating 0.20 ml of the homogenate (20 mg) with 2.000 ng of the substrate preparation, and 0.3 ml of 0.01 M 1,10-phenanthroline and 0.1 ml of 0.3 M sodium EDTA to inhibit kininases. The volume was adjusted to 2.5 ml with citrate-phosphate buffer (pH 5.3). The samples were incubated at 37°C for the specified periods, and the reaction stopped by placing the samples in a boiling water bath for 5 minutes. The kinins formed were assayed with the isolated cat jejunum and blood pressure response of the anesthetized rat. The enzyme activity was expressed in nanograms of bradykinin released per milligram tissue per hour of incubation.

Subcellular Fractionation

Differential centrifugations were carried out at 4°C in a Sorvall RC2-B centrifuge with a rotor type SS-34. The homogenate (20 ml) prepared with 2 g of cardiac tissue was fractionated as described by Tolnai and Beznak. Nuclear, mitochondrial, lysosomal, and supernatant fractions were obtained by centrifugation for 10 minutes at 700 g, 10 minutes at 12,700 g, and 45 minutes at 37,000 g respectively. All pellets were resuspended in 0.25 M sucrose containing 0.1 mM EDTA and buffered with 10 mM Tris buffer to pH 7.4.

To perform a density gradient centrifugation, a portion (0.25 ml) of lysosomal fraction was layered on the top of a freshly prepared continuous density gradient, consisting of sucrose (30% to 61.7%) and centrifuged at 90,000 g for 3 hours in a 3 X 5 ml swinging-bucket rotor type SW-39 in a Beckman ultracentrifuge at 4°C. After centrifugation the tubes were individually separated into 10 fractions by aspiration from bottom to top, and a pellet which was resuspended in 0.25 M sucrose. Acid phosphatase was assayed with sodium glycerophosphate as substrate, cytochrome oxidase by the method of Cooperstein and Lazarow, CKGE by bioassay, and proteins by the method of Lowry et al.

Molecular Weight Determination

A K 15/30 column (Pharmacia Fine Chemicals) with Ultrogel AcA54 (LKB), for the gel filtration medium, was used to determine the molecular weight of the CKGE. The active fraction was eluted with 50mM phosphate buffer pH 7.4 at a constant flow rate of 0.11 ml/min. Three protein standards (lysozyme, trypsin, and bovine serum albumin) were used to calibrate the column. Fractions (3.3 ml) were collected for protein and CKGE determination. Results were expressed as $K_v$ ($V_e - V_o/V_t - V_o$) versus the logarithm of the molecular weight ($V_o$ is the elution volume, $V_o$ is the void volume, and $V_t$ the total volume).

Isoelectric Point Estimation

The test-tube method was chosen to estimate the approximate isoelectric point of CKGE. A series of test-tubes with 0.2 g of DEAE-Sephadex A-50 (Pharmacia Fine Chemicals) were equilibrated with acetate buffers of different pHs and constant ionic strength (0.1). A constant amount of active fraction was added to each tube and mixed. After 30 minutes, the supernatant was assayed for the substance.

Drugs

The following drugs were used: synthetic bradykinin (Sigma); trypsin inhibitor (Sigma); aprotinin (Trasylol, Bayer); pentobarbitone sodium (Abbott), and pepstatin (Sigma, prepared at 2 mg/ml dissolved at pH 9-11). Antiserum to bradykinin was donated by Dr. O. Carretero, Detroit, Michigan.

Results are expressed as mean ± SEM and statistical significance was determined by Student's $t$ test.

Results

Subcellular Fractionation

Kallikrein-like activity was found in all fractions isolated. Specific activity was highest in the lysosomal fraction and lowest in the mitochondrial and supernatant fractions. Figure 1 shows that the CKGE had a distribution pattern similar to that of phosphatase and different from that of cytochrome oxidase. Osmotic shock of the lysosomal fraction induced liberation into the supernatant of approximately 75.5% ± 7% of the total CKGE activity. The nature of the sedimentable...
material associated with the CKGE activity was further investigated by density gradient centrifugation of the lysosomal fraction. Maximum activity was found associated with acid phosphatase activity. Evidence of absorption to other subcellular structures was not found. The equilibrium density for the CKGE-containing particle was 1.164 ± 0.002 g/ml.

**Molecular Weight**

Figure 2 shows the calibration curves obtained for determining the molecular weight. With Ultrogel Aca-54 for the gel medium, CKGE eluted between trypsin (23,800) and bovine serum albumin (66,500). The cardiac enzyme shows an approximate molecular weight between 30,000 and 35,000.

**Other Properties of the Cardiac Kinin-Generating System**

Table 1 shows some other characteristics of the kinin-forming enzyme present in rat cardiac tissue. The kinin-releasing substance is nondialyzable and heat labile. It shows an isoelectric point of 4.5 ± 0.2. It releases a kinin from a substrate present in rat plasma at a rate similar to that released by kallikrein and trypsin. The release is unaffected, however, by incubation of the enzyme with trasyloil or with soybean trypsin inhibitor. Pepstatin, an inhibitor of cathepsin D, leukokinin-forming enzyme, renin, and pepsin, do not inhibit CKGE in the concentrations of 10⁻²-10⁻⁶ M. The kinin released by cardiac homogenate resembles bradykinin and kallidin in its chemical and pharmacological properties. Figure 3 shows that the contractions of smooth muscle were inhibited by antibodies that specifically inhibit the biological effect of kinins.

**Discussion**

This study demonstrates that a cardiac kinin-generating enzyme (CKGE) is present in the rat. Using differential centrifugation of the cardiac homogenate we have found that the system occurs primarily in the lysosomal fraction. During density gradient centrifugation, the CKGE displayed a distribution similar to that of the lysosomal fraction. The equilibrium density for the CKGE-containing particle was 1.164 ± 0.002 g/ml.

**Table 1. Characteristics of the Kinin-Forming Enzyme in Rat Cardiac Tissue**

<table>
<thead>
<tr>
<th>Properties</th>
<th>Plasma kallikrein</th>
<th>Glandular kallikrein</th>
<th>CKGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>100,000</td>
<td>24,000-44,000</td>
<td>30,000-35,000</td>
</tr>
<tr>
<td>Substrate</td>
<td>HMWK</td>
<td>LMWK and HMWK</td>
<td>LMWK (and/or?) HMWK</td>
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<tr>
<td>Kinin released</td>
<td>Bk</td>
<td>Lys-Bk</td>
<td>Bk or Lys-Bk</td>
</tr>
<tr>
<td>Inhibited by SBTI</td>
<td>yes</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Inhibited by Trasyloil</td>
<td>no</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>8.7</td>
<td>4.0</td>
<td>4.5 ± 0.2</td>
</tr>
</tbody>
</table>

LMWK = low molecular weight kininogen; HMWK = high molecular weight kininogen; CKGE = cardiac kinin-generating enzyme; SBTI = Soybean trypsin-inhibitor; Bk = bradykinin; Lys-Bk = lys-bradykinin.
to that of marker enzyme used for lysosomes. Rat cardiac enzyme acting on a protein plasma substrate releases an active peptide that has similar pharmacological properties to bradykinin and its analogs. In isolated tissue preparations, the active product contracts the rat uterus and the cat jejunum. In vivo, it is vasoactive and depresses blood pressure probably by relaxing arteriolar smooth muscle. By using antibodies that specifically inhibit the action of bradykinin and lysyl-bradykinin, the biological effect was almost completely abolished.

The kinin-forming enzyme of cardiac tissue differs clearly from plasma kallikreins as follows:

1. It has an optimum pH in the acid range (pH 5.3). It may be noted that about 30% of the optimal activity is still present at neutral pH. Thus, the formation of kinin will proceed at normal body pH as well as in the acid range.

2. It shows an isoelectric point of 4.5 ± 0.2 and an apparent molecular weight about 30,000 to 35,000.

3. It is not inhibited by soybean trypsin inhibitor. Thus, the contribution of the CKGE to peptide release may be differentiated from that of plasmatic kallikrein. The fact that CKGE is not inhibited by aprotinin (Trasylol) indicates that it is also different from other known glandular kallikreins.

These results indicate that the cardiac enzyme is able to release a vasoactive peptide similar to bradykinin or lysyl-bradykinin. The characteristics of the cardiac enzyme are similar to those of a lysosomal protease of the cathepsin-like type of enzyme. It is uncertain whether the CKGE of the rat participates in the genesis of kinin in the heart in physiological conditions. Greenbaum11 has suggested that an alternate kinin-generating system is present in the body and the presence of leukokinin-forming enzymes in white cells obviously points to the potential formation of leukokins as potent inflammatory agents, especially in terms of increasing vascular permeability. The current study offers no information on the functional significance of the CKGE in the physiological or pathological process. However, such possibility cannot be excluded, and it would probably provide an alternative pathway of kinin generation.

References

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