Identification, purification, and localization of tissue kallikrein in rat heart

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A tissue kallikrein has been isolated from rat heart extracts by DEAE-Sepharose and aprotinin-affinity column chromatography. The purified cardiac enzyme has both *N*-tosyl-L-arginine methyl ester esterolytic and kinin-releasing activities, and displays parallelism with standard curves in a kallikrein radioimmunoassay, indicating it to have immunological identity with tissue kallikrein. The enzyme is inhibited by aprotinin, antipain, leupeptin and by high concentrations of soybean trypsin inhibitor, but stimulated by lima-bean or ovomucoid trypsin inhibitor and low concentrations of soybean trypsin inhibitor. By using a specific monoclonal antibody to tissue kallikrein in Western blot as well as active-site labelling with [¹⁴C]di-isopropyl fluorophosphate, the cardiac enzyme was identified as a protein of 38 kDa, a molecular mass identical with that of tissue kallikrein. Immunocytochemistry at the electron-microscopic level localized this enzyme to the sarcoplasmic reticulum and granules of rat atrial myocytes. Two cardiac kallikrein precursors, (38 and 40 kDa) were identified from the translation *in vitro* of heart mRNA by immunoprecipitation and electrophoresis of [³⁵S]methionine-labelled cell-free translation products. Kallikrein mRNA in the rat heart was also demonstrated by dot-blot analysis using a tissue kallikrein cDNA probe. These results indicate that the tissue kallikrein gene is expressed in the rat heart and that the purified enzyme is indistinguishable from tissue kallikrein with respect to enzymic and immunological characteristics.

INTRODUCTION

Tissue kallikreins (EC 3.4.21.35) are serine proteinases that cleave kininogen substrates to produce potent vasoactive kinin peptides (Schachter, 1980). *In vitro*, kinins are able to cause either smooth-muscle contraction or relaxation depending on the specific target tissue (Johnson, 1979). Many tissue sites, not previously recognized as kallikrein-containing, have been found to have a proteinase that is enzymically and immunologically identical with the tissue kallikrein in urine, kidney and salivary gland. These sites include spleen (Chao *et al.*, 1984), vasculature (Nolly *et al.*, 1985), pituitary (Powers & Hatala, 1986; Chao *et al.*, 1987), brain (Chao *et al.*, 1983) and skeletal muscle (Shimojo *et al.*, 1987). In addition to kininogen, recent studies have expanded the list of potential natural substrates to include apolipoprotein B-100 (Cardin *et al.*, 1984), renin (Sealey *et al.*, 1978), insulin (ole-MoiYoi *et al.*, 1979) and pro-(atrial natriuretic peptide) (pro-ANP) (Currie *et al.*, 1984). These findings suggest the possibility that kallikreins present in these endocrine and exocrine tissues may have a different functional significance as well as different regulatory mechanisms (Chao *et al.*, 1983, 1987; Nolly *et al.*, 1985; Powers & Hatala, 1986).

Studies indicate that kinins play an important regulatory role in cardiac function by acting directly on vascular resistance or by inducing prostaglandin release (Needleman *et al.*, 1975). The kinin-generating system is thought to have a great impact on the pathophysiology of heart disease. Evidence for this is shown in elevated kinin levels in the coronary sinus blood of dogs after occlusion of the anterior descending coronary artery (Kimura *et al.*, 1973), and also in an activated kinin-generating system observed in the coronary sinus and aortic blood in seven of eleven patients with myocardial ischaemia (Pitt *et al.*, 1970). Reports have shown that enzymic activity capable of producing kinins from kininogen is present in crude extracts of human,

bovine, dog and rat cardiac tissue (Nolly *et al.*, 1981; Britos & Nolly, 1981); that the enzyme is tissue kallikrein has not been confirmed. We have recently identified another member of the tissue kallikrein gene family, namely arginine esterase A, in cardiac tissue by immunocytochemistry at the optical- and electron-microscopic level (Simson *et al.*, 1989). Several recent studies have suggested that the processing enzymes for pro-ANP are present in heart tissues (Cantin & Genest, 1985; Schwartz *et al.*, 1985; Sugawara *et al.*, 1985; Thibault *et al.*, 1986). The identification of tissue kallikrein in heart, added to our previous findings that exogenous tissue kallikrein *in vitro* can activate ANP from pro-ANP in rat heart extracts (Currie *et al.*, 1984), would also support the notion that tissue kallikrein may be one of the regulatory enzymes involved in ANP processing.

The present study describes the identification, isolation and localization of tissue kallikrein in the rat heart and demonstrates kallikrein biosynthesis in the cell-free translation products encoded by heart mRNA.

EXPERIMENTAL

Materials

The following materials were obtained from commercial sources: DEAE-Sepharose CL-6B (Pharmacia); Affi-Gel 10, low-molecular-mass protein standards and prestained molecular-mass protein standards (Bio-Rad); bovine serum albumin, bradykinin, sodium deoxycholate, Coomassie Blue and Triton X-100 (Sigma); *N*-tosyl-L-arginine [³H]methyl ester ([³H]Tos-Arg-OMe) (Amersham); [¹⁴C]di-isopropyl fluorophosphate ([¹⁴C]DFP), [³⁵S]methionine, [³²P]ATP, [¹²⁵I]NaI and EN³HANCE (New England Nuclear); acrylamide, *NN'*-methylenebisacrylamide (International Biotechnologies, New Haven, CT, U.S.A.); SDS (Boehringer Mannheim Biochemicals, Indian-

Abbreviations used: ANP, atrial natriuretic peptide; Tos-Arg-OMe, *N*-tosyl-L-arginine methyl ester; DFP, di-isopropyl fluorophosphate; SBTI, soybean trypsin inhibitor; OMTI, ovomucoid trypsin inhibitor; LBTI, lima-bean trypsin inhibitor; e.u., esterase unit.

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apolis, IN, U.S.A.); trypsin (from bovine pancreas, type IV, twice-crystallized), soybean, ovomucoid and lima-bean trypsin inhibitors (SBTI, OMTI and LBTI) and carboxypeptidase B (Worthington); dithiothreitol (Aldrich); complete Freund's adjuvant (Difco); nitrocellulose (Schleicher and Schuell; 0.45 μ m pore size); antibody-gold complexes (E. Y. Laboratories, San Mateo, CA, U.S.A.). All other reagents used were of analytical grade.

Enzyme purification and monoclonal-antibody development

Rat urinary or submandibular kallikrein (tissue kallikrein) (Chao & Margolius, 1979) and rat arginine esterase A (Chao, 1983) were purified to homogeneity, and antisera to purified rat kallikreins were generated. Specific monoclonal antibodies to rat tissue kallikrein were developed, characterized and purified along with polyclonal antibodies by kallikrein affinity column chromatography (Woodley *et al.*, 1985). Purified kallikrein and the affinity-purified monoclonal antibody (V_4D_{11}) were labelled with 125 I by the lactoperoxidase method (Shimamoto *et al.*, 1980).

Cardiac kallikrein purification

Step 1: tissue-extract preparation. The rats (Sprague-Dawley; 250 g body wt.) were anaesthetized with pentobarbital (50 mg/kg). The vena cava was cut and the circulation was perfused via cardiac puncture with normal saline (0.9% NaCl) until tissues appeared blood-free. Heart (both atrium and ventricle) was removed, minced and homogenized in 0.25 M-sucrose, pH 7.0, at 4 °C. Homogenates were centrifuged at 600 g for 8 min, and the supernatant was treated with sodium deoxycholate (0.5% w/v) for 30 min at room temperature. The supernatants were collected after centrifugation at 20000 g for 30 min, and further centrifuged at 105000 g at 4 °C for 1 h. The supernatants were collected and dialysed against 0.02 M-Tris/HCl, pH 8.0, containing 1 mM-EDTA. The protein concentrations were determined by the Lowry (Lowry *et al.*, 1951) method, bovine serum albumin being used as the standard.

Step 2: DEAE-Sepharose CL-6B chromatography. The dialysed sample was then passed through a DEAE-Sepharose column (2.2 cm $\times$ 20 cm) previously equilibrated with the same buffer and washed until the A_{280} of the effluent fell to below 0.05 unit. Adsorbed proteins were eluted with a linear gradient of NaCl (0–0.6 M). Fractions containing esterolytic activity were measured with Tos-Arg-OMe as the substrate. The conductivity of the eluate was measured at 25 °C with a Yellow Springs Instruments conductivity bridge (model 31) and a conductivity cell (type 3403). Fractions containing kallikrein-like esterase activity were combined and dialysed against 0.2 M-NaCl in 0.02 M-Tris/HCl, pH 8.0.

Step 3: aprotinin-agarose affinity chromatography. The dialysed enzyme solution was applied to an aprotinin-agarose affinity column (Shimamoto *et al.*, 1980) equilibrated with 0.2 M-NaCl in 0.02 M-Tris/HCl, pH 8.0. The column was washed with the same buffer until protein was not detected in the effluent. Adsorbed material was eluted with 0.1 M-glycine/HCl, pH 3.0. Fractions (5 ml each) were collected into tubes containing 1 ml of 0.5 M-Tris/HCl, pH 9.0.

Enzymic activity assays

Kallikrein-like Tos-Arg-OMe esterase activity was measured as described previously (Chao, 1978). One esterase unit (e.u.) is defined as the amount of enzyme that hydrolyses 1 μ mol of Tos-Arg-OMe/min at pH 8.0 and 30 °C. For the pH-optimum study, the Tos-Arg-OMe esterase activity of the cardiac enzyme was assayed in 0.2 M-Tris/HCl buffer (up to pH 9.0) or 0.2 M-

glycine/NaOH buffer (beyond pH 9.0) over the pH range 7.0–10.0. The kininogenase assay (Shimamoto *et al.*, 1979a) was used to determine the kinin-generating activity of the rat cardiac enzyme, and the kinin generated was measured by a kinin radioimmunoassay (Shimamoto *et al.*, 1978). Purified bovine low-molecular-mass kininogen was used as the substrate. The direct radioimmunoassay of rat tissue kallikrein was carried out as previously described (Shimamoto *et al.*, 1979b).

Active-site labelling

For active-site labelling, cardiac enzyme (about 4 μ g) was incubated with [14 C]DFP (0.2 μ Ci; sp. radioactivity > 100 mCi/mmol) in 0.01 M-sodium phosphate, pH 7.0, with a total volume of 30 μ l for 1 h at 37 °C, followed by 16 h at 4 °C as previously described (Chao *et al.*, 1983). The mixture was then added to 30 μ l of extraction buffer containing 0.0625 M-Tris/HCl, pH 6.8, 20% (v/v) glycerol and 0.2% SDS. SDS/PAGE was performed in a 7.5–15% gradient gel in Tris/glycine buffer, pH 8.8. The gels were fixed and stained for 30 min in 50% (w/v) trichloroacetic acid containing 0.2% Coomassie Blue and destained in solution containing 7% acetic acid and 18% methanol. The gels were then treated with EN³HANCE and dried on Whatman no. 3MM filter paper. Fluorography was carried out by placing the gels in direct contact with Kodak X-Omat film. The film was developed after 2 days of exposure.

Western-blot analyses

An aliquot of the cardiac enzyme (1 μ g), submandibular kallikrein (2 μ g) or submandibular-gland extract (50 μ g) were subjected to SDS/PAGE under reducing conditions on a gradient (7.5–15%) gel as described above. Proteins were then electrophoretically transferred on to nitrocellulose membrane by using a Trans-Blot Cell (Bio-Rad) as described by Burnette (1981). The nitrocellulose was incubated in 125 I-labelled monoclonal antibodies to tissue kallikrein (V_4D_{11}) as described by Woodley *et al.* (1985) and autoradiographed.

Kallikrein mRNA analysis

Total RNA was isolated from whole rat heart by the guanidinium thiocyanate extraction method of Chirgwin *et al.* (1979), and dot-blot analysis was performed as described previously (Chao *et al.*, 1987). Briefly, RNA samples were denatured in 1 M-glyoxal/10 mM-sodium phosphate, pH 7.0, at 50 °C for 1 h. The reaction mixture was cooled and spotted on to nitrocellulose with the aid of a slight vacuum by means of an aspirator. The filtration time was about 1 min for each set of samples. After sample application, the nitrocellulose was dried and baked under vacuum at 80 °C for 2 h. It was prehybridized and hybridized at 42 °C according to the method of Thomas (1980), except that the mixture included 10% (w/v) dextran sulphate. A tissue kallikrein cDNA (Gerald *et al.*, 1986) was labelled with 32 P by nick-translation to a specific radioactivity of $(1-2) \times 10^8$ c.p.m./ μ g of DNA. The probe was denatured at 100 °C for 2 min, cooled, and added to the hybridization buffer at a concentration of $(2-3) \times 10^5$ c.p.m./ml to hybridize with the blot for about 24 h. The blot was then washed successively for 20 min at 65 °C with solutions of $2 \times$ SSC [$1 \times$ SSC = standard saline citrate (0.15 M-NaCl/0.015 M-sodium citrate, pH 7.0)], $0.3 \times$ SSC, and $0.1 \times$ SSC containing 0.1% SDS. It was then exposed to Kodak X-Omat AR film.

Translation of heart mRNA in a rabbit reticulocyte system

Rabbit reticulocyte lysate was prepared and used as described previously (Roberts & Paterson, 1973). The reaction mixture contained, in a final volume of 50 μ l, 20 μ l of lysate, 20 μ l of 'master mix' (2.5 mM-ATP, 1 mM-GTP, 1.5 mM-spermidine,

20 mM-creatine phosphate, 64 mM-Hepes, 5 mM-dithiothreitol, 64 μ M amino acids (except methionine), 300 mM-potassium acetate, 4.5 mM-magnesium acetate), 20–30 μ Ci of [35 S]methionine (400–800 Ci/mmol), 3 μ g of total RNA in water, and 0.6 μ g of creatine phosphokinase. The reaction mixtures were incubated at 30 °C for 1 h and the reaction was terminated by placing the samples on ice. Insoluble material was removed from translation products by centrifuging at 12000 *g* for 15 min in a Microfuge. The supernatants were diluted in 3 vol. of wash buffer containing 10 mM- NaH_2PO_4 , pH 7.6, 1 mM-EDTA, 1% Nonidet P40, 1% sodium deoxycholate, 0.3% SDS and 2 mM-dithiothreitol. Immunoprecipitation was carried out by adding 40 μ g of affinity-purified polyclonal antibody against tissue kallikrein from sheep origin or normal sheep IgG and 2 μ g of purified urinary kallikrein as carrier protein. The mixtures were incubated overnight at 4 °C and precipitates were collected by centrifugation for 15 min in a Microfuge. The pellets were washed three times in wash buffer and were analysed by SDS/7.5–15% PAGE. The radioactive protein bands were then detected by fluorography.

Immunocytochemical localization

Samples of atria from Sprague–Dawley rats were taken after perfusion fixation with 2% calcium acetate/buffered 4% paraformaldehyde and minced into slivers (1 mm thick). Tissues were then dehydrated in dimethylformamide and embedded in Lowicryl K4M. The immunogold method used has been described previously (Simson *et al.*, 1989). Briefly, after a blocking step with 0.5% albumin in phosphate-buffered saline, pH 7.0, thin sections (100 nm) on stainless-steel grids were incubated in the primary antibody at dilutions ranging from 1:50 to 1:1000 for 2 h at room temperature, rinsed thoroughly, then incubated with goat anti-mouse immunoglobulin coated on to gold particles (5 or 10 nm diameter) for 1 h, again rinsed thoroughly, then stained with uranyl acetate and lead citrate. Sections were examined with a JEOL 100S electron microscope. In control sections, the primary antibody was replaced by normal mouse ascitic fluid of the same dilution as the monoclonal antibody.

RESULTS

Purification of tissue kallikrein from rat heart

Rat heart extracts were determined to contain 0.35 ± 0.05 ng of kallikrein/mg of protein (mean $\pm$ S.E.M., $n = 8$) by a direct radioimmunoassay. Kallikrein-like activity in the rat heart extracts was monitored by a radiochemical method using Tos-Arg-OMe as the substrate. After dialysis against 0.02 M-Tris/HCl, pH 8.0, the tissue extract was passed through a DEAE-Sephacel CL-6B column. Two peaks of esterolytic activities were eluted from the column with a NaCl gradient. The fractions containing tissue kallikrein (the second peak) were eluted at about 0.3 M-NaCl/0.02 M-Tris/HCl, pH 8.0. The pooled fractions were applied to an aprotinin-affinity column (1.5 cm $\times$ 15 cm) equilibrated with 0.2 M-NaCl in 0.02 M-Tris/HCl, pH 8.0. As shown in Fig. 1, most of the proteins flowed through the column, and the adsorbed esterolytic activity was eluted with 0.1 M-glycine/HCl, pH 3.0.

Enzymic characteristics

Appropriate dilutions of the aprotinin-affinity column eluate were used to obtain a range of antigen concentrations comparable with those of the rat tissue kallikrein standard (0.08–10 ng/tube). Fig. 2 shows typical log–logit transformations of a radioimmunoassay standard curve of rat tissue kallikrein, serial dilutions of the rat cardiac enzyme and rat arginine esterase A (Chao, 1983). A linear curve which paralleled the standard curve was obtained from the purified enzyme, indicating the

Fig. 1. Aprotinin-affinity chromatography at rat cardiac kallikrein

The column dimensions were 1.5 cm $\times$ 15 cm; the starting material was 90 A_{280} units of protein from a DEAE-Sephacel CL-6B column in 60 ml of 0.02 M-Tris/HCl, pH 8.0; Fractions 1–30 had volumes of 8 ml each; fractions 31–42 had volumes of 5 ml; the flow rate was 30 ml/h.

immunological identity of the cardiac enzyme and tissue kallikrein. Arginine esterase A, a kallikrein-related kininogenase which has been localized in rat atria (Simson *et al.*, 1989), does not cross-react with tissue kallikrein in this assay. The cardiac enzyme has Tos-Arg-OMe esterolytic activity of 120 e.u./mg of kallikrein. It also releases kinin from purified bovine low-molecular-mass kininogen at a rate of 35.7 μ g of kinin generated/30 min per mg of kallikrein. The pH profile of Tos-Arg-OMe esterase activity of the enzyme was identical with that of tissue kallikrein, with an optimum pH of 9.0 (results not shown). The results showed that cardiac enzyme and tissue kallikrein have a similar specific activity against both synthetic and natural substrates and have identical immunological characteristics. The effects of various trypsin inhibitors on the activity of the rat cardiac enzyme are shown in Fig. 3. The

Fig. 2. log–logit transformation of a typical radioimmunoassay standard curve of rat tissue kallikrein (▲), rat cardiac kallikrein (○) and rat arginine esterase A (■)

B/B_0 is the amount of bound radioactivity in the presence, divided by that in the absence, of unlabelled kallikrein using sheep anti-(rat urinary kallikrein) antiserum, the ratio being expressed in percentage terms.

Fig. 3. Effect of trypsin inhibitors on rat cardiac kallikrein esterase activities

A 20 μ l portion of rat cardiac kallikrein (4×10^{-2} e.u./ml in water) and 10 μ l of inhibitor (final concentrations as indicated) were incubated at 37 °C for 30 min; at which time 30 ml of 0.2 M-Tris/HCl buffer, pH 8.0, was added and the solution was left at room temperature for 10 min. [3 H]Tos-Arg-OMe (3.0×10^4 c.p.m., 10 μ l) was added to the solution, mixed and incubation was allowed to proceed for 30 min at room temperature. The [3 H]methanol released was measured by a Beckman LS-355 liquid-scintillation spectrometer. Enzyme activity is expressed as a percentage of a control incubated in the absence of inhibitor. Each value represents the average for three experiments, each done in duplicate. ▲, Aprotinin (units/ml); □, antipain (μ M); ○, leupeptin (μ M); △, SBTI (μ M); ●, LBTI (μ M).

cardiac enzyme is inhibited by aprotinin, leupeptin, antipain and high concentrations of SBTI. Furthermore, similar to tissue kallikrein (Chao, 1979), the cardiac enzyme is stimulated by low concentrations of SBTI, and by LBTI or OMTI over a broad range of concentrations. The data indicates that cardiac kallikrein and tissue kallikrein have similar susceptibility to various trypsin-like-serine-proteinase inhibitors.

Western-blot analyses

In order to determine whether there was specific recognition by a monoclonal antibody to tissue kallikrein, the enzyme isolated from rat heart was subjected to SDS/PAGE under reducing conditions and electrophoretically transferred to the nitrocellulose membrane. In Fig. 4, after incubation with an 125 I-labelled monoclonal anti-kallikrein antibody (V_4D_{11}), the cardiac enzyme (lane 1), submandibular kallikrein (lane 2), as well as submandibular-gland extract (lane 3) were bound to the antibody and revealed by autoradiography. The Western blot shows that the cardiac enzyme is recognized by specific monoclonal antibody to tissue kallikrein, and the purified enzymes from heart and submandibular gland have identical molecular masses (38 kDa).

Active-site labelling

Cardiac kallikrein was labelled with [14 C]DFP, and revealed by fluorography of a SDS/PAGE under reducing conditions. The molecular mass of the 14 C-labelled cardiac kallikrein is 38 kDa (Fig. 5). A lower-molecular-mass 14 C-labelled protein band was also revealed that may represent a partially cleaved fragment of cardiac kallikrein. However, this protein band is not recognized by a specific monoclonal antibody to tissue kallikrein (Fig. 4, lane 1). The result shows that the cardiac enzyme is a serine proteinase, since it can form a covalent linkage with [14 C]DFP.

Fig. 4. Western-blot analysis of cardiac kallikrein and submandibular kallikrein

Electrophoresis was performed on a 7.5–15% linear-gradient polyacrylamide/SDS gel and the proteins were electrophoretically transferred on to nitrocellulose. The blot was incubated with 125 I-labelled monoclonal anti-(rat tissue kallikrein) antibody (V_4D_{11}), and the antibody-binding sites were revealed by autoradiography. Lane 1, 1 μ g of cardiac kallikrein; lane 2, 2 μ g of submandibular kallikrein; lane 3, 50 μ g of submandibular-gland extract.

Cell-free translation of rat heart mRNA

Cell-free translation products directed by heart mRNA were immunoprecipitated and subjected to SDS/PAGE under reducing conditions. Fig. 6 shows total translation products (lane 1), immunoprecipitates with normal sheep immunoglobulin (lane 2) and immunoprecipitates with affinity-purified sheep anti-(rat urinary kallikrein) antibody (lane 4). Kallikrein antibodies recognized the translation products directed by heart mRNA with molecular-mass values of 38 and 40 kDa. 125 I-labelled rat urinary kallikrein is shown in lane 3.

Dot-blot analysis of tissue kallikrein mRNA in rat heart

Fig. 7 shows the results of dot-blot hybridization with a kallikrein cDNA. The intensities of signals from heart are slightly higher than those from liver (results not shown) when using equal amounts of RNA. The combined data from RNA hybridization of dot-blot analysis and the kallikrein synthesis in cell-free translation system indicate the existence of kallikrein mRNA in the heart. As positive controls, kallikrein mRNA were also identified in other tissues, such as kidney, spleen and pancreas, sites known to synthesize tissue kallikrein (Chao *et al.*, 1986).

Fig. 5. Active-site labelling of cardiac kallikrein

The enzyme (4 μ g) was incubated with [14 C]DFP and analysed on a 7.5–15% linear-gradient polyacrylamide/SDS gel under reducing conditions. The protein bands with binding activity were revealed by fluorography. (a) Molecular-mass standards: rabbit muscle phosphorylase b, 97 kDa; bovine serum albumin, 66 kDa; hen's-egg-white ovalbumin, 43 kDa; bovine carbonic anhydrase, 31 kDa; SBTI, 21.5 kDa; and hen's egg-white lysozyme, 14 kDa; (b) protein staining of cardiac kallikrein; (c) fluorography of cardiac kallikrein.

Fig. 6. Electrophoretic analyses of rat heart mRNA-dependent rabbit reticulocyte-lysate translation products

Cell-free translation products were electrophoresed on a 7.5–15% linear-gradient polyacrylamide/SDS gel after immunoprecipitation. Lane 1, total cell-free translation products; lane 2, translational products immunoprecipitated with normal sheep IgG; lane 3, 125 I-labelled rat tissue kallikrein; lane 4, translational products immunoprecipitated with affinity-purified sheep anti-(rat tissue kallikrein) antibody.

Fig. 7. Dot-blot analyses of kallikrein mRNA in rat heart and other tissues

Total RNA was extracted from various tissues and dotted on to nitrocellulose. The kallikrein mRNA in each spot was measured by hybridization to 32 P-labelled rat tissue-kallikrein cDNA probe, followed by exposure of the nitrocellulose blot to X-ray film. Serial dilution of total RNA was performed: 1, 40 μ g; 2, 20 μ g; 3, 10 μ g; 4, 5 μ g.

Immunocytochemical localization

At the electron-microscopic level, immunostaining using the specific monoclonal antibody (V_4D_{11}) for tissue kallikrein was observed primarily in regions between myofibrils that correspond to sites of sarcoplasmic reticulum of rat atrial myocytes (Fig. 8a). The most intense labelling was seen near the cell periphery, beneath the membrane. A low level of staining was also localized in atrial granules with this antibody. In sites where T-tubules were recognizable, immunostaining was not seen within the tubule, but was present nearby. The control sections showed low background staining when normal mouse ascitic fluid was used in place of the primary antibody (Fig. 8b).

DISCUSSION

The present report describes the first demonstration that tissue kallikrein mRNA and protein are present in rat heart. The cardiac enzyme was isolated from rat heart extracts by DEAE-Sepharose and aprotinin-affinity column chromatography and shown to be an active serine proteinase with both Tos-Arg-OMe esterase and kininogenase activities. The enzyme is indistinguishable from tissue kallikrein and clearly differentiable from other members of the kallikrein gene family with respect to molecular mass, charge, immunological properties, pH optimum, substrate specificity and susceptibility to inhibitors. Furthermore, it is recognized by a specific monoclonal antibody to purified tissue (urinary) kallikrein which does not cross-react with arginine esterase A, tonin or other kallikrein-like enzymes (Woodley *et al.*, 1985).

Molecular analysis of the kallikrein gene products was initiated by examining the primary biosynthetic products directed by total RNA from rat heart in a cell-free translation system.

Fig. 8. Electron photomicrographs of calcium acetate-buffered paraformaldehyde-fixed, Lowicryl K4M-embedded rat atrium sections

Ⓐ Immunostained with V_4D_{11} as primary antibody followed by goat anti-mouse immunoglobulin coated on to 10 nm-diameter colloidal gold particles. Note gold particles associated with cytoplasmic elements (arrows) between myofibrils as well as with granules (G). Mitochondria (Mi) and myofibrils (Mf) are unstained, as are structures believed to be T-tubules (arrowhead) (magnification $35000\times$). Ⓒ Ascites fluid substituted for the primary antibody, showing low background distribution of gold particles in control sections. A few gold particles are seen on granules, even in controls, but the surrounding sarcoplasm is essentially free of staining. T-tubules are again marked with arrowheads (magnification $35000\times$).

Immunoprecipitates of ^{35}S -labelled preprokallikrein, using affinity-purified anti-kallikrein antibodies, were identified by fluorography of SDS/PAGE gels as two bands with molecular masses of 38 and 40 kDa. The ^{125}I -labelled purified active urinary kallikrein has a molecular mass ranging from 36 to 38 kDa. The difference in electrophoretic mobilities between translation

products and ^{125}I -labelled active kallikrein is not surprising, since the products synthesized *in vitro* are prepro forms and as such are not processed or glycosylated in the reticulocyte system. The presence of kallikrein mRNA in the heart was further supported by dot-blot analysis using a tissue-kallikrein cDNA probe. The low intensity of signals from heart is consistent with the data

from a direct radioimmunoassay for rat tissue kallikrein, showing a low cardiac kallikrein content of 0.35 ± 0.05 ng/mg of protein (mean $\pm$ S.E.M., $n = 8$) as compared with that in kidney (36.1 ± 2.1 ng/mg of protein; mean $\pm$ S.E.M., $n = 6$) or other tissues (Chao & Margolius, 1983).

By using electron microscopy the subcellular localization of tissue kallikrein was examined with a specific monoclonal antibody to tissue kallikrein. With the calcium acetate-buffered paraformaldehyde fixative used in the present study, membranes were poorly preserved. The fact that positive staining was mainly localized between myofibrils at the cellular sites of sarcoplasmic reticulum, and to a lesser extent, in granules, suggests a functional diversity of tissue kallikrein in the heart. Previously, we have localized another kallikrein-like enzyme, namely arginine esterase A (Chao, 1983), in rat heart at both the optical- and electron-microscopic level and have co-localized arginine esterase A with pro-ANP in granules of atrial myocytes by double-label electron-microscopic immunocytochemistry (Simson *et al.*, 1989). The functional roles of these two related enzymes in the heart has yet to be determined, although both have been shown to release ANP from prohormone *in vitro* (Currie *et al.*, 1984; Simson *et al.*, 1989).

Recent studies have reported several potential processing enzymes of ANP in bovine, porcine and rat heart (Seidah *et al.*, 1986; Wypij & Harris, 1987; Imada *et al.*, 1988). A tissue-kallikrein-like enzyme has been implicated in the catabolism of ANP (Thibault *et al.*, 1983). Our previous studies showed that tissue kallikrein is capable of processing pro-ANP to produce the active form *in vitro* (Currie *et al.*, 1984) and that the activation can be abolished by aprotinin, a potent inhibitor of tissue kallikrein. Studies suggested that pro-ANP is converted into the active form during, or just after, secretion from the atrium (Cantin & Genest, 1985; Schwartz *et al.*, 1985; Sugawara *et al.*, 1985; Thibault *et al.*, 1986). The present study shows the localization of tissue kallikrein in the atrial myocytes, where active ANP is formed. Although the relationship between tissue kallikrein and ANP is unknown, together these findings suggest that cardiac kallikrein is a candidate for one of the regulatory factors in pro-ANP processing or ANP metabolism.

Tissue kallikrein releases vasoactive substances, kinins, which are known to regulate local blood flow within the heart through its effect on coronary arteries (Lochner & Parratt, 1966). This has also been demonstrated in studies showing increased kinin-like activity in dog coronary sinus blood after coronary-artery occlusion (Kimura *et al.*, 1973), a condition resembling coronary heart disease. Furthermore, activation of kallikrein-like enzymic activity has been reported in coronary sinus blood of patients with myocardial ischaemia (Pitt *et al.*, 1970). A recent study (Scholkens & Linz, 1989) has shown that, in isolated perfused working rat hearts with regional myocardial ischaemia, bradykinin, in concentrations as low as 1 nM, increased coronary flow and reduced the incidence and duration of the ventricular fibrillation following reperfusion. In addition, the venous effluent of these hearts showed reduced lactate dehydrogenase and creatine kinase activities and decreased lactate output as well as improved cardiodynamic and metabolic parameters. These effects could be competitively reversed by specific bradykinin antagonists (Scholkens & Linz, 1989). These findings suggest that the kinin system may play an important role in pathological conditions such as ischaemic heart disease and/or act as a protective or compensatory system by increasing the blood supply to cardiac tissue through vasodilatation. Tissue kallikrein, which produces kinins from kininogen, may be one of the key factors in the regulation of this system. Further studies need to be carried out to elucidate the role of cardiac kallikrein, especially in conditions like ischaemic heart diseases.

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