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DIRECT EVIDENCE FOR THE LOCATION OF KALLIKREIN IN THE STRIATED DUCTS OF THE CAT'S SUBMANDIBULAR GLAND BY THE USE OF SPECIFIC ANTIBODY

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SUMMARY

Kallikrein was located in the apical portion of the striated duct cells of the cat's submandibular gland by an immunohistochemical technique. This portion only of these cells showed an intense band of specific fluorescence. There was no evidence of specific fluorescence in the acinar and demilune cells nor in the interstitial tissue or blood vessels. In some sections the collecting ducts showed a very fine fluorescent luminal rim.

INTRODUCTION

The kallikreins, or kininogenases, are enzymes which belong to the larger group of serine proteases. They are present in many glandular organs and in body fluids of mammals (Frey, Kraut & Werle, 1968; Schachter, 1969). The cellular and subcellular location of glandular kallikrein has been studied recently in the salivary glands where its possible physiological significance has been the subject of much speculation and discussion (Hilton, 1970; Schachter, 1970).

The experiments of Bhoola *et al.* (Bhoola & Ogle, 1966; Bhoola, 1969; Bhoola & Heap, 1970) and of Erdös *et al.* (Chiang, Erdös, Miwa, Tague & Coalson, 1968; Erdös, Tague & Miwa, 1968; Geipert & Erdös, 1971) showed that kallikrein is present in organelles resembling zymogen granules in the submandibular salivary gland of many mammals. These conclusions were arrived at by isolating populations of organelles by differential and density gradient centrifugation of tissue homogenates, measuring the kallikrein concentrations of the fractions, and then examining them under the electron

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microscope. From such studies inferences were also made by Bhoola and co-workers in regard to the location of kallikrein in the cells of the intact gland. They suggested that the secretory granules of the acinar cells were the specific sites of kallikrein location in the submandibular gland of most laboratory mammals, including the cat (Bhoola, 1969, 1970). The same suggestion was made by Beraldo, Siqueira, Rodriguez & Machado (1972) from other indirect evidence, viz. the correlation of acini formation and kallikrein content of the rat's submandibular gland during post-natal development.

More recently, another approach to this problem was attempted independently in the cat by Barton, Sanders, Schachter & Uddin (1975) and by Garrett & Kidd (1975), who correlated ultrastructural changes with kallikrein content of the submandibular gland following stimulation of its autonomic nerves. Both groups made use of the fact that parasympathetic nerve stimulation causes marked secretion but relatively little reduction in kallikrein content of the gland, whereas sympathetic nerve stimulation which causes less secretion can practically deplete the gland of its kallikrein (Beilenson, Schachter & Smaje, 1968). Both Barton *et al.* (1975) and Garrett & Kidd (1975) came to the same conclusion, viz. that although sympathetic nerve stimulation had no obvious effect on the content of the acinar cell granules it practically depleted the 'light' cells of the striated ducts of their granules. Parasympathetic nerve stimulation, on the other hand, which had no significant effect on the granules of the striated duct cells, markedly reduced the number of acinar cell granules. Barton *et al.* (1975), who measured the kallikrein content of the gland not only after nerve stimulation but after degenerative section of the chorda lingual nerve and after duct obstruction, found a close correlation between the kallikrein content of the gland and the concentration of apical granules in the cells of the striated ducts after all these procedures. These results provided circumstantial evidence that, in the cat, salivary kallikrein is present in secretory organelles or granules in these specialized ducts, rather than as might have been anticipated in the secretory granules of the acinar cells. The apical duct cell granules are about one-quarter the size of the acinar cell granules.

The present experiments using specific antibody to kallikrein and a fluorescein-labelled marker provide direct evidence that kallikrein is in fact located in the apical region of the cells of the striated ducts. These cells showed an intense, uneven band of specific fluorescence. In some sections the collecting ducts also showed a distinct but very fine fluorescent luminal rim. There was no indication of any specific fluorescence in the acinar or other elements of the gland including interstitial tissue and blood vessels.

METHODS

Pure cat salivary gland kallikrein (SGK) was prepared as described by Moriwaki, Hojima & Schachter (1976). The final preparation had a very high kininogenase activity by chemical (24·5 BAEe u./mg protein) and bio-assay tests (1260 Ku./mg protein) and yielded a single band on disk electrophoresis. Antibody to pure kallikrein was obtained from serum of rabbits 15 days after three consecutive weekly injections of SGK in 0·9 % NaCl, emulsified with complete Freund's adjuvant for the first injection and with incomplete adjuvant for the other two. At each weekly injection of SGK a total volume of 1·5 ml. was injected s.c. at the base of the back of the neck at several distinct but adjacent sites so that the solution was injected over an area of approximately 5 cm square. Control serum was obtained from the same rabbits before immunization with SGK in the same manner as the antibody-containing serum, viz. from blood obtained by ear vein puncture. Immunodiffusion tests (Ouchterlony, 1958) yielded single precipitation arcs for immune sera whereas control sera were unreactive.

Fixation and embedding of the submandibular gland were based on the procedure of Sainte-Marie (1952). Small (2 × 2 mm) portions of the gland were dissected out from cats anaesthetized with chloroform followed by chloralose (100 mg kg^{-1} I.V.). The tissue was rinsed quickly in cold 0·9 % NaCl and fixed in 95 % ethanol for 18 hr at 4° C. The tissue blocks were cleared with xylene and embedded in paraffin under vacuum. Sectioning was performed within a few hours whenever possible; if not, embedded blocks were kept at 4° C. The sections, 6 and 8 μm thick, were floated on water at 40° C and collected on albuminized slides. The slides were drained and the sections dried at room temperature. The tissue sections were kept at 4° C if the staining was not done immediately. Prior to staining, deparaffinization was carried out as usual in cold xylene followed by 95 % cold ethanol and cold phosphate-buffered saline (PBS) at pH 8·0.

Fluorescent staining by the indirect method was based on Coons' sandwich technique (1958). The general procedure described by Kawamura (1969) was employed as follows: Sections were incubated at 37° C for 30 min with undiluted immune and non-immune (control) sera, washed for 15 min in PBS, counterstained for 2 min with Evans blue (0·1 % in PBS) and washed for 5 min in PBS. They were then incubated at 37° C for 30 min with FITC (fluorescein isothiocyanate)-labelled rabbit anti-IgG (Miles-Yeda, Israel). This labelled anti-IgG had previously been diluted to one tenth of its original concentration (1 % IgG solution with O.D. ratio at 280/495 nm = 1·7) and then treated with cat liver powder. The section was then washed for 15 min in PBS, dried at room temperature and mounted with fluormount (Edward Gurr Ltd, London).

A typical slide had four serial sections on it placed side by side. The sections were delineated on the slide with wax pencil (Revlon, blue eye-liner was found most satisfactory) to prevent spread of the added solutions. The first two sections were treated with non-immune and the last two with immune serum. One section from each pair was then stained with FITC-labelled rabbit anti-IgG while the other was covered with PBS. The slide thus had one section showing specific immunofluorescence with three others as controls.

A Zeiss photomicroscope II with HBO high-pressure mercury lamp was used for fluorescence microscopy. An FITC filter was used as an exciter filter while a combination of barrier filters (65, 50 and 44, Zeiss) were employed. Dark field condenser and objectives were used to improve the contrast. Photographs were taken with spot reading on Anscochrome 200 ASA from which Cibachrome prints were made. The use of Cibachrome, a process for printing directly from colour slides, made it

possible to reproduce accurately the appearance of the tissue as seen under fluorescence microscopy.

Ethanol fixation was more satisfactory than frozen tissue and cryostat sectioning, giving better microscopic resolution and reducing only slightly the antigenicity of SGK in the fixed tissue section. Other fixatives such as formaldehyde, glutaraldehyde, methyl alcohol and acetone resulted in poor structural detail and/or loss of antigenicity of SGK in the section.

RESULTS

Specificity of antibody to kallikrein

The purification of cat salivary kallikrein and the preparation of its antibody in the rabbit are referred to or described under Methods. The antibody contained in rabbit serum produced only a single precipitation arc with our preparation of kallikrein, which further indicates its purity. Antigen (kallikrein) failed to show any reaction under the same conditions with control serum obtained from the same rabbit prior to immunization with the enzyme. Pl. 1 shows a gel diffusion plate illustrating single precipitation reaction bands over a fivefold range of concentrations of antigen. In this experiment the serum protein was concentrated fivefold by fractionation with ammonium sulphate. The same result was obtained with neat serum and with lower concentrations of antigen although the precipitation arc was fainter.

Immunohistochemical localization of kallikrein

When sections of submandibular tissue had reacted with antibody to kallikrein and with flourescent label and were viewed under the fluorescence microscope (see Methods), an intense band of yellow-green fluorescence was seen which began at the apical (or luminal) border of the cell and extended nearly to the nucleus with little loss of intensity. Pl. 2A shows the typical appearance of a section that has reacted with antibody and fluorescent marker. Note the marked contrast of specific to non-specific fluorescence in the apical portion of the striated duct cells compared to the surrounding tissues. This enhanced contrast was in part achieved by the use of Evans blue (see Methods) as a counterstain. Other structures such as acinar, demilune or myoepithelial cells, blood vessels and interstitial tissue, all failed to exhibit significant specific fluorescence. In some sections however, the collecting ducts showed a very fine but distinct fluorescent luminal rim. The control section of tissue shown in Pl. 2*B* was prepared in the same way except that the serum containing antibody to kallikrein was not added. It shows that the fluorescence in the control is quite different from the experimental tissue. The acinar cells again show little or no fluorescence and the striated ducts show only a mild diffuse fluorescence

without significant contrast from surrounding tissues. A similar degree of diffuse fluorescence was seen even without the fluorescent label itself, indicating some 'natural' or non-specific fluorescence of the untreated tissue.

DISCUSSION

Our studies in the cat are of special interest in view of the pioneer physiological studies on the submaxillary gland of this animal (Barcroft & Piper, 1912; Barcroft, 1914; Bayliss, 1923). Also, more recent studies have dealt with the possible role of salivary kallikrein in functional hyperaemia in this same organ (Hilton & Lewis, 1955; Bhoola, Morley, Schachter & Smaje, 1965). The present experiments provide direct evidence for the specific location of kallikrein in the apical region of the striated duct cells of the cat's submandibular gland. They support recent suggestions based on indirect evidence that kallikrein is present in organelles or small granules near the lumen of the light cells of these ducts (Barton *et al.* 1975; Garrett & Kidd, 1975). Our results are also similar to those recently obtained in the rat by Ørstavik, Brandtzaeg, Nustad & Halvorsen (1975) and by Brandtzaeg, Gautvik, Nustad & Pierce (1976), who found, also by immunohistochemical fluorescence microscopy, that kallikrein is present in the convoluted granular tubules and to a lesser extent in striated duct cells, but is absent from acinar cells. It is also possible, however, that in some mammals kallikrein is present in the acinar cell granules of this gland (Bhoola, 1969; Bhoola, Dorey & Jones, 1973).

The concentration of specific fluorescence near the apical region of the striated duct cells indicates that secretion of kallikrein is into the lumen of the duct, apparently exclusively so. This finding speaks against its role as the metabolic mediator of functional hyperaemia (Hilton & Lewis, 1955; Hilton, 1970). It is difficult to conceive of a mechanism whereby kallikrein, secreted into the duct, could find its way back to the blood vessels, release kinin, and then cause a vasodilatation to begin with a latency of only 350–900 msec such as occurs after stimulation of the chorda lingual nerve (Karpinski, Barton & Schachter, 1971). The fine rim of luminal fluorescence occasionally seen in the collecting ducts could be due to the adsorption of kallikrein along the duct system where it might exert some action relating to electrolyte transport. This observation and its significance, however, require further study.

Renin-like enzymes have been described in the submandibular gland of the mouse (Werle, Trautschold & Schmal, 1963; Oliver & Gross, 1966; Bhoola *et al.* 1973) and rat (Gutman, Levy & Shorr, 1973; Garcia, Boucher & Genest, 1976). This finding is of interest since both renin and kallikrein are also present in the kidney and each releases a specific active peptide,

viz. angiotensin and kinin respectively (see Schachter, 1969). Recently it has been suggested that kidney kallikrein may have a role as a natriuretic hormone corresponding to the sodium-retaining role of renin (Carretero & Scicli, 1976). The salivary renin-like enzymes are secreted in high concentrations in the saliva and are probably located in the zymogen-like granules of the convoluted or striated ducts (Cozzari, Angeletti, Lazar, Orth & Gross, 1973; Michelakis, Yoshida, Menzie, Murakami & Inagami, 1974). Also, whereas the angiotensinogen concentration of plasma falls markedly after bilateral nephrectomy it is unaffected after bilateral submandibular sialectomy (Gutman *et al.* 1973). These observations suggest that unlike kidney renin, salivary renin is not secreted into the blood circulation, at least not primarily.

Nerve growth factor (NGF), another protein, is also present in high concentrations in the submandibular gland of the mouse (Levy-Montalcini & Hamburger, 1951). In this instance, however, the evidence suggests that the secretion of this substance is into the blood circulation since its concentration in plasma falls rapidly after bilateral sialectomy (Hendry & Iversen, 1973). Like renin and kallikrein, however, NGF has been located in granular tubules and duct cells of the mouse submandibular gland. However, unlike our results for kallikrein, fluorescent immunohistochemical studies indicate that NGF is concentrated at the basal rather than at the apical cell membrane (Levy-Montalcini & Angeletti, 1961).

In view of the above considerations, without dismissing other possibilities, we are inclined to the view that the physiological significance of kallikrein and renin in the submandibular gland is likely to be related to their secretion into the duct system. In recent years some interesting new properties of the kallikreins (or kininogenases) have been described, e.g. natriuretic activity (Carretero & Scicli, 1976), involvement in blood coagulation (Cochrane, Revak & Wuepper, 1973), enhancement of sperm motility (Fritz, 1975) and stimulation of cell proliferation (Rixon & Whitfield, 1973). It is likely that the kallikreins in different tissues do not have a common physiological role, and, as previously suggested, some of their significant functions may be unrelated to the release of kinin (see Schachter, 1962). Similarly, renin may have significant actions independent of, or unrelated to the release of angiotensin. Themselves a large group of enzymes, the kallikreins or kininogenases belong to the wider group of serine proteases, all of which have a common and very specific configuration of amino acids at their active site. It seems likely, therefore, that like other serine proteases such as trypsin, thrombin, fibrinolysin and elastase (see Stroud, 1974), the different kallikreins may turn out to have diverse physiological roles. The possibilities exist that salivary kallikrein may play a role in sodium transport in the salivary duct, as a digestive enzyme, in absorption, or in

cell proliferation and repair in the digestive tract. The presence in the submandibular gland of known active macromolecules like kallikrein, renin, NGF, and of a new one like sialotonin (Barton, Karpinski, Moriwaki & Schachter, 1976), suggests that the physiological significance of the salivary glands extends beyond those relatively simple functions which are generally assigned to then.

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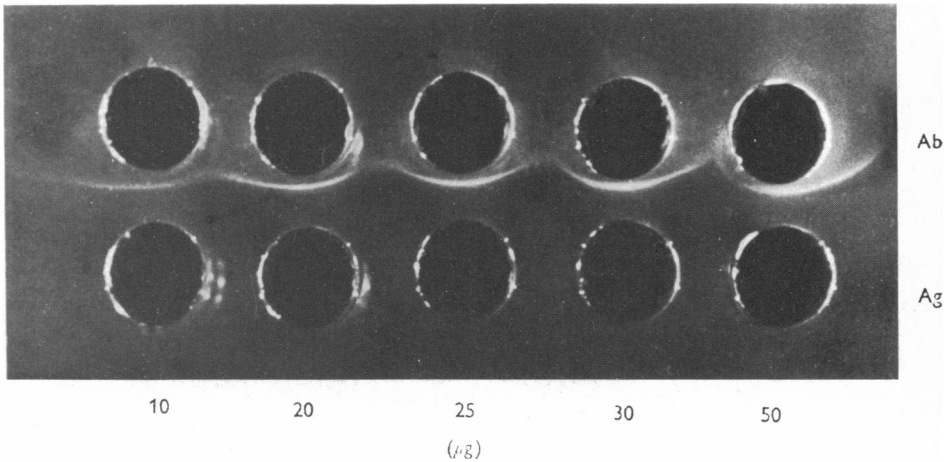
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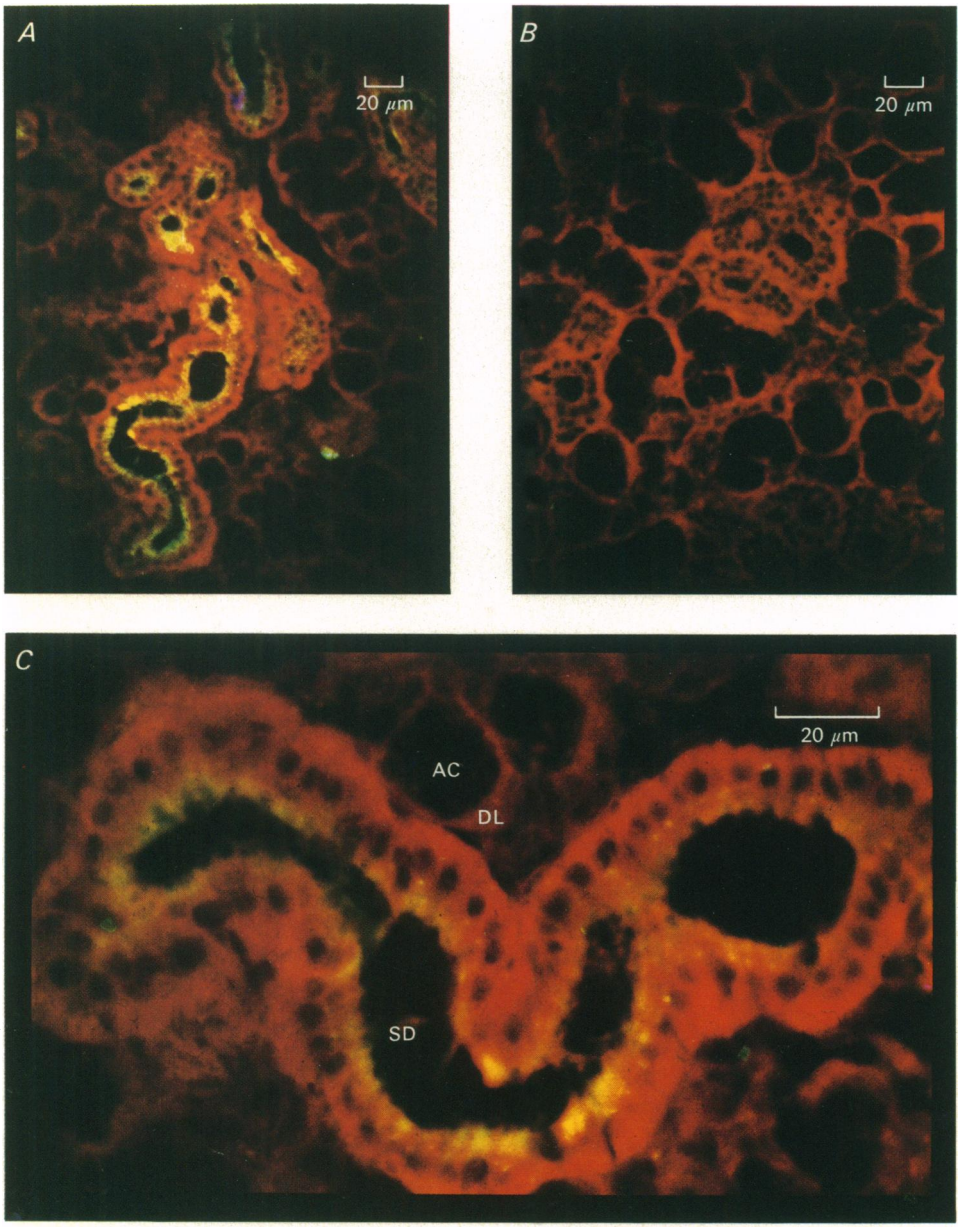
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EXPLANATION OF PLATES

PLATE 1

Gel-immunodiffusion plate showing precipitation arcs of pure cat submaxillary gland kallikrein (Ag) with its specific antibody (Ab). Numbers indicate total amount of pure antigen in each trough (see text for details).

PLATE 2

Sections of cat's submandibular gland showing localization of kallikrein by immunofluorescence microscopy (see text for details). SD, striated duct. AC, acinus. DL, demilune. *A*, after reaction of tissue section with antibody to kallikrein. Note the intense band of yellow-green fluorescence in the apical region of striated duct cells. The reddish background is due to counterstaining with Evans blue dye. Fluorescence is absent in acinar and demilune cells as well as in surrounding tissue. Original magnification, × 65. *B*, control section prepared as *A* but without addition of antibody to kallikrein. Note absence of any fluorescence in acinar cells which appear black and the mild diffuse fluorescence of striated ducts showing little or no contrast with surrounding tissues. Original magnification, × 65. *C*, part of area of specific fluorescence of duct cell shown in *A* but at higher magnification. Note the absence of fluorescence in the basal portion of the cells. Original magnification, × 160.