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Atrial natriuretic peptide in the heart and pancreas

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Summary. We used antisera to pure atrial natriuretic peptide to localise this peptide by immunocytochemistry in rat and human tissue. We showed that both rat and human atrial cardiocytes gave a positive reaction while ventricular cardiocytes were consistently negative. Peripheral islet cells in rat but not in human pancreas also showed positive staining for ANP. We showed by double labelling techniques that the ANP was present in the glucagon containing cells.

Key words: Atrial natriuretic peptide - Immunocytochemistry

Introduction

Atrial cardiocytes of the mammalian heart contain granules which are morphologically similar to those in peptide secreting endocrine cells (Huet and Cantin, 1974; Cantin, 1975). These secretory granules were discovered by Kisch (1956), a finding confirmed by several investigators (Posche, 1957; Bompiani et al., 1959). The granularity of the atria can be altered experimentally by salt and water loading or deprivation (De Bold, 1979) and by procedures which cause perturbation of salt and water balance; these include adrenal regeneration hypertension (Martinez, 1966) and adrenalectomy (Cantin, 1973).

Further investigations (Sonnenberg et al., 1980) have shown that crude extracts of rat atria have a powerful diuretic and natriuretic effect. The isolation of specific granules from crude extracts of rat atria by differential and density gradient centrifugation yields an even greater natriuresis and diuresis in experimental animals (De Bold, 1979). There is evidence from biological assays that the left atrium responds to distension with release of material having a diuretic action (Kappagoda, 1979).

The above findings have led to the isolation of various atrial polypeptides with different biological actions. The gene sequence of these compounds has been determined and the amino acid sequence deduced and pure peptides prepared by cell free translation of mRNA (Atlas, 1984).

Previous immunocytochemical studies of the localisation of these peptides have been performed using antibodies to partially purified atrial extracts (Cantin, 1984). We have raised an antibody to pure synthetic peptide (1-28 hANP) and the aim of the present paper is to use this highly specific ANP antiserum to study the tissue distribution of this important new peptide.

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Materials and methods

ANP Antisera

a) **Preparation of immunogen.** Human ANP (1-28 hANP) prepared by solid phase synthesis (Cambridge Research Biochemicals, Cambridge, England) was conjugated to bovine thyroglobulin using a water-soluble carbodiimide as a coupling agent. 100 µl of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDPAC) solution (40 mg/ml) was added to a solution consisting of 0.5 mg hANP and 5 mg bovine thyroglobulin dissolved in 300 µl of distilled water. 37 pg of radiolabelled tracer peptide (50,000 cpm [^{125}I] - hANP) was included to determine the efficiency of coupling. The solution was incubated overnight at 4° C, after which 100 µl of freshly prepared EDPAC solution (40 mg/ml) was added and

the mixture kept at 20" C for a further 2 hours. The conjugate was dialysed for 24 hours against 0.9% (w/v) NaCl, then divided into aliquots sufficient to immunise three rabbits and stored at 20" C. In this procedure a molar ratio of hANP to thyroglobulin of 13.5:1 was obtained with a coupling efficiency of 60%.

b) **Immunisation.** New Zealand white rabbits (2-3 kg) were immunised by injecting into each popliteal lymph node (Sigel et al., 1983) a 0.1 ml emulsion consisting of equal volumes of immunogen (1 mg/ml) and freund's complete adjuvant. Immunity was boosted by similar injections given intramuscularly at intervals of 5-6 weeks.

The antiserum used in this study gave 50% binding of 2 pg of [^{125}I] - hANP (Amersham International, specific radioactivity 1850 Ci/mmol) at a concentration of 1/33,000. The cross reactivity of the antiserum *in vitro* was greater than 90% when tested with a variety of synthetic atrial natriuretic peptides (5-28 ANP, 7-28 ANP, atriopeptin I, atriopeptin I1 and atriopeptin 111).

Other antisera

The antiserum to human glucagon was obtained from Miles Laboratories (UK) Ltd. and the antiserum to bovine thyroglobulin was a gift (Professor K. Whaley).

Tissue fixation and processing

a) **Light microscopy.** Tissues from six young male Wistar rats were fixed by immersion, some in Bouin's fluid and others in neutral buffered formol saline. They were then embedded in paraffin wax and 3 µ sections stained with routine histological stains. Adjacent sections were used for immunocytochemistry. Equivalent sections of human tissues were obtained from the files of the Western Infirmary Pathology Department. These had all been fixed in buffered formol saline and embedded in paraffin wax.

b) **Electron microscopy.** For ultrastructural examination 1-2 mm pieces of tissue were fixed in glutaraldehyde

Fig. 2. Atrial rnyocytes in rat showing granular immunostaining mainly in the perinuclear space. The rest of the cytoplasm is unstained. ANP irnrnunoperoxidase, haematoxylin counterstain; interference contrast microscopy. × 350

Fig. 3. Endocrine granules in the perinuclear space of a rat atrial rnyocyte. ANP irnrnunogold preparation; uranyl acetate and lead citrate contrast enhancement. x 9,675

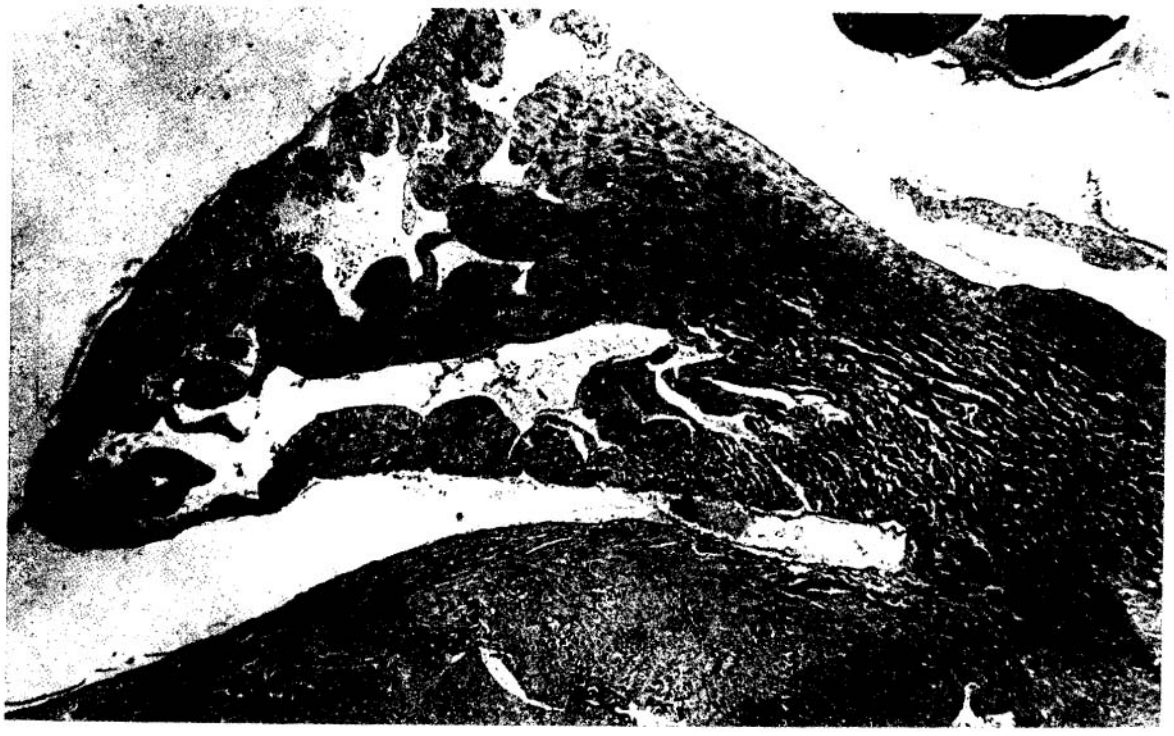


Fig. 1. Auricular appendage and right ventricle of a rat heart. The atrium shows strong staining for ANP while the ventricle (below)is negative. Paraffin section stained with the irnrnunoperoxidase method for ANP and counterstained with haernatoxylin. x 80

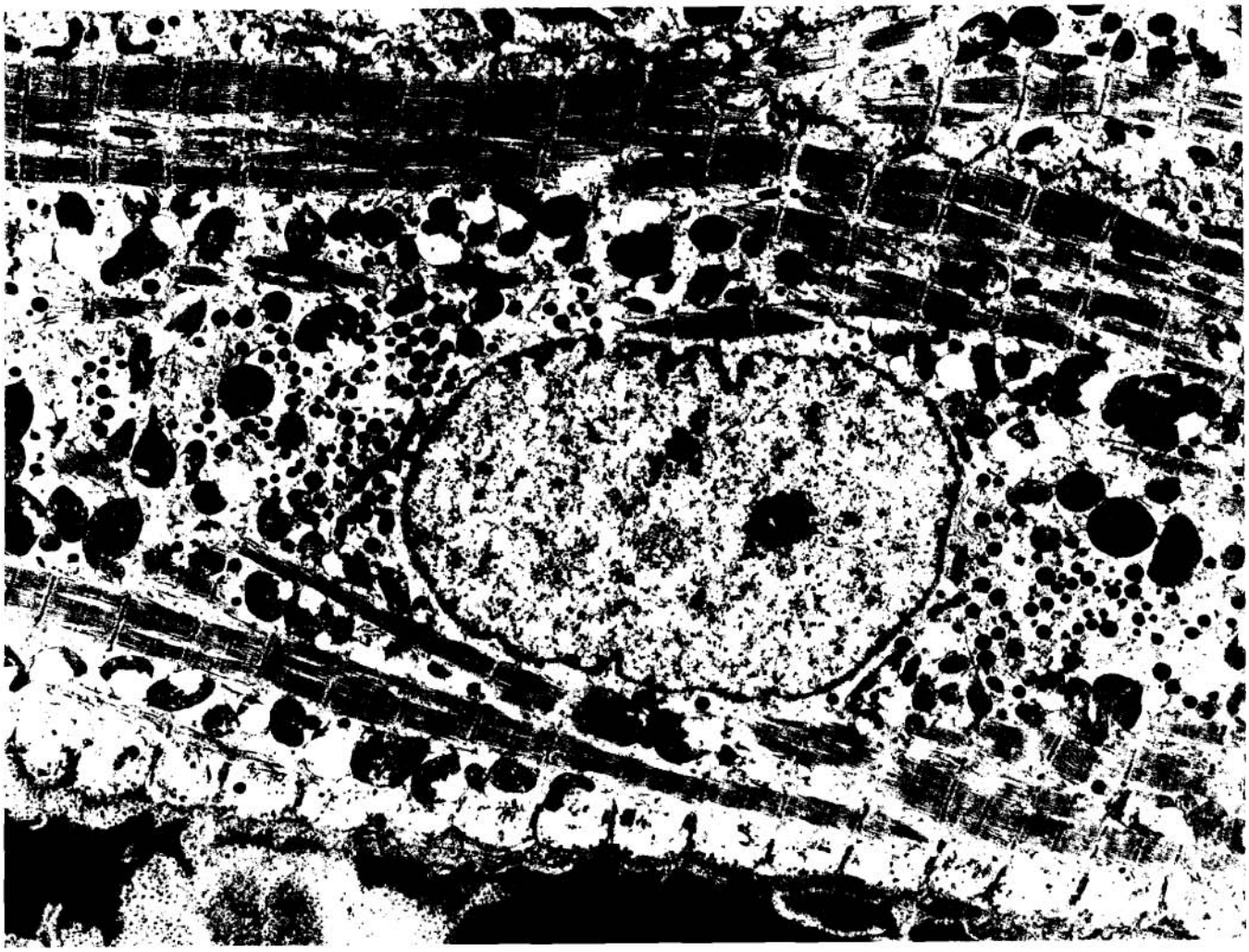
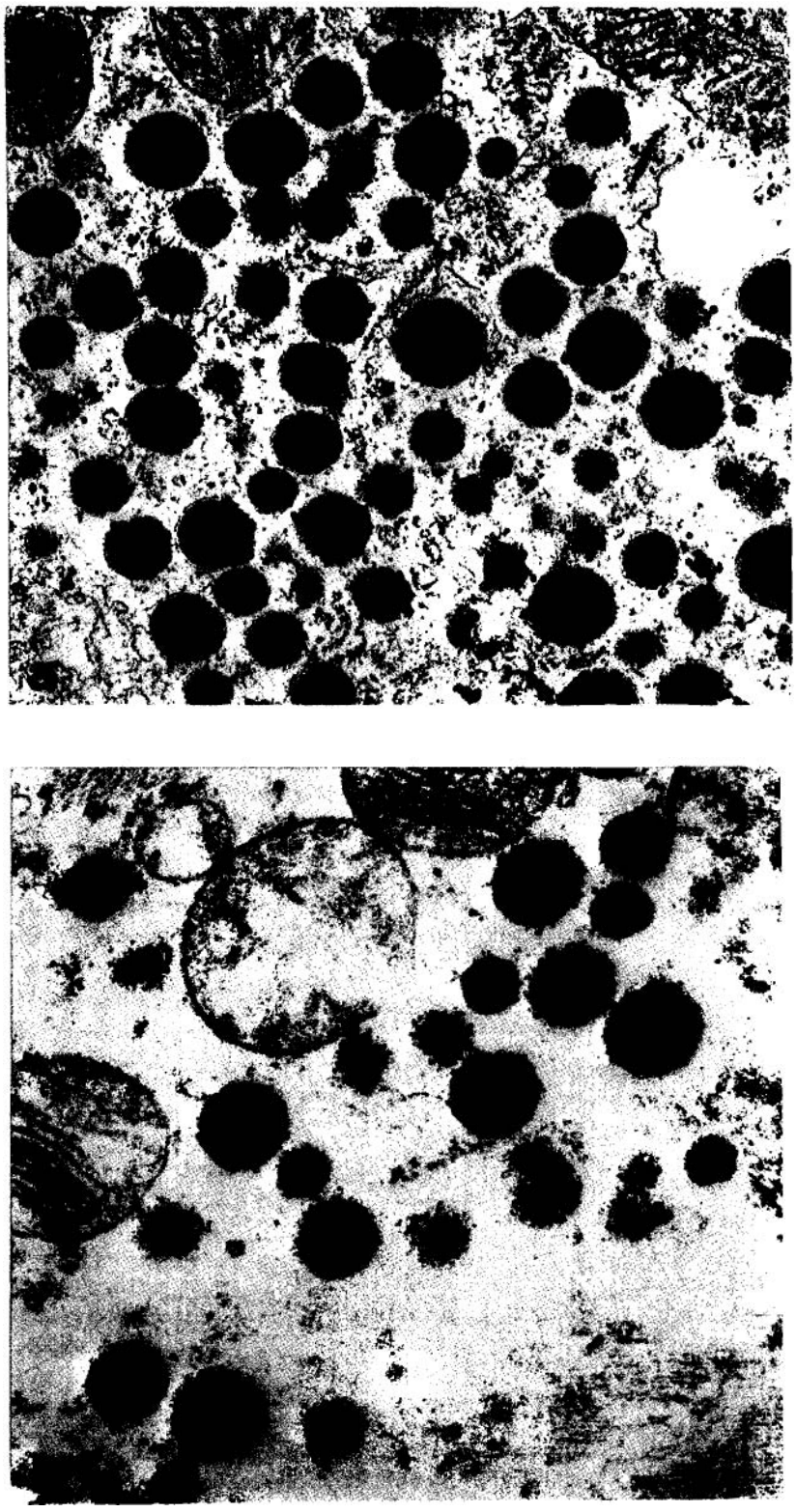


Fig. 4. Endocrine granules specifically stained by immunogold (a) and immunoperoxidase (b) **methods. Uranyl** acetate/lead **citrate contrast enhancement; both ×40,500**



in 15% picric acid followed by fixation in osmium tetroxide. They were then embedded in araldite and 90 nm ultrasections mounted on stainless steel grids.

Irnrnunocytochernistry

a) **Light microscopy.** We used the peroxidase-antiperoxidase method (Sternberger, 1979). A 1/1000 dilution of **ANP** antiserum was incubated on the sections for 14 hours at 4° C. The glucagon antiserum was used at a 1/500 dilution. Double labelling on the same section was performed using a modified double immunoperoxidase method (Nakane, 1968).

b) **Electron microscopy.** The grids were exposed to a 1/500 dilution of antiserum for 48 hours, then the antibody binding sites were visualised by two techniques:

1) Peroxidase - antiperoxidase (PAP) method. This carried out as previously reported (Lindop et al., 1983).
2) Immunogold Method - After thorough washing, the grids were incubated with antirabbit colloidal gold complex (GARG-20, Janssen Pharmaceuticals Ltd) for 1 hour at 20" C.

All grids were thoroughly washed, then subjected to contrast enhancement with uranyl acetate and lead citrate. They were examined in a Phillips 301 G electron microscope at 80 KV.

c) **Negative controls.** Negative controls for both light and electron microscopy included sections stained with non-immune rabbit serum, antiserum to bovine thyroglobulin, and the ANP antiserum which had been incubated with an excess of free **ANP.**

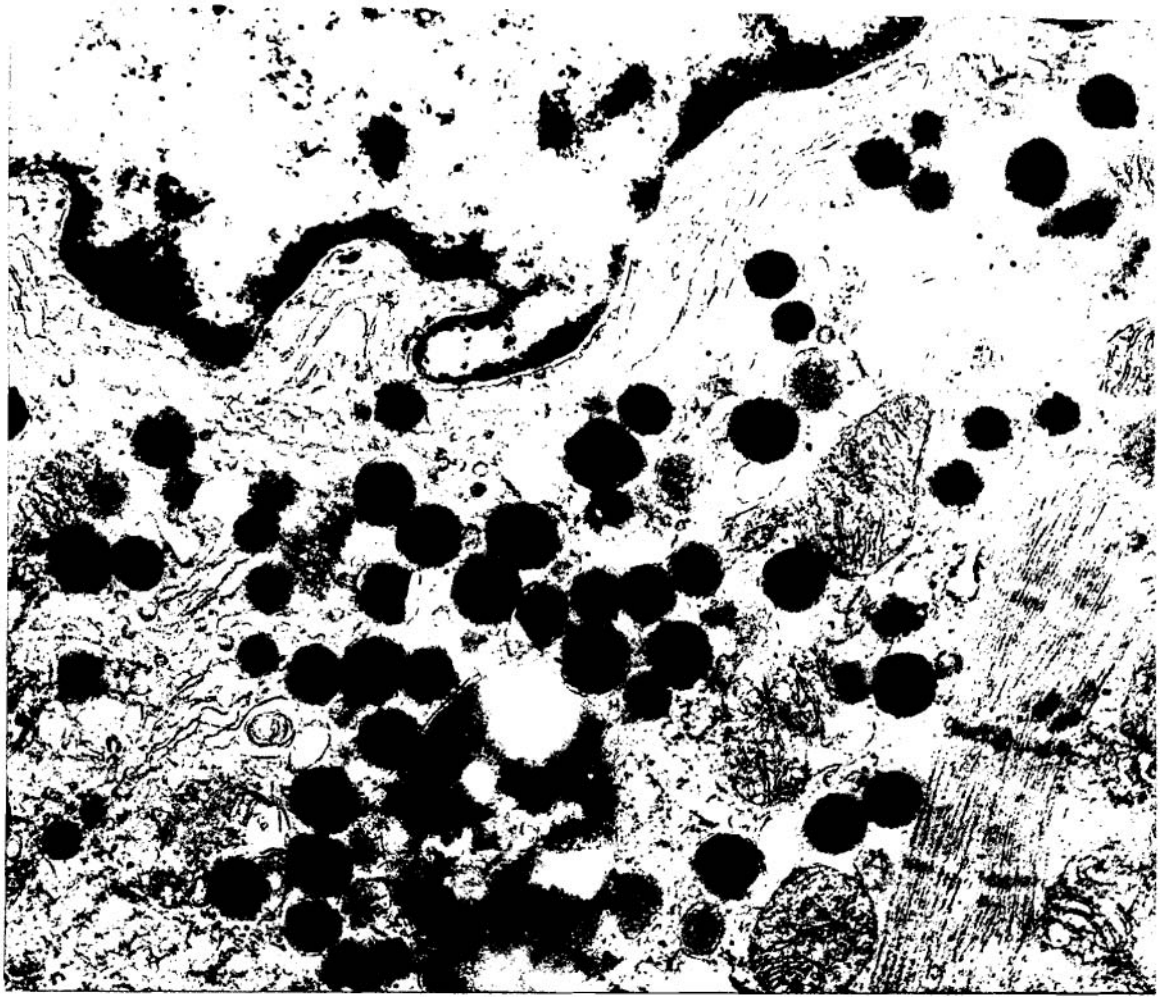


Fig. 5. A human myocyte showing the perinuclear area with a prominent Golgi apparatus and membrane bound granules stained by the immunogold technique. Uranyl acetatellead citrate. × 29,250

Results

a) Heart

Light microscopy. We found positive staining for atrial natriuretic peptide in both atria and the interatrial septum. The ventricular muscle was negative (Fig. 1). The human auricular appendages also stained positively while ventricular muscle did not. The histological appearance was similar in both species. The atrial staining was granular and confined to the perinuclear area of cardiocytes. The positive reaction was confined to granules (Fig. 2). In random sections we were unable to observe a difference in staining between the right and left atria of the rat. There was strong staining of cardiocytes in all layers of the atrium with no gradient of staining intensity across the thickness of the chamber wall.

Electron microscopy. Ultrastructural immunocytochemistry showed that all morphological types of granules stained positively for ANP. The antibody binding sites were visualised equally well with the PAP (Fig. 3) and with the immunogold technique (Fig. 4) which gave identical results. The ultrastructure appearances of human myocytes were similar to those of the rat (Fig. 5).

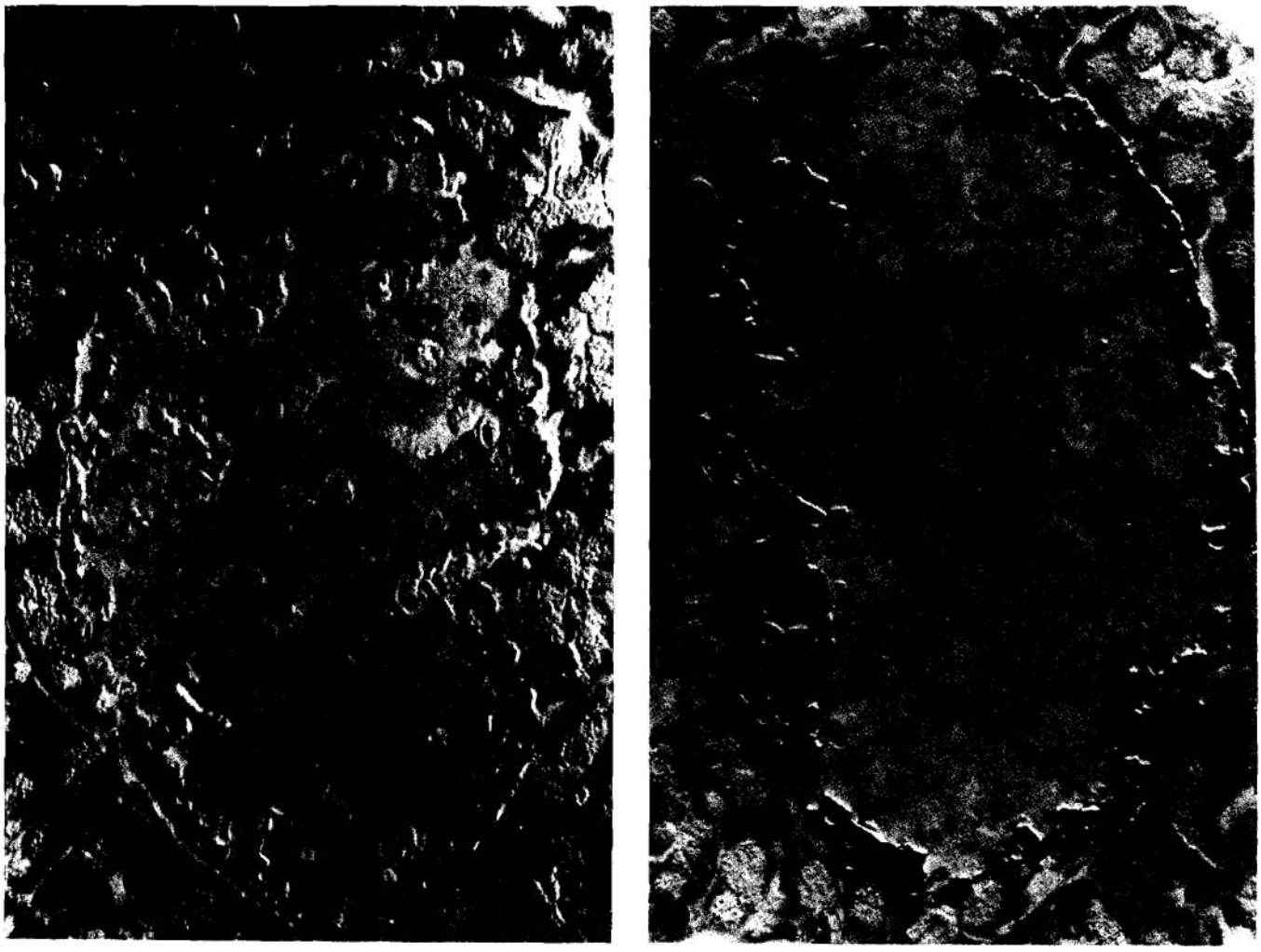


Fig. 6. (a) and (b) Opposite faces of two adjacent paraffin sections stained for ANP by the imrnunoalkaline phosphatase method (a) and glucagon (b) (irnrnunoperoxidase method). These show co-localisation of ANP and glucagon in the same cell. At most of the gaps in the mantle the cells are closely related to blood vessels. Both counterstained with haernatoxylin. x 180

b) Pancreas

Cells in the pancreatic islets stained positive for ANP. Positive staining was present in islets of all sizes in all parts of the pancreas but the ducts and acinar cells were negative. These ANP positive cells formed an almost complete mantle one cell thick round the outside of the islets with only occasional small gaps. These gaps were the sites of entry and of egress of the islet blood vessels. Using both double labelling techniques and single staining of adjacent "face to face" sections (Fig. 6) we confirmed that glucagon and ANP were contained within the same cells in the pancreatic islets. The staining for both substances was granular. No positive staining could be demonstrated when the glucagon antiserum was reacted with the heart sections. We found no positivity in formalin fixed human pancreatic tissue.

Discussion

In this study we have used an antibody to pure ANP. The specificity of this antibody was demonstrated by testing the binding of the antibody with a battery of peptides *in vitro*. Specificity of tissue staining was shown by negative controls which included normal rabbit serum, bovine thyroglobulin antiserum, and ANP antiserum incubated with an excess of pure ANP. Because of the close similarity in amino acid sequence of atrial peptides across the phylogenetic scale, antibodies raised in one species cross react with other species (Cantin et al., 1984; Metz et al., 1984).

Our findings using this antiserum to pure ANP confirm some of the results of previous studies using antibodies to the partially purified peptides (Cantin et al., 1984). The presence of specific granules containing ANP in the perinuclear area of atrial cardiocytes has been previously described by others (Cantin et al., 1984; MacKenzie et al., 1985). However, unlike these authors, in random sections we were unable to detect a difference between the staining intensity of the right and left atria. The subendocardial cardiocytes also stained as strongly as those in the outer layers of the atria. Since the granularity of atria varies with water and electrolyte balance (de Bold, 1979) this could be due to the difference in the physiological state of the animals in our experiments. The positive staining for ANP on light microscopy was shown by two different techniques of ultrastructural immunocytocliemistry localised in the membrane bound storage granules. As previously noted, using an antiserum to partially purified peptides (Cantin et al., 1984) all granules were stained.

Imnunoreactive ANP has been found by others in brain (Jacobowitz et al., 1985; Saper et al., 1985) salivary gland (Cantin et al., 1984) kidney and adrenal gland (MacKenzie et al., 1985). ANP released from the heart has systemic effects but it seems likely that ANP may also have local functions within the tissues (MacKenzie et al., 1985). In this respect it may resemble other peptides such as somatostatin and angiotensin II which have both systemic and local actions.

Our finding of ANP containing cells within the pancreas has not been previously described. We have shown by double labelling techniques that the immunostainable ANP was located exclusively within glucagon containing cells of the pancreatic islets. The staining of the cells was granular but we cannot say from our preliminary study whether the ANP was synthesised or taken up by these cells, as polypeptide hormones can be taken up by their target cells (King and Cuatrecasas, 1981) and other proteins can be taken up by endocytosis and concentrated in cells where they can be seen as granular staining with immunoperoxidase techniques (Taugner et al., 1982).

The situation of these cells at the periphery of each islet and the knowledge that ANP relaxes blood vessel tone in constricted vessels suggests the possibility that any ANP released from these cells could influence blood flow through the pancreas. Further morphological and experimental studies will be necessary to elucidate the significance of ANP within the pancreas. This is clearly an important avenue of further investigation.

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