Selective localization of calpain I (the low-Ca^{2+}-requiring form of Ca^{2+}-dependent cysteine proteinase) in B-cells of human pancreatic islets

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Received 13 March 1985

An immunohistochemical study was performed to localize two distinct Ca^{2+}-proteases (low-Ca^{2+}-requiring calpain I and high-Ca^{2+}-requiring calpain II) and their specific inhibitor (calpastatin) in human pancreas using the respective monospecific antibodies. Strongly positive staining by anti-calpain I antibody was found in pancreatic islets, specifically in B-cells, whereas the exocrine pancreatic tissue showed essentially no positive immunostaining. No such specific staining was found with anti-calpain II antibodies or anti-calpastatin antibodies. The results suggest that the Ca^{2+}-dependent proteolysis in B-cells can be triggered by a small rise of the intracellular Ca^{2+} concentration without serious interference by the endogenous inhibitor.

Ca^{2+}-dependent cysteine proteinase *Calpain* *Calpastatin* *Pancreas* *B-cell* *Insulin*

1. INTRODUCTION

Calpain (EC 3.4.22.17) is the collective name for Ca^{2+}-dependent cysteine proteinase [1], one of the most important intracellular nonlysosomal proteases found mainly in the cytosolic fraction. Calpain is known to be widely distributed in mammalian and avian tissues [2–6], but its physiological function remains unclear. Two forms of calpains are found which differ in Ca^{2+}-requirement: calpain I, the low-, and calpain II, the high-Ca^{2+}-requiring form. Some immuno-cyto- and/or histo-chemical methods had previously been applied to study the distribution and localization of calpain in the skeletal muscle [7,8], but the interpretation of the results was difficult due to the cross-reactivity between conventionally prepared anti-calpain I and calpain II antibodies [9,10]. Two-step affinity chromatography is required to obtain specific antibodies which can discriminate calpain I and II, as described in [11,12].

Calpain usually coexists with its specific inhibitor, calpastatin, and consequently calpain activity is regulated by calpastatin as well as by Ca^{2+} levels. A specific antibody against this inhibitor was successfully obtained as described in our recent report [13].

Here, we have used these monospecific antibodies for immunohistochemical localization of calpain and calpastatin in human pancreas.

2. MATERIALS AND METHODS

2.1. *Preparation of antigens and antibodies*

Calpain I was purified from porcine erythrocytes, calpain II from porcine kidney and calpastatin from human erythrocytes as described in [13,14]. The respective 83-kDa subunit protein

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

of calpain I, 80-kDa subunit protein of calpain II, and 70-kDa calpastatin were extracted from SDS-gels after electrophoresis and used to immunize rabbits. The IgG fraction was isolated from antiserum by ammonium sulfate fractionation and DEAE-cellulose chromatography. The anti-calpain I 83-kDa subunit IgG (anti-calpain I) was purified by subjecting the material sequentially to calpain I 83-kDa subunit-Sepharose and calpain II 80-kDa subunit-Sepharose column chromatographies. For the anti-calpain II 80-kDa subunit IgG (anti-calpain II), the reverse sequence of the two columns was used. These chromatographies made the final products completely devoid of the cross-reaction between the two types of calpains; by immunoelectrophoretic blotting analysis only the homologous heavy subunit band was recognized without any immunological cross-reactivity to other proteins. The details of these affinity chromatographies were previously described [11,12]. The purified anti-calpastatin IgG was obtained by affinity chromatography [13]. The following antisera against pancreatic hormones were used: guinea pig anti-porcine insulin (1:300, Miles, Elkhart, IN); rabbit anti-glucagon (1:3750, JIMRO, Japan); rabbit anti-bovine pancreatic polypeptide (1:800, a gift from Dr Chance, Lilly); rabbit anti-somatostatin (1:3750, JIMRO, Japan).

2.2. *Immunohistochemical staining of human pancreas*

Human pancreas was taken at autopsy, fixed in 10% neutral formalin for 12 h at room temperature and embedded in paraffin by routine methods. Series of consecutive and non-consecutive paraffin sections from the different portions of pancreas were immunostained by the peroxidase-anti-peroxidase (PAP) method of Sternberger et al. [15]. Briefly, the sections were treated with 0.3% H_2O_2 in methanol for 30 min, to block endogenous peroxidase activity and incubated with normal porcine serum (1:10, DAKO, Copenhagen, Denmark) or normal rabbit serum (1:10, DAKO) for 30 min at room temperature. In the next step, sections were incubated overnight at 4°C with the following antibodies: affinity-purified anti-calpain I (10 μg/ml), anti-calpain II (28 μg/ml), anti-calpastatin (30 μg/ml), anti-insulin (1:300), anti-glucagon (1:3750), anti-somatostatin (1:3750), and anti-pancreatic polypeptide (1:800). Further incubation with porcine anti-rabbit IgG (1:20, DAKO) and PAP complex (soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase, 1:80, DAKO) was performed for 30 min at 4°C, except for insulin staining where the sections were incubated with peroxidase-labeled rabbit anti-guinea pig IgG. Peroxidase staining was developed using diaminobenzidine as the substrate in 0.05 M Tris-HCl buffer (pH 7.6) with 0.003% H_2O_2. The following controls were taken: (a) incubation with purified anti-calpain I previously absorbed with calpain I (320 μg/ml anti-calpain I), calpain II (640 μg/ml anti-calpain I) and bovine insulin (200–400 μg/ml anti-calpain I); (b) incubation with homologous non-immune serum.

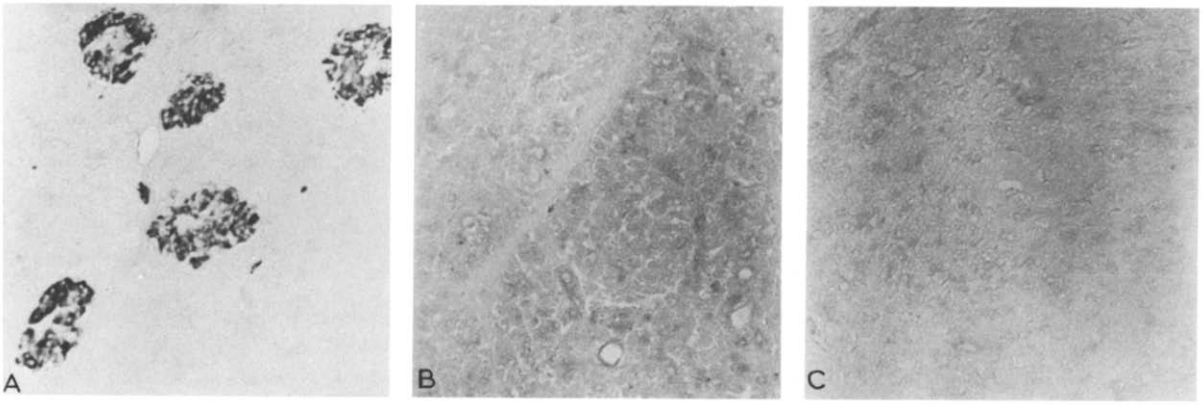

Fig.1. PAP staining of human pancreas by anti-calpain I, anti-calpain II and anti-calpastatin in non-consecutive paraffin sections. A, B and C are stainings obtained by anti-calpain I, anti-calpain II, and anti-calpastatin, respectively.

3. RESULTS

The staining patterns obtained with anti-calpain I, anti-calpain II and with anti-calpastatin were different in human pancreas as shown in fig.1. Calpain I showed positive immunoreactive material as indicated by the presence of the reaction product for peroxidase in the islets of Langerhans, but no positive staining was seen in the exocrine glands (fig.1A). No positive immunostaining was observed for calpain II or calpastatin as shown in figs 1B and 1C, respectively.

Fig.2A–E shows the staining patterns of an islet which contains predominantly B-cells and a significant number of A- and pp-cells. The distribution pattern of calpain I-positive cells coincides with that of B-cells, as is clear from fig.2A and B. A-cells and pp-cells are essentially negative for calpain I. Fig.2F–J shows the staining pattern of an islet which contains A-, B- and D-cells. Again, the distribution pattern of calpain I-positive cells coincides with that of B-cells: A- and D-cells are essentially negative for calpain I. Four pancreases, including one from a 7-year-old child, showed essentially the same staining pattern. From these data, it is clear that calpain I is selectively localized in B-cells of pancreatic islets. The specific staining with anti-calpain I was blocked only by purified calpain I (fig.3A), but not by purified calpain II (not shown) or insulin (fig.3B). The same anti-calpain II or anti-calpastatin used in our experiments showed intense immunostaining in other tissues.

4. DISCUSSION

Here, we localized calpain I, a Ca^{2+}-dependent protease, almost exclusively in the human endocrine pancreas, especially in B-cells of islets, by immunostaining of consecutive tissue sections using specific antibodies. No significant immunostaining was obtained in the exocrine pancreas. The localization pattern is rather unexpected, since it is not the endocrine pancreas, but the exocrine pancreas that has long been known as a rich source of proteases.

Judging from the intensity of immunostainings, it is obvious that the amount of calpain I, which requires only a micromolar level of Ca^{2+} for activation, in islet B-cells far exceeds that of calpain II, which requires a millimolar level of Ca^{2+} for activation. The predominance of calpain I over cal-

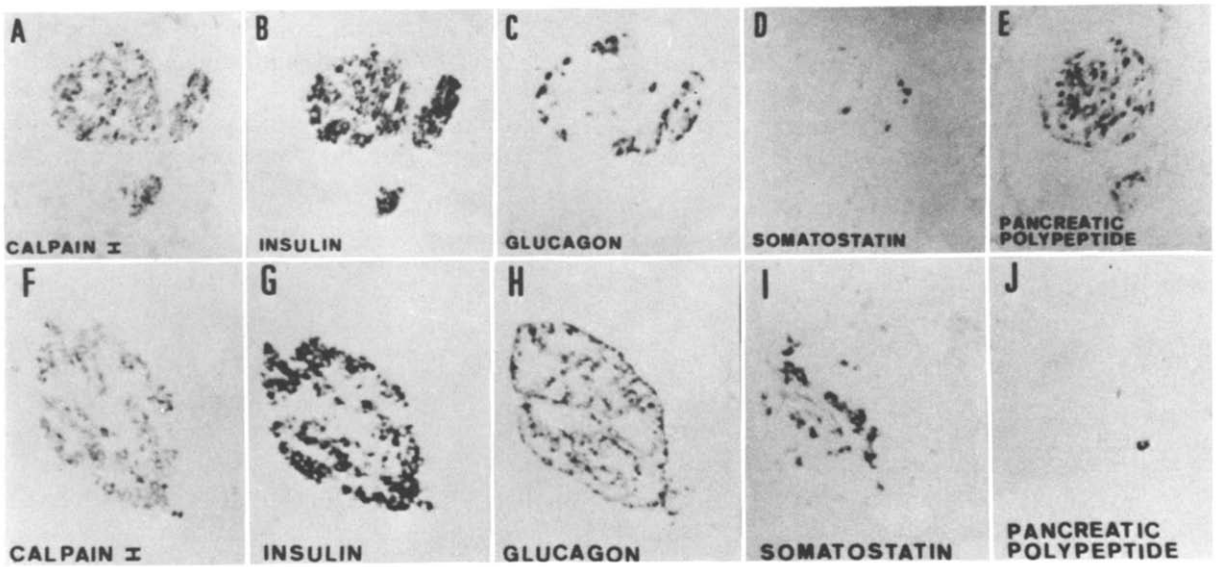

Fig.2. Immunohistochemical localization of calpain I and pancreatic hormones in a series of consecutive paraffin sections of human pancreas. A and F, stainings for calpain I; B and G, for insulin; C and H, for glucagon; D and I, for somatostatin; E and J, for pancreatic polypeptide. The islet in the upper panel contains few D-cells (D), whereas the islet in the lower panel contains few pp-cells (J).

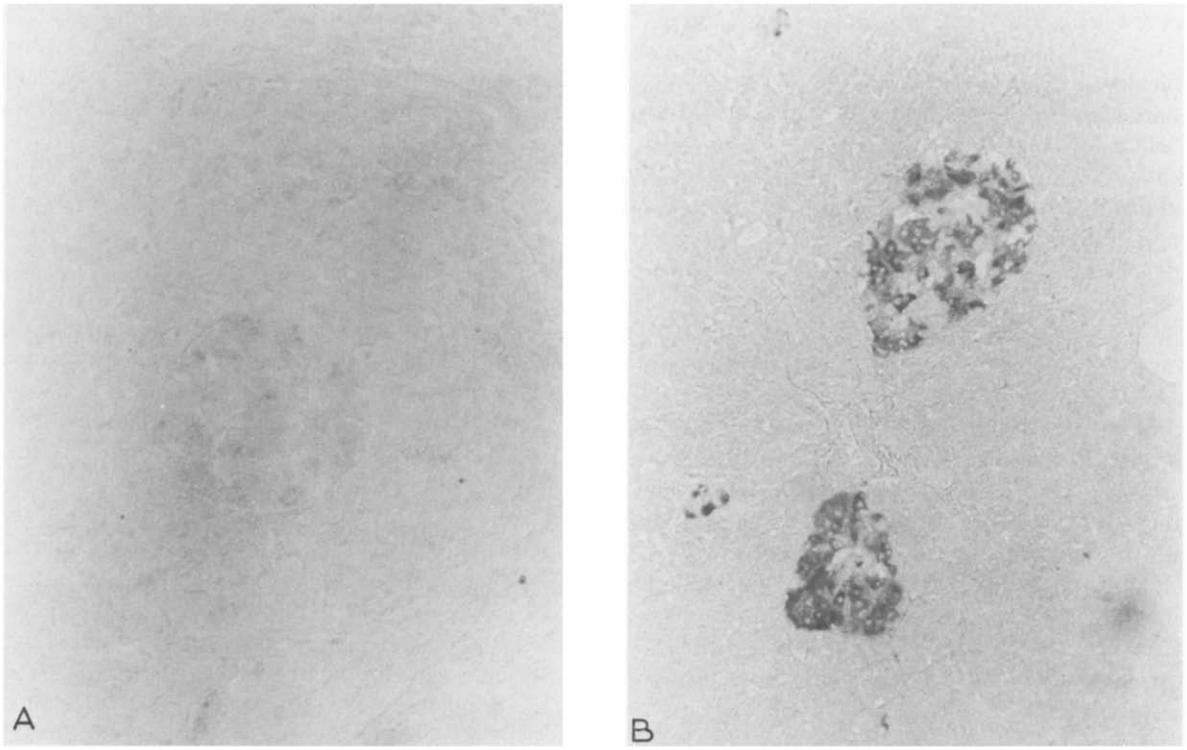

Fig.3. Immunostaining pattern for calpain I after preabsorption of the anti-calpain I antibody with calpain I (A) and insulin (B).

pain II is a characteristic feature of islet B-cells, for most of the cells and tissues usually contain more calpain II than calpain I as has been described in several distribution studies, leaving erythrocytes and the submandibular gland as rare exceptions [16].

Various cells and tissues are known to contain a specific proteinous inhibitor of calpain, namely calpastatin [1,16]. Distribution studies showed that the inhibitory activity of calpastatin in various cells and tissues is usually significant enough for it to easily cope with enzymatic activities of endogenous calpains [1,16], and this gives another regulatory mechanism for the Ca^{2+}-dependent proteolytic system in various cells and tissues. However, the amount of calpastatin in islet B-cells is shown to be remarkably low, which is another characteristic feature of islet B-cells.

These data collectively indicate that the Ca^{2+}-dependent proteolytic system in human pancreatic B-cells can readily be activated functionally by only a small rise in the intracellular Ca^{2+} concentration at the micromolar level, without any great interference by calpastatin.

It is well known that Ca^{2+} plays an important role in the process of insulin secretion by B-cells (review [17]). A significant influx of Ca^{2+} has been detected in the process of glucose-mediated insulin secretion [17]. The presence of some Ca^{2+}-binding proteins such as calmodulin [18] and vitamin D-dependent calcium-binding protein [19] in islet B-cells has been demonstrated and suggested to be implicated in the process of insulin secretion. Calpain may also be implicated in the process, though the precise role played by the enzyme remains unclear.

Ca^{2+}-dependent protein kinase (C-kinase) is reported to be activated during the course of glucose-induced insulin secretion by B-cells [20,21]. It is tempting to suggest that one of the physiological functions of calpain I in B-cells could be the activation of C-kinase, since it has already been demonstrated that calpain I has a strong ability to activate C-kinase in rat brain [22]. However,

calpain is not the sole factor which can activate C-kinase; another mechanism proposed for the activation of C-kinase is mediated by phosphatidylinositol-specific phospholipase C [23]. Which mechanism plays the central role in the activation of C-kinase during the course of glucose-mediated secretion of insulin will be the subject of ongoing studies.

ACKNOWLEDGEMENTS

We thank Dr Y. Seino, H. Imura, K. Takahashi and H. Yamabe for helpful discussions and Miss K. Sano, K. Ikeda and A. Kimoto for skillful technical assistance. This work was supported in part by Grants-in-Aid for Scientific and Cancer Research, Ministry of Education, Science and Culture, Japan.

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