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Serum calcium-decreasing factor (caldecrin) from porcine pancreas has proteolytic activity which has no clear connection with the calcium decrease

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We purified a serum calcium-decreasing factor, which showed chymotrypsin-like protease activity, from porcine pancreas to homogeneity. The factor administered to mice intravenously at a dose of 20 μg/kg b.w. decreased serum calcium by 15%. Treatment of the factor with the serine protease inhibitor, PMSF, caused a leftward shift in the dose–response curve, showing strengthened activity. It also caused a decrease in serum calcium and hydroxyproline levels in rats. At a dose of 10 ng/ml, the factor inhibited ^{45}Ca release from cultured fetal long bone stimulated by parathyroid hormone (PTH) and PTH-related protein, but not by interleukin-1α, prostaglandin E_1 and 1,25-dihydroxy vitamin D_3. No other well-known pancreatic proteases had these effects. In view of the results of experiments using protease inhibitor and pancreatic proteases, and in view of the specificity of this factor in vitro, we propose that the factor exerts its serum calcium-decreasing activity most probably not through proteolytic degradation of PTH, but through an inhibition of PTH action on bones by a yet undefined mechanism.

Hypocalcemia; Pancreas; Protease; PTH; Bone resorption

1. INTRODUCTION

Takaoka has proposed that the porcine pancreas contains the (new) factor(s), named PX, with many biological activities including the reduction of serum calcium, amino acids and blood urea nitrogen levels, and the increase of leukocytes [1,2]. Acute pancreatitis is accompanied by hypocalcemia, which suggests that the pancreas has serum calcium-controlling factor(s) [3]. We purified a serum calcium-decreasing factor from porcine pancreas which we have named caldecrin. In this report we show that caldecrin has a serine protease activity, but that the caldecrin function is fully expressed even after the inhibition of protease activity.

Abbreviations: caldecrin, serum calcium-decreasing factor; TFA, trifluoro acetic acid; FPLC, fast-protein liquid chromatography; DFP, diisopropyl fluorophosphate; PMSF, phenylmethylsulfonyl fluoride; APMSF, 4-amidino-phenyl-methanesulfonyl fluoride; E-64, L-*trans*-3-carboxy-oxiran-2-carbonyl-L-leucylagmatine; PTH, parathyroid hormone; PTHrP, parathyroid hormone related protein; IL, interleukin; PGE, prostaglandin E; $1,25(OH)_2D_3$, 1,25-dihydroxy vitamin D_3; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

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2. EXPERIMENTAL

2.1. *Bioassay for serum calcium-decreasing activity and the measurement of serum hydroxyproline concentration*

Male balb/c mice weighing 20–30 g and male Wistar rats weighing 60–70 g were injected with 200 μl/20 g body weight of the test sample in 0.1 M Tris–HCl (pH 7.4) into the tail vein after starvation for 18–20 h, and were assayed for serum calcium and hydroxyproline concentrations 4 h after the injection. The calcium concentration was measured using the *o*-cresolphthalein complexone method [4]. The serum hydroxyproline concentration was measured using an amino acid analyzer (Hitachi type 835).

2.2. *Bioassay for bone resorption using long-bone culture*

A long-bone organ culture was performed as described by Sato et al. [5]. The bone-resorption activities of hormones and test materials were expressed as the percentage of ^{45}Ca released in the culture medium of the total ^{45}Ca incorporated in the bone.

2.3. *Purification of serum calcium-decreasing factor from porcine pancreas*

Acetone powder of porcine pancreas was extracted with 0.1 M cold Tris–HCl buffer (pH 7.5) in 2% of NaCl for 1 h. After centrifugation, the supernatant was fractionated with acetone (30–60%), dialyzed against water and fractionated with saturated ammonium sulfate (45–60%). The precipitate was dissolved in 50 mM sodium acetate (pH 5.5) and dialyzed overnight against the same buffer. The dialyzate was applied to a Q Sepharose Fast-Flow (Pharmacia) ion-exchange column (4.5 × 16 cm). After washing, the absorbed materials were eluted by a stepwise increase of NaCl from 0.1 to 0.5 M in the starting buffer at a flow rate of 5.3 ml/min, and 1.5 min fractions were collected. The third peak at 0.2 M NaCl elution was concentrated by precipitation with 80%-saturated ammonium sulfate, and the concentrate was applied to a Superdex 75 FPLC column (Pharmacia), equilibrated with 0.2 M ammonium acetate (pH 6.8). The fractions were eluted at a flow rate of 0.5 ml/min and were collected every 2 min. Fractions of molec-

ular weights between 22,000 and 15,000 Da were combined, dialyzed against 50 mM sodium acetate (pH 5.5) and then applied to a Mono Q FPLC column (Pharmacia). Absorbed materials were eluted on a NaCl gradient from 0 to 0.5 M in the starting buffer at a flow rate of 0.5 ml/min, and 2 min fractions were collected. The fractions of the main peak eluted at 0.2 M NaCl from the Mono Q FPLC column were applied to a reverse-phased HPLC column (Wakosil 5C18-200), equilibrated with 0.1% TFA and eluted on a 25 min linear gradient of acetonitrile in 0.1% TFA from 0 to 50% and a 5 min linear gradient from 50 to 80%. The factor was eluted at 50% acetonitrile.

2.4. *Other Procedures*

SDS-PAGE was performed by tricine-SDS-PAGE on 16.5% gels and stained with Coomassie brilliant blue [6]. The isoelectric point was determined using IEF-PAGE on 4% gels [7]. Antiserum to purified caldecrin was raised in female rabbits. Protease activity was assayed using azocasein as a substrate [8].

3. RESULTS AND DISCUSSION

We purified serum calcium-decreasing factor (caldecrin) from the porcine pancreas as described in section 2. As shown in Fig. 1A, purified caldecrin treated with 1 mM PMSF showed a main band migrating at 28,000 Da and minor bands with smaller molecular weight on SDS-PAGE analysis. But on IEF-PAGE analysis, purified caldecrin revealed one band and the pI was calculated to be 4.5 (Fig. 1B). The starting extract and purified caldecrin were then compared by SDS-PAGE and Western blotting immunostained with polyclonal antibody against purified caldecrin. Fig. 1C shows that only the major band of 28 kDa was detected with the starting extract. These results suggest that the minor components of purified caldecrin were attributable to the degradation of caldecrin during purification.

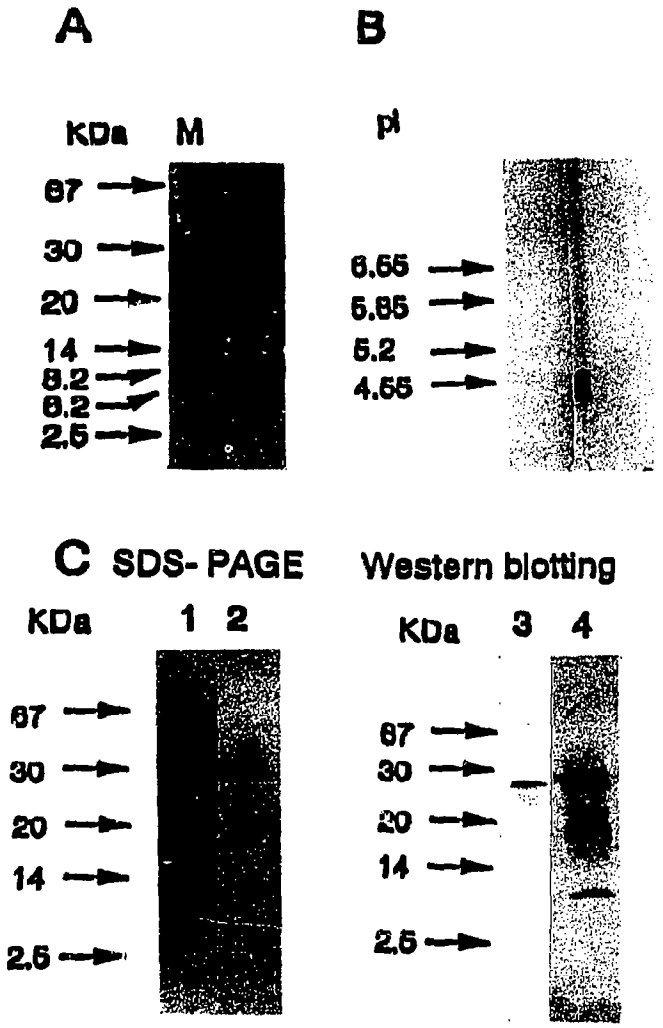

Fig. 1. (A) SDS-PAGE and (B) IEF-PAGE analysis of purified caldecrin (A, 20 μg/lane: B, 14 μg/lane). (C) SDS-PAGE and Western blotting analysis of extract (lane 1 and 3, 21 μg/lane) and purified caldecrin (lane 2 and 4, 8 μg/lane).

As shown in Fig. 2, the injection of purified caldecrin into mice caused a dose-dependent decrease of serum calcium and the effect reached a maximum decrease of 15% at 20 μg/kg body weight. The bioactivity of caldecrin was almost the same as that of porcine calcitonin [9], but the partial amino-acid sequence of caldecrin showed no similarity to the calcitonin sequence (data not shown). Thus, the activity of purified caldecrin could not have resulted from calcitonin contamination.

During the purification of caldecrin, we found that the appearance of some minor bands on SDS-PAGE analysis was suppressed by pretreatment of samples with protease inhibitor, PMSF. We thus measured the protease activity of purified caldecrin using azocasein as a substrate. Table I shows that the protease activity of caldecrin was almost completely inhibited by DFP, PMSF, soybean trypsin inhibitor and chymostatin, indicating that caldevrin is a chymotrypsin-like serine protease. The specific protease activity of caldecrin was 37% of that of chymotrypsin. Polyclonal antibodies against purified caldecrin did not react with chymotryp-

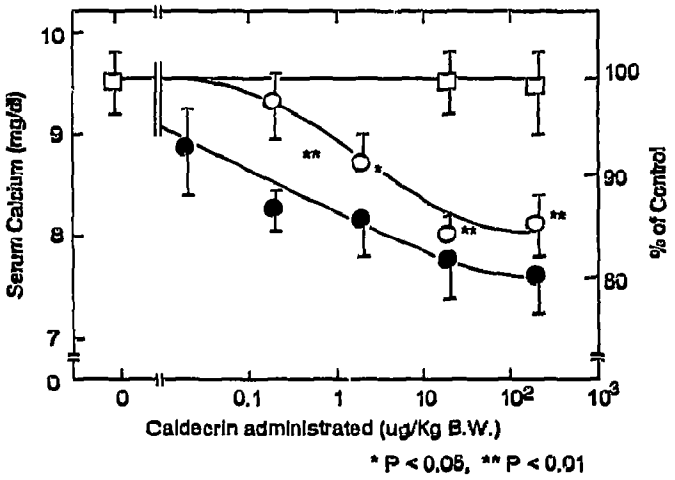

Fig. 2. Dose–response curve of caldecrin on the serum calcium concentration and evidence for calcium-decreasing activity with no relation to protease activity. Various concentrations of purified caldecrin without (○) or with (●) pretreatment of 1 mM PMSF, or PMSF control vehicle alone (□) were injected in mice. Values were expressed as mean ± S.D. of mice (n=5).

Table I

The effects of various inhibitors on the protease activity of the serum calcium-decreasing factor, caldecrin. Purified caldecrin (4 μg) was preincubated with various protease inhibitors at 37°C for 5 min. Then azocasein hydrolysis activity was measured with incubation at 37°C for 30 min. Values are expressed as means ± S.D. of five experiments

Protease inhibitors	Proteolytic activity of caldecrin (% of control)
DFP (0.1 mM)	10.6± 0.4*
PMSF (1 mM)	3.1± 3.4
APMSF (50 μM)	97.6± 9.2
Leupeptin (100 μM)	94.4± 7.6
Chymostatin (100 μM)	12.2± 3.3
E-64 (2.5 μM)	104.2± 3.7
Pepstatin (100 μM)	55.8±11.3
Soybean trypsin inhibitor (100 μg/ml)	1.8± 2.6

*Protease activity preincubated with DFP for 10 min was completely diminished (0.5 ± 0.02% of control).

sin (data not shown). These results suggest that the chymotrypsin-like protease activity of caldecrin is not derived from chymotrypsin contamination. Furthermore, purified caldecrin treated with the irreversible serine protease inhibitor, PMSF, still showed serum calcium-decreasing activity, the maximum activity of which (20% decrease) was much greater than the activity of the untreated sample, and the dose-response curve shifted to the left. PMSF in the vehicle did not

Table II

The effect of the serum calcium-decreasing factor (caldecrin) on serum calcium and hydroxyproline levels. Purified caldecrin or vehicle were injected into starved rats at a dose of 1 mg/kg b.w. and assayed 4 h after injection. Values were expressed as means ± S.D. of rats (n=5)

	Control vehicle	Caldecrin
Serum calcium (mg/dl)	8.59±0.38	7.83±0.08*
Serum hydroxyproline (nmol/ml)	58.0±7.8	42.1±6.8**

*P<0.05
**P<0.01

show any effect (Fig. 2). When incubated with mouse serum, the protease activity of caldecrin became undetectable (data not shown). These results suggest that the bioactivity of caldecrin is not connected with protease activity.

Next, serum hydroxyproline was measured to find out whether caldecrin directly interacts with the bone tissue [10]. Table II shows that caldecrin decreased not only the serum calcium concentration but also the serum hydroxyproline levels in rats, suggesting that caldecrin inhibits not only calcium release from the bone but also hydroxyproline degradation. The plasma level of PTH in rats was 153% of the controls 2 h after caldecrin administration when the calcium concentration was 92.0%, suggesting that caldecrin function is antagonistic to PTH action but does not act by degrad-

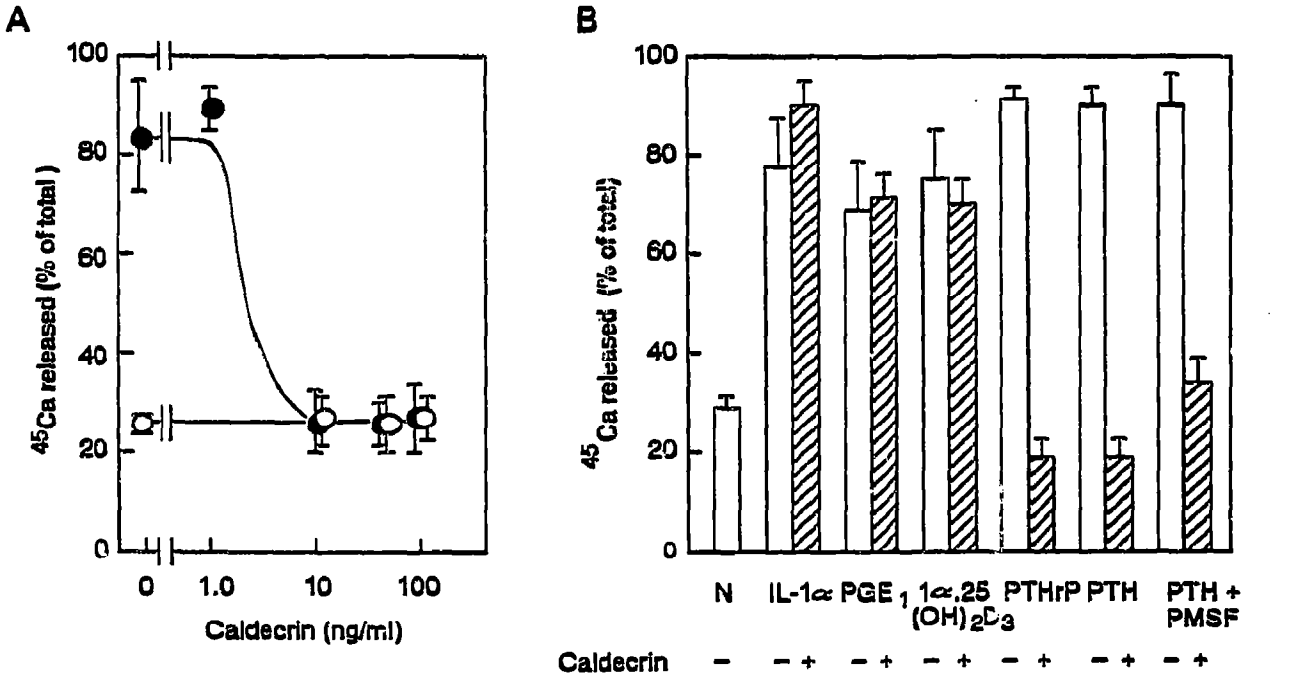

Fig. 3. Effect of caldecrin on fetal bone resorption in primary culture. (A) Fetal long bones were cultured for 3 days in α-minimum essential medium including various concentrations of the factor without (○) or with PTH (10^{-8} M) (●). Values are means ± S.D. for triplicate experiments. (B) Fetal long bones were cultured with IL-1α (5 ng/ml), PGE_1 (20 ng/ml), $1,25(OH)_2D_3$ (10^{-9} M), PTHrP (50 ng/ml) and PTH (10^{-8} M) with or without caldecrin (10 ng/ml). Values are means ± S.D. for triplicate experiments with two cultures.

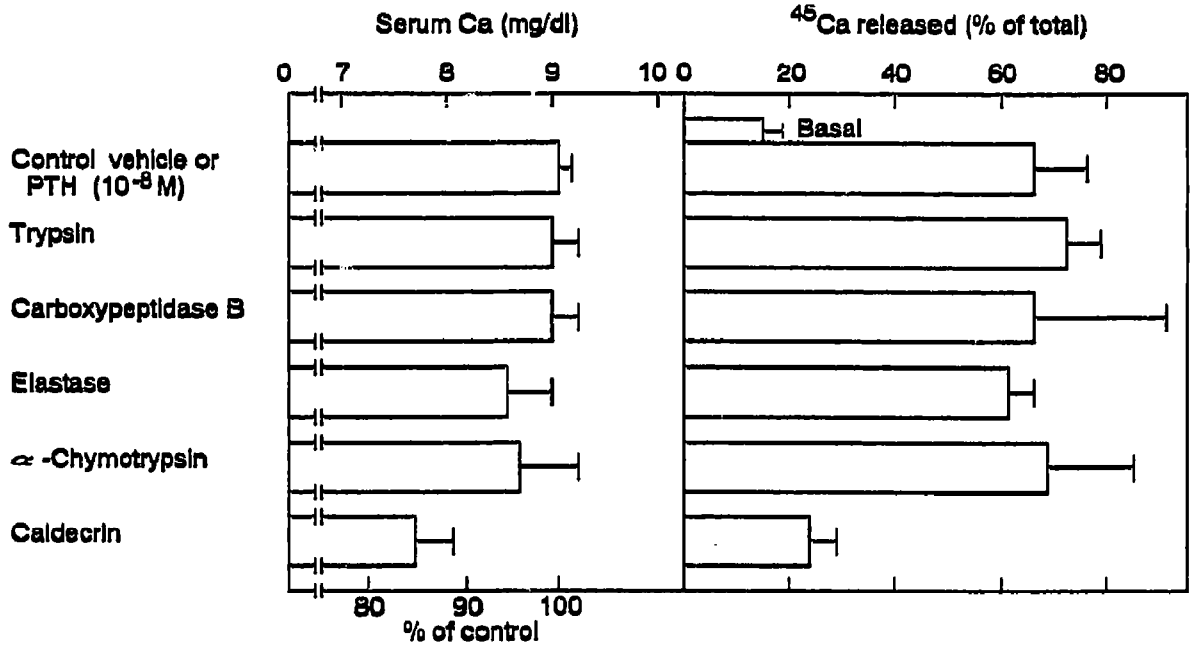

Fig. 4. Effect of pancreatic proteases and caldecrin on serum calcium-decreasing activity and PTH-blocking activity on long bone culture. Each protease was used at a concentration of 1 mg/kg b.w. for mouse serum calcium-decreasing activity and 10 ng/ml for PTH-blocking activity.

ing PTH. Next, caldecrin was tested on an in vitro long-bone culture system. Although caldecrin did not influence the basal calcium release from the bone, the release enhance by PTH to about 80% of the total was completely blocked in the presence of 10 ng/ml of caldecrin (Fig. 3A). This antagonistic activity was further tested with other bone-resolving hormones and cytokine. The results showed that caldecrin specifically inhibited ^{45}Ca release stimulated by PTH and PTHrP, but did not inhibit the release stimulated by PGE_1 and $1,25(OH)_2D_3$. This suggests possible involvement of the PTH receptor for the specific inhibition by caldecrin. It is notable that caldecrin did not inhibit the action of IL-1α, a peptide stimulant like PTH and PTHrP. The bioactivity of caldecrin was not affected by PMSF treatment either, again suggesting that the antagonistic effect against the calcium releasing activity of the PTH family is not related to protease activity (Fig. 3B). While this study was in progress, Yoneda et al. reported that a partially purified porcine pancreas extract (PX) decreased blood calcium in mice, and inhibited bone resorption which was not only stimulated by PTH but also by IL-1α, PGE_2, tumor necrosis factor (TNF), transforming growth factor (TGF-β) and $1,25(OH)_2D_3$. PX also inhibited osteoclast formation [11]. The discrepancy in antagonistic function between PX and caldecrin in bone resorption may come from the difference in purity between the preparations.

Finally, well-known pancreatic proteases tested were not effective in decreasing serum calcium and inhibiting PTH action in long bone culture (Fig. 4), suggesting that caldecrin specifically counteracts PTH in bone and decreases the serum calcium concentration and that caldecrin functions independently of proteolytic degradation of PTH.

Hypocalcemia is frequently observed in patients with acute pancreatitis [13]. The mechanism of hypocalcemia in acute pancreatitis is still unclear. One possible mechanism is an abrupt release of some pancreatic calcium controlling factor(s) into the circulation. Only amylin in the pancreatic islets has been shown to have hypocalcemic activity [12]. Caldecrin is different from amylin in molecular weight and sequence. We conclude that caldecrin, a new calcium-controlling factor purified from pancreas, is a possible agent which causes hypocalcemia in acute pancreatitis.

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