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Pronase Effect on Pancreatic Beta Cell Secretion and Morphology

Abstract. *Pronase at low concentration (4 micrograms per milliliter) produces a reversible increase of glucose-stimulated insulin release in isolated islets of Langerhans. Pronase also affects the ultrastructure of the beta cells by inducing extensive development of tight junctions as well as the accumulation of secretory product within the extracellular spaces.*

Proteolytic enzymes have been widely employed in the study of the molecular architecture and function of the cell membrane in various tissues. Incubation of intact cells with trypsin or Pronase has been reported to induce changes ranging from destruction of the cell membrane to subtle unmasking of previously unaccessible receptor sites located within the plasma membrane (*1*).

We have studied the effects of proteolysis on the pancreatic beta cells by incubating isolated rat islets of Langerhans with Pronase (*2*) at low concentration and observing concomitantly the morphology of the cells and the insulin release at low and high glucose concentration. The results show that such proteolytic treatment causes a reversible increase of the insulin response to glucose stimulation and peculiar ultrastructural alterations. These alterations include extracellular sequestration of secretory product and the appearance of extensive tight junctions (*3*) between islet cells.

Isolated islets were obtained by collagenase digestion of pancreases (*4*) removed from fed male albino rats. The incubation medium consisted of Krebs-Ringer bicarbonate buffer (KRB) equilibrated with 94 percent O_2 and 6 percent CO_2, and supplemented by 1 percent bovine serum albumin and, unless otherwise mentioned, 50 mg of glucose per 100 ml.

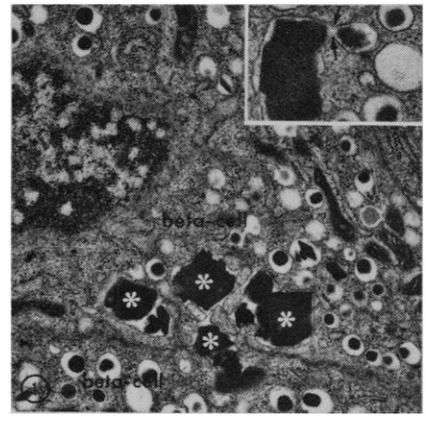

Fig. 1. Pronase-treated islet exposed to high glucose concentration (300 mg/100 ml). Several masses of electron dense material (asterisks) are seen within the intercellular space between two beta cells. The ultrastructure of the cellular organelles appears normal (× 11,600). The inset shows a secretory granule undergoing exocytosis (arrows) in proximity to a dense mass. The frequency of such images suggests that the dense masses are formed by coalescence of extruded granule cores (× 20,400).

In all experiments, the islets were divided in two pools and first incubated for 90 minutes at 37°C, either in 5 ml of KRB alone for the control pool or with Pronase E (*5*) at a concentration of 4 μg/ml.

After this first incubation period, both pools of islets were thoroughly washed in KRB. Groups of five islets were distributed in 1.5 ml of KRB containing glucose (either 50 or 300 mg/100 ml) and incubated for 90 minutes at 37°C. Portions of incubation media for measurement of immunoreactive insulin (IRI) content (*6*) were taken at 15, 30, 60, and 90 minutes.

Pelleted islets were briefly fixed in 2 percent phosphate-buffered glutaraldehyde and processed either for conventional thin-section electron microscopy or for freeze-etching by the method of Moor and Mühlethaler (*7*). Release of IRI from Pronase-treated islets incubated in a medium containing 50 mg of glucose per 100 ml did not differ significantly from that of the controls during the first 60 minutes of incubation (Table 1). After 90 minutes, however, IRI release by the Pronase-treated islets was approximately twice that of the controls. In a medium containing 300 mg of glucose per 100 ml, a twofold augmentation in the insulin response of Pronase-treated islets was evident after 30 minutes (Table 1).

In order to determine whether the enhancing effect of Pronase on glucose-induced IRI release was a reversible phenomenon rather than an irreversible leak of intracellular constituents, Pronase-treated islets were first incubated for 60 minutes in a medium containing 300 mg of glucose per 100 ml and then transferred to a medium with 50 mg of glucose per 100 ml and incubated for 60 minutes. Production of IRI, which was 41.4 ± 1.7 ng per milliliter per five islets (mean ± standard error of mean) after incubation at high glucose concentration, declined to a near basal level of 6 ± 0.3 ng per milliliter per five islets ($P < .001$), virtually identical to that observed in control islets.

In thin sections of Pronase-treated

Table 1. Effect of prior incubation with Pronase (4 μg/ml) for 90 minutes on immunoreactive insulin (IRI) release during incubation of isolated islets at various glucose concentrations. The statistical significance (*P*) of the difference between experimental and control values is given; S.E.M., standard error of the mean; NS, not significant; *N*, number of observations.

Incubation time (minutes)	Glucose (mg/100 ml)	IRI release per five islets (ng/ml)				
		Controls		*P*	Pronase-treated (4 μg/ml)	
		Mean ± S.E.M.	*N*		Mean ± S.E.M.	*N*
15	0	0.77 ± 0.13	15	NS	0.92 ± 0.14	16
15	50	1.20 ± 0.33	15	NS	1.05 ± 0.17	16
15	300	3.06 ± 0.70	13	NS	4.75 ± 0.94	12
30	0	1.20 ± 0.23	15	NS	1.80 ± 0.24	16
30	50	1.18 ± 0.33	15	NS	1.72 ± 0.31	16
30	300	10.62 ± 0.97	13	< .001	20.44 ± 2.19	12
60	0	1.98 ± 0.32	11	NS	2.30 ± 0.37	16
60	50	1.52 ± 0.44	15	NS	2.43 ± 0.59	15
60	300	24.31 ± 2.50	13	< .001	46.67 ± 4.06	12
90	0	1.45 ± 0.21	14	< .001	2.86 ± 0.32	16
90	50	2.20 ± 0.63	15	= .05	3.97 ± 0.67	15
90	300	50.54 ± 4.06	13	< .01	72.12 ± 6.26	12

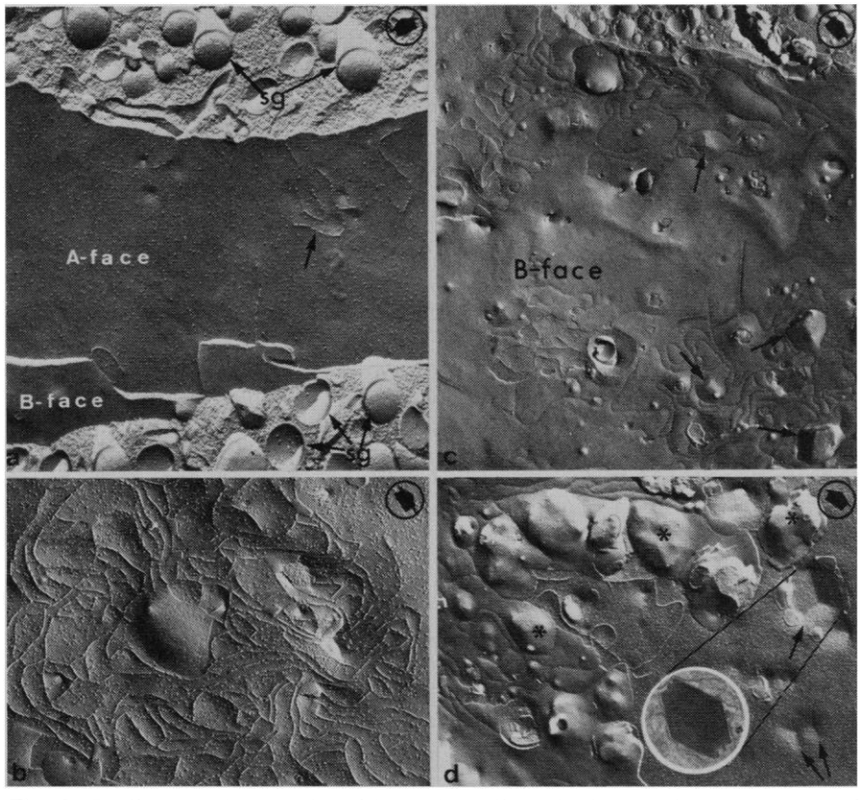

Fig. 2. Replicas of freeze-etched islet cells (*10*). All photographs were processed to reveal shadows in white. Circled arrows indicate the direction of shadowing. (a) Control islet. The fracture plane has passed through two adjacent cells, revealing the cytoplasm containing secretory granules (*sg*) and exposed two membrane faces, one facing the extracellular spaces (A-face), the other facing the interior of the cell (B-face). The arrow indicates a small tight junction consisting of linear ridges (× 22,000). (b) Islet treated with Pronase and exposed to low glucose concentration (50 mg/100 ml). Area of islet cell membrane (A-face) shows the complex pattern of a highly developed tight junction network (× 22,000). (c and d) Cell membrane faces of Pronase-treated islet exposed to high glucose concentration (300 mg/100 ml). (c) The upper and lower parts show extensive, ramifying, and compartmentalized tight junctions on the B-face of cell membrane. Arrows indicate the polygonal-shaped protuberances (× 8,000). (d) The asterisks indicate protuberances enclosed within the network of tight junctions that appear as anastomosing furrows on the B-face. Complementary concavities (arrows) of varying size are seen on the A-face (× 10,000). The inset is a thin section of conventionally fixed and embedded material showing the polygonal shape of accumulated secretory product within the intercellular space (× 16,000).

islets exposed to a high glucose concentration (300 mg/100 ml), focal accumulations of dense material, often polygonal in shape, were noted (Fig. 1). These accumulations were located in the extracellular spaces surrounding beta cells exclusively and seemed to deform the cell membranes. They are presumed to represent masses of fused secretory material released by means of apparently normal exocytosis (inset, Fig. 1). In Pronase-treated islets exposed to a low glucose concentration (50 mg/100 ml), these extracellular masses were not found, which suggests that the masses are related to stimulated insulin secretion. Except for these masses, the Pronase-treated islet cells did not differ morphologically from controls.

To characterize further the morphological alterations resulting from Pronase treatment, we studied islets by the freeze-etching technique, which allows visualization of extensive areas of membrane. According to Branton (*8*), freeze-etching splits cell membranes so as to produce two complementary fracture faces, one facing the extracellular space (A-face), the other facing the interior of the cell (B-face) (*9*).

In replicas of islet cells (*10*) incubated with high glucose concentration, numerous polygonal deformations were observed on both faces of cell membranes. These deformations are assumed to represent the imprints of the extracellular accumulations of secretory product described in thin sections. Accordingly, these imprints are seen as angular concavities on the A-faces of membranes (Fig. 2d), whereas they appear as angular protuberances on the B-faces (Fig. 2, c and d). No such deformations of the membranes were observed in replicas of islets exposed to low glucose concentrations.

An unexpected finding in the freeze-etch replicas was a considerable increase of tight junctions in Pronase-treated islets, both at low (Fig. 2b) and high glucose concentrations (Fig. 2, c and d) (*11*). Replicas of junctional areas corresponding to tight junctions appeared as linear branching and anastomosing ridges on the A-faces (Fig. 2b) and as linear branching and anastomosing furrows on the complementary B-faces (Fig. 2, c and d) (*9, 12*). These membrane specializations were also present in control islets but were far less numerous (Fig. 2a). In Pronase-

treated islets incubated in a high glucose concentration, the extensive network of tight junctions often encircled the polygonal protuberances or cancavities (Fig. 2, c and d), believed to represent masses of extracellular secretory product.

These data indicate that the incubation of islets of Langerhans with a low concentration of Pronase caused (i) a reversible increase of glucose-stimulated IRI release; (ii) the development of extensive tight junction networks at both low and high glucose concentrations; and (iii) at high glucose level only, the conspicuous accumulation of secretory product in the extracellular spaces between beta cells. The fact that mild Pronase treatment increases glucose-stimulated IRI release is in accord with studies in other tissues in which it has been proposed that membrane proteolysis can unmask relatively inaccessible receptor sites located within the membrane (*1*). The increased IRI output after Pronase treatment could be due to a heightened sensitivity of the cells to glucose. Although passive "leakage" of insulin through an altered cell membrane cannot be completely excluded by our experimental results, the normal ultrastructural appearance of the cells, including evidence of normal exocytosis, the preservation of the secretory response to glucose, and the absence of comparable masses between other islet cell types, speak against this possibility.

The presence of undissolved secretory product within the extracellular space is an infrequent phenomenon (*13*). Whether its abundance in Pronase-treated islets is the consequence of damage to the normal solubilization mechanism of the secretory product of beta cells or massive augmentation of insulin release, or else represents mechanical trapping of released product within the extensive tight junctional network (*14*) remains to be determined. Tight junctions might compartmentalize the intercellular spaces into relatively closed areas, from which large amounts of secretion products cannot easily escape and diffuse into the open intercellular space (*15*).

The proliferation of tight junctions following Pronase treatment demonstrates for the first time that these differentiations of the cell membrane are not stable structures but are, under certain conditions, capable of major development. Conceivably, the capacity to stimulate the proliferation of tight junctions may provide a means for further investigating their function.

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10. In descriptions of freeze-etch replicas in both text and figure legends, we used the term islet cell rather than beta cell. However, these terms are probably always equivalent because in freeze-etching we studied the center of the islet, shown to consist mostly of beta cells in the rat [H. Ferner, *Das Inselsystem des Pancreas* (Thieme, Stuttgart, 1952), p. 119].
11. The amount of tight junctions in treated and untreated islets was determined by stereological measurements including only the exposed membrane faces containing tight junctions. After Pronase treatment, the density of boundary length of tight junction per unit of surface area is 817 ± 40 nm/μm^2 compared to only 148 ± 14 nm/μm^2 in the controls ($P < .005$). Whereas the enhanced IRI release is reversed upon removal of the glucose stimulation, the tight junctions apparently remain over 2.5 hours of incubation. The possibility that insulin released during the first incubation period with Pronase may have acted by itself as an inducer of tight junctions cannot be ruled out and remains to be investigated.
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14. The tight junction or zonula occludens has a sealing role in many epithelia, in which the junction usually separates two different environments (*3*) [A. Matter, L. Orci, C. Rouiller, *J. Ultrastruct. Res.*, Suppl. 11 (1969)]. Particularly extensive tight junctions between the epithelial cells of the epididymis have been described [D. S. Friend and N. B. Gilula, *J. Cell Biol.* **53**, 758 (1972)]; they resemble in extension and configuration those produced by Pronase between islet cells.
15. In view of the secretory product trapped between Pronase-treated islet cells, the measured IRI release during glucose stimulation probably represents only a fraction of the total output.
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