Hormone-Induced Cyclic Guanosine Monophosphate Secretion from Guinea Pig Pancreatic Lobules

Abstract. *Carbamylcholine (30 μM) increased the concentration of guanosine 3',5'-monophosphate (cyclic GMP) in guinea pig pancreatic lobules about eight- to tenfold over the basal concentration in 30 seconds with a concomitant increase in the rate of amylase secretion. The concentration of cyclic GMP rapidly declined to a plateau value of about 16 percent of the peak level in 10 minutes. Cellular cyclic GMP decreased, mostly because the nucleotide was secreted into the medium; cellular adenosine 3',5'-monophosphate (cyclic AMP), however, did not change, nor was this nucleotide secreted into the medium. An immunocytochemical technique showed that cyclic GMP was distributed in the apical plasmalemma membrane and lumen of the pancreas. Carbamylcholine increased the cyclic GMP fluorescence in the apical plasmalemma membrane within 30 seconds, and in zymogen granules and the plasma membrane in the apical part of acinar cells in 10 minutes. The islets of Langerhans did not show any change in cyclic GMP. Fluorescence of cyclic AMP in pancreatic lobules was not altered by carbamylcholine and was localized along the apical portion of plasmalemma and cytoplasm. Cyclic GMP may thus participate either in the process of exocytosis or in the activation of enzymes secreted from the pancreas.*

Pancreatic acinar cells synthesize and secrete a number of digestive enzymes by a process of exocytosis which involves fusion of membranes of secretory zymogen granules with that of the plasmalemma membranes facing the lumen (*1*). Under hormonal stimulation, acinar cells continuously secrete enzymes from stored zymogen granules. Previous studies have implicated adenosine 3',5'-monophosphate (cyclic AMP) as a mediator for enzyme release in the pancreas, but the experimental evidence has not been very convincing (*2*). Such evidence was obtained by adding cyclic AMP or its dibutyryl derivative directly to tissue slices; results obtained by these means can lead to erroneous conclusions (*3*).

Recently it was shown that changes in the concentrations of guanosine 3',5'-monophosphate (cyclic GMP) rather than changes in cyclic AMP are associated with the activity of various secretagogues in pancreatic slices and isolated pancreatic acinar cells (*4, 5*). However, the effects of hormonal stimulation on amylase secretion was greater with pancreatic lobules than with isolated pancreatic acinar cells (*4*).

To explore the mechanism by which cyclic GMP is involved in enzyme secretion from pancreas, we have related the rate of amylase secretion with the time course of cyclic GMP accumulation in pancreatic lobules as measured by a recently developed fluorescent immunocytochemical technique (*6, 7*) and by direct measurement of cyclic nucleotides.

Hartley guinea pigs (male, NIH strain) weighing 250 to 300 g were fasted overnight. Pancreatic lobules were prepared according to the procedure of Scheele and Palade (*8*). The lobules were incubated at 37°C for 30 minutes in Krebs-Ringer bicarbonate buffer, *p*H 7.4, containing 1 mg/ml of glucose. The medium was decanted and the lobules were washed and resuspended in the same medium. About four to eight lobules weighing 20 to 40 mg were incubated with carbamylcholine and other agents in 2 ml of Krebs-Ringer bicarbonate buffer (*p*H 7.4) saturated with 95 percent O_2 and 5 percent CO_2 in a Dubnoff metabolic shaker at 37°C for various times. At the end of the incubation period, the medium was removed and frozen, and the lobules remaining in the flask were rapidly frozen in liquid nitrogen or Dry Ice. The cyclic nucleotides present in the lobules were extracted by homogenization of the tissue in 5 percent trichloroacetic acid. The extract was centrifuged at 3200*g* for 20 minutes. The precipitate was dissolved in 1*N* NaOH and a portion was used for determination of proteins, bovine serum albumin being used as a standard (*9*). Trichloroacetic acid was removed by ether extraction, and the ether was removed by incubating the samples at 50°C. Portions were taken from the assay of cyclic nucleotides. A portion of the frozen medium was heat-denatured and used for the assay of cyclic nucleotides. The cyclic GMP and cyclic AMP were assayed (without purification) by the radioimmunoassay method of Frandsen and Krishna (*10*) on Dowex 1 column. Identical values were obtained with or without purification.

Amylase released into the medium was determined according to the method of Rinderknecht *et al.* (*11*), amylase azure blue (Calbiochem) being used as a substrate.

In order to determine the cytochemical localization of cyclic nucleotides, pieces of pancreas were incubated in Krebs-Ringer bicarbonate buffer (*p*H 7.4) for various periods of time as described above. At the end of the incubation period, pieces of pancreas were removed from the medium and frozen immediately on a cardboard pad by immersion in 2-methyl butane (Eastman

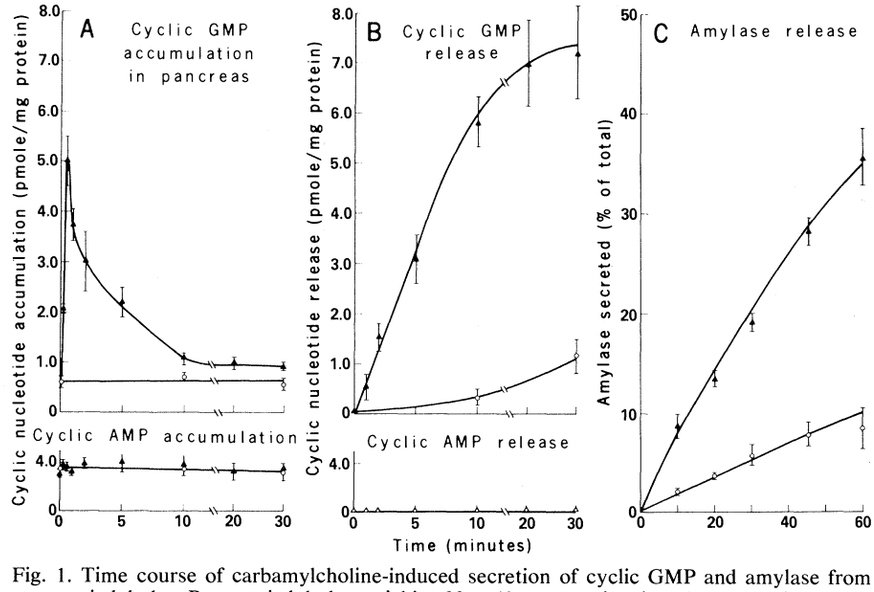

Fig. 1. Time course of carbamylcholine-induced secretion of cyclic GMP and amylase from pancreatic lobules. Pancreatic lobules weighing 20 to 40 mg were incubated at 37°C with 30 μ*M* carbamylcholine in 2 ml of Krebs-Ringer bicarbonate buffer, *p*H 7.4. The tissue and medium concentration of cyclic GMP and cyclic AMP were determined in triplicate separately after various times of incubation (A and B). Amylase released into the medium was determined at various times of incubation and expressed as the percentage of total amylase (tissue plus medium) present at the end of the incubation (C). Control (○); carbamylcholine (▲). Values are mean of four experiments ± standard error.

Kodak); they were then cooled in liquid nitrogen, and sections 4 to 6 μm thick were cut in a cryostat microtome (Slee International). These sections were transferred to cover slips and dried. Antiserums to cyclic GMP and cyclic AMP were raised in rabbits as described by Steiner *et al.* (*12*) and were diluted 1:8 and 1:10, respectively, with phosphate-buffered saline (5.4 m*M* Na_2HPO_4 in 150 m*M* NaCl, adjusted to *p*H 7.4 with KH_2PO_4). The sections were covered with 25 μl of diluted antiserum to either cyclic GMP or cyclic AMP for 30 minutes at 25°C. The sections on the cover slips were immersed in phosphate-buffered saline for 30 minutes and were covered with a 1:10 dilution of goat antiserum to rabbit IgG labeled with fluorescein isothiocyanate (Cappel) for 30 minutes. The sections on the cover slip were again immersed in phosphate-buffered saline for 30 minutes and mounted with 50 percent glycerine dissolved in the buffer. Sections were examined with a Zeiss dark-field fluorescence microscope through a fluorescein isothiocyanate filter with a 200-watt xenon lamp and a 53/44 barrier filter. Sections were photographed for a fixed time with Kodak Tri-X 35-mm film (*6,7*).

During incubation of pancreatic lobules with carbamylcholine (30 μ*M*) the concentration of cyclic GMP in the tissue increased markedly within seconds, reaching a peak of about eight- to tenfold over the basal concentration in 30 seconds with a concomitant increase in the rate of amylase secretion. The concentration of cyclic GMP then declined rapidly to about 50 percent of the peak level in about 3.5 minutes and to about 16 percent of the peak level in 10 minutes (Fig. 1). This reduction in tissue cyclic GMP was mostly due to its being secreted into the medium; the accumulation of cyclic GMP in the medium occurred slowly during the first minute of incubation with carbamylcholine, then rapidly until it reached a plateau in 20 minutes. Half maximal cyclic GMP release was obtained in 5.5 minutes. In the absence of carbamylcholine the basal tissue concentration of cyclic GMP was not changed during the entire period of incubation, but a small amount of cyclic GMP was secreted continuously into the medium, indicating a constant turnover of this nucleotide. Concentrations of cyclic AMP in the tissue were not changed during the incubation with carbamylcholine, nor was cyclic AMP secreted into the medium during this period. To investigate the possibility that the cyclic GMP released into the medium was bound to the digestive enzymes, we passed the medium containing cyclic GMP through an Amicon CF-25 filtration device or serum albumin was added as a carrier and the proteins were precipitated with 80 percent ethanol at 0°C (*10*). More than 90 percent of the cyclic GMP was found in the filtrate or in the supernatant; very little was associated with proteins. When the tissue was homogenized in 90 percent ethanol at 0°C, most of the cyclic GMP was associated with the proteins. However, this finding does not rule out the possibility that cyclic GMP is bound to proteins during its release and becomes dissociated in the incubation medium because of changes in the environment. Although most of the cyclic GMP in pancreatic acinar cells was released into the medium, we were not able to detect the secretion of either guanylate cyclase or cyclic GMP phosphodiesterase into the medium. A similar accumulation and secretion of cyclic GMP occurred during incubation with the polypeptide agent, caerulein. Whether cyclic GMP was formed in cytoplasm and then taken up into the granules (as occurs with the digestive enzymes) or was formed in the granules remains to be elucidated.

To gain further insight into the possible role of cyclic nucleotides in the hormone-induced secretion of enzymes by pancreatic acinar cells, we used immunocytochemistry to examine the localization of these nucleotides in non-

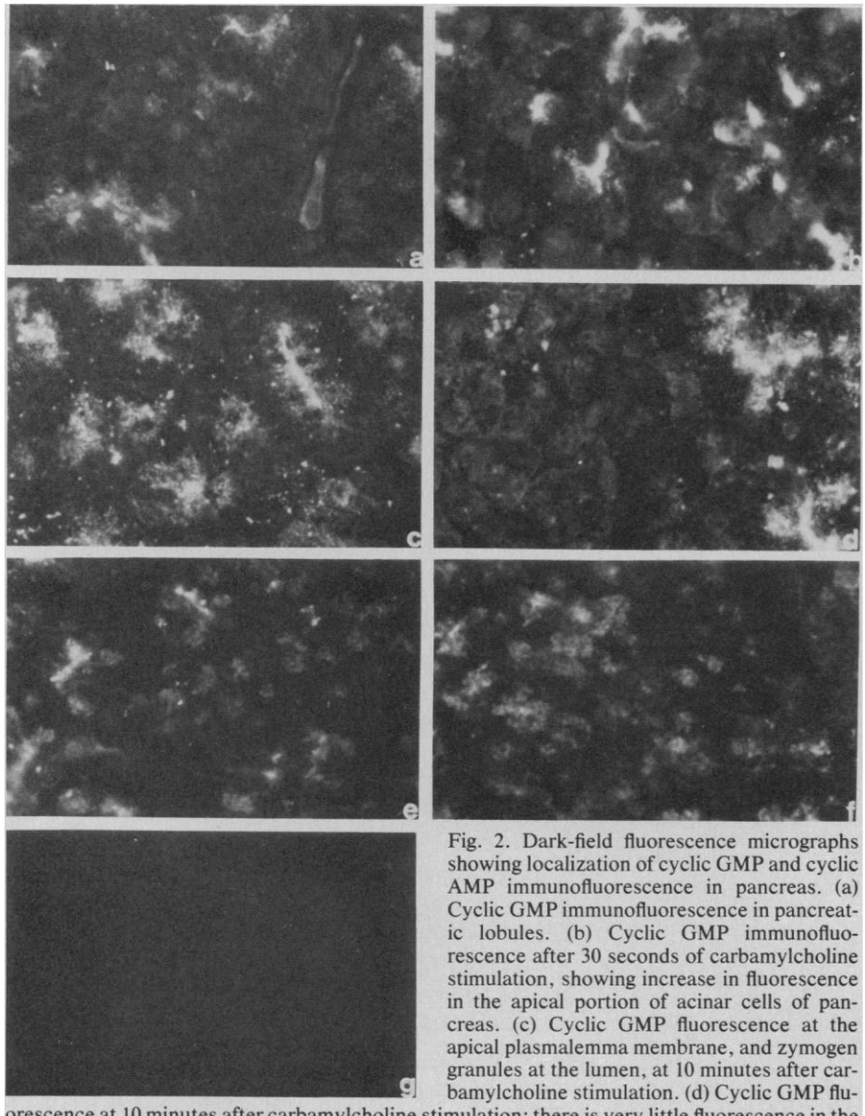

Fig. 2. Dark-field fluorescence micrographs showing localization of cyclic GMP and cyclic AMP immunofluorescence in pancreas. (a) Cyclic GMP immunofluorescence in pancreatic lobules. (b) Cyclic GMP immunofluorescence after 30 seconds of carbamylcholine stimulation, showing increase in fluorescence in the apical portion of acinar cells of pancreas. (c) Cyclic GMP fluorescence at the apical plasmalemma membrane, and zymogen granules at the lumen, at 10 minutes after carbamylcholine stimulation. (d) Cyclic GMP fluorescence at 10 minutes after carbamylcholine stimulation; there is very little fluorescence in the islets of Langerhans but the acinar cells show intense fluorescence. (e) Cyclic AMP immunofluorescence in pancreatic lobules localized diffusely in the apical region of acinar cells and cytoplasm. (f) Cyclic AMP immunofluorescence at 10 minutes afer carbamylcholine stimulation, showing no change in the fluorescence in the acinar cells. (g) Nonspecific fluorescence in a section of pancreas treated with nonimmune serum and the fluorescein-labeled goat antibody against rabbit IgG.

stimulated and carbamylcholine-stimulated acinar cells of the pancreas.

Cyclic GMP in the pancreas appeared to be localized predominantly in the apical plasmalemma membrane and the lumen of the acinar cells (Fig. 2a). After carbamylcholine stimulation, the cyclic GMP immunofluorescence was markedly increased in the apical plasmalemma membrane and in the lumen within 30 seconds (Fig. 2b), and in the lumen and zymogen granules in the apical part of the acinar cells after 10 minutes (Fig. 2c). However, the cells in the islets of Langerhans, which are involved in endocrine secretion of glucagon and insulin, did not show any significant change in cyclic GMP immunofluorescence (Fig. 2d). Concentrations of cyclic AMP in the resting and stimulated pancreatic acinar cells were not altered and cyclic AMP was diffusely localized along the apical portion of plasma membrane and cytoplasm (Fig. 2, e and f). No significant immunofluorescence was observed in pancreatic acinar cells after treatment of the sections with nonimmune serum and fluorescein-labeled goat antiserum to rabbit IgG (Fig. 2g), or with antiserum to cyclic GMP which had been incubated with 5 μM cyclic GMP.

Since this immunocytochemical procedure is performed on unfixed cryostat sections, it seems probable that the free cyclic nucleotide would be lost during the staining procedure and that only the nucleotide bound to cellular compartments would be visible. We have found that, even though most of the cyclic GMP in the medium is in a free form, most of the cyclic GMP in the tissue appears to be bound. Thus, it seems that most of the cyclic GMP present in the tissue is associated with the apical portion of the plasma membrane which gives rise to an intense cyclic GMP immunofluorescence in the lumen during exocytosis of zymogen granules.

Ong *et al.* (*6*) reported that cyclic GMP was present on the nuclear element and plasma membranes of a number of cells in liver, small intestinal brush border, thyroid, and testis. These authors suggested that this nucleotide may serve a regulatory function in nuclear directed events and membrane functions. Our observation of cyclic GMP in the lumen, the apical plasmalemma membrane, and the granules of pancreatic acinar cells suggests another role for this nucleotide in the secretion of enzymes.

The mechanism by which cyclic GMP is released is not known, but we postulate that it may be released by exocytosis as are the pancreatic enzymes. In support of this hypothesis is the finding that the zymogen granules contain a high concentration of cyclic GMP, and the release of the cyclic GMP parallels the release of the amylase after stimulation with carbamylcholine and other agents. Even though more than 90 percent of cyclic GMP in the medium is not associated with proteins, most of this nucleotide is apparently associated with macromolecules in the cells. In contrast, cyclic AMP is not released into the medium. It appears that cyclic GMP becomes dissociated during or after secretion into the medium. This dissociation may be brought about by differences in the ionic composition between the medium and the cytoplasm of the cells. The role of the cyclic GMP present in the pancreatic secretion is not clear. Whether it is involved in the activation of enzymes after they are released requires further investigation.

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Duchenne Dystrophy: Alteration in Muscle Plasma Membrane Structure

Abstract. *Freeze fracture studies of skeletal muscle from eight patients with Duchenne dystrophy showed nonuniform distribution and depletion of particles on both protoplasmic and extracellular faces of the muscle plasma membrane. The findings support the view that the muscle surface membrane is abnormal in this disorder and indicate that alterations are present in the internal molecular architecture of the membrane.*

Biochemical studies have suggested a muscle cell surface membrane abnormality in Duchenne muscular dystrophy (*1*). Moreover, electron microscopic studies have shown focal defects of the muscle cell surface membrane in nonnecrotic fibers (*2, 3*), and electron cytochemical studies have demonstrated peroxidase permeation through the defects (*3*). It has been suggested that this defect is an early and possibly basic one (*3*).

Table 1. Particle distribution per square micrometer on plasma membranes. Values are mean and standard deviation for each subject, except the values in italics, which are group means and standard errors of the means.

P face		E face	
Duchenne	Control	Duchenne	Control
112 ± 11	257 ± 6	44 ± 8	159 ± 7
123 ± 43	284 ± 11	89 ± 40	159 ± 7
129 ± 18	275 ± 20	74 ± 13	155 ± 11
93 ± 45	239 ± 43	50 ± 24	133 ± 12
123 ± 50	262 ± 18	31 ± 17	151 ± 10
134 ± 25		36 ± 20	
50 ± 15		23 ± 12	
113 ± 9		17 ± 8	
*110 ± 27**	*262 ± 18*	*45 ± 9**	*151 ± 4.3*

*$P < .001$ (Duchenne value versus corresponding control value).

The freeze fracture technique provides the microscopist with the opportunity to visualize and quantitate fine details of membrane ultrastructure. The work of Branton and colleagues has demonstrated that the fracture preferentially passes along the midline of the membrane through the hydrophobic phospholipid interior. Both fracture faces are covered with particles, and it is now accepted that these particles are proteins and structural components of the membrane (*4*). Utilizing this technique we report widespread alterations in the internal molecular architecture of the muscle plasma membrane in Duchenne muscular dystrophy.

Quadriceps muscle plasma mem-