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Structural and Functional Characterization of Isolated Pancreatic Exocrine Cells

(tissue dissociation/collagenase/hyaluronidase/protein synthesis/secretogogue response)

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ABSTRACT **Viable isolated exocrine cells have been obtained from guinea-pig pancreas by a tissue dissociation procedure using crude collagenase (EC 3.4.4.19) and hyaluronidase (EC 3.2.1.35), chelation of divalent cations, and mild shearing forces. Cell yields are 50–60%, based on recovered DNA, and about 90% of the population consists of exocrine cells which, although rounded up, retain their *in situ* polarity with regard to regional distribution of zymogen granules and specialization of the former luminal plasmalemma. The isolated cells incorporate labeled amino acids into proteins at linear rates for at least 4 hr at levels comparable to pancreatic slices *in vitro*; more than 95% of the exocrine cell population is active in this process, as shown by autoradiography. In response to secretogogues at optimal doses (100 μM carbamylcholine; 0.1 μM pancreozymin, or 0.01 μM caerulein), the cells discharge up to 30% of their content of pulse-labeled secretory proteins to the medium over a 3-hr period; in the same time, the controls release about 5% of their content. The results indicate that isolated exocrine cells are capable of synthesizing the processing secretory proteins, and of responding directly to cholinergic and peptidic secretogogues.**

Recent studies in our laboratory on the synthesis, intracellular transport, and release of secretory proteins have been performed with guinea-pig pancreatic slices or parotid lobules incubated *in vitro* at 37° (1, 2). Since certain aspects of our work require an analysis of the secretory process at the level of individual cells, we have developed a tissue dissociation procedure with which it is possible to isolate single exocrine cells from the guinea-pig pancreas (3). For this purpose, three requirements had to be met. The technique should (*a*) produce a high yield of viable cells stable to subsequent manipulations; (*b*) be simple and reproducible, and (*c*) avoid prolonged exposure to protease activity, common to many tissue dissociation procedures. This last requirement is particularly important if hormone receptors on the cell surface are to undergo minimal damage (4, 5).

With these requirements in mind, we have developed a tissue dissociation procedure by use of sequential crude collagenase (EC 3.4.4.19) and hyaluronidase (EC 3.2.1.35) digestion, chelation of divalent cations, and mild shearing forces. The starting point was the procedure used by Howard *et al.* (6) and Berry and Friend (7) to obtain viable, single hepatic-parenchymal cells. Since all of the steps in our procedure are potentially harmful, each was optimized to produce maximal cell yields with minimal damage. Cell integrity was assessed by morphology and by determination of their ability to synthesize, transport, and release secretory proteins in response to hormonal stimulation.

MATERIALS AND METHODS

Reagents were obtained from the following sources: Crude collagenase (*Clostridium histolyticum;* EC 3.4.4.19), 137–200 U/mg, chromatographically purified collagenase, 250–600 U/mg, crude bovine-testis hyaluronidase (EC 3.2.1.35) 467 U.S.P. U/mg, and chromatographically purified soybean trypsin inhibitor, Worthington Biochemical Corp., Freehold, N.J.; L-[4,5-^{3}H]leucine, 40 Ci/mmol, L-[*U*-^{14}C]leucine, 270–320 Ci/mol, and carbamylcholine·HCl, Schwarz–Mann, Orangeburg, N.Y.; bovine-plasma albumin, fraction V, Miles Laboratories Inc., Kankakee, Ill.; Azocoll, Calbiochem., Los Angeles, Calif.; Pancreozymin, 6000 Crick, Harper, and Raper U/mg, and synthetic caerulein were the kind gifts of Dr. E. Jorpes, Karolinska Institute, Stockholm, and Prof. B. Camerino, Farmitalia, Milano, respectively. Male albino guinea pigs, 350–400 g (8 to 9 weeks old), were obtained from the Rockefeller University colony. They were starved overnight before use and killed by a blow to the head.

Dissociation and Incubation Media. For all steps in the procedure, we used a Krebs–Ringer bicarbonate solution equilibrated with 95% O_2 and 5% CO_2 (to pH 7.4) and containing 14 mM glucose, a complete L-aminoacid supplement (1), and 0.1 mg of soybean trypsin inhibitor per ml, which was added to prevent tryptic activation of endogenous zymogens; it does not affect the protease activity in crude collagenase. $MgCl_2$ and $CaCl_2$ concentrations were adjusted as required and are indicated in the text. Crude collagenase or bovine-plasma albumin were dissolved in and dialyzed at 4° against Krebs–Ringer bicarbonate solution containing the appropriate concentrations of divalent cations, and centrifuged at 1000 × *g*

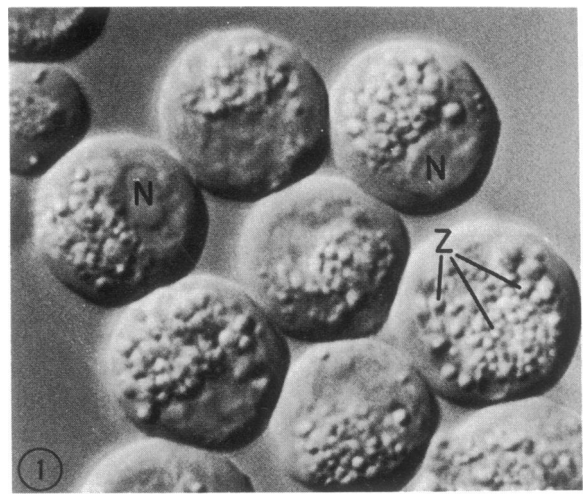

FIG. 1. Differential interference-contrast micrograph of living pancreatic exocrine cells. *N*, nucleus; *Z*, zymogen granules. ×1600.

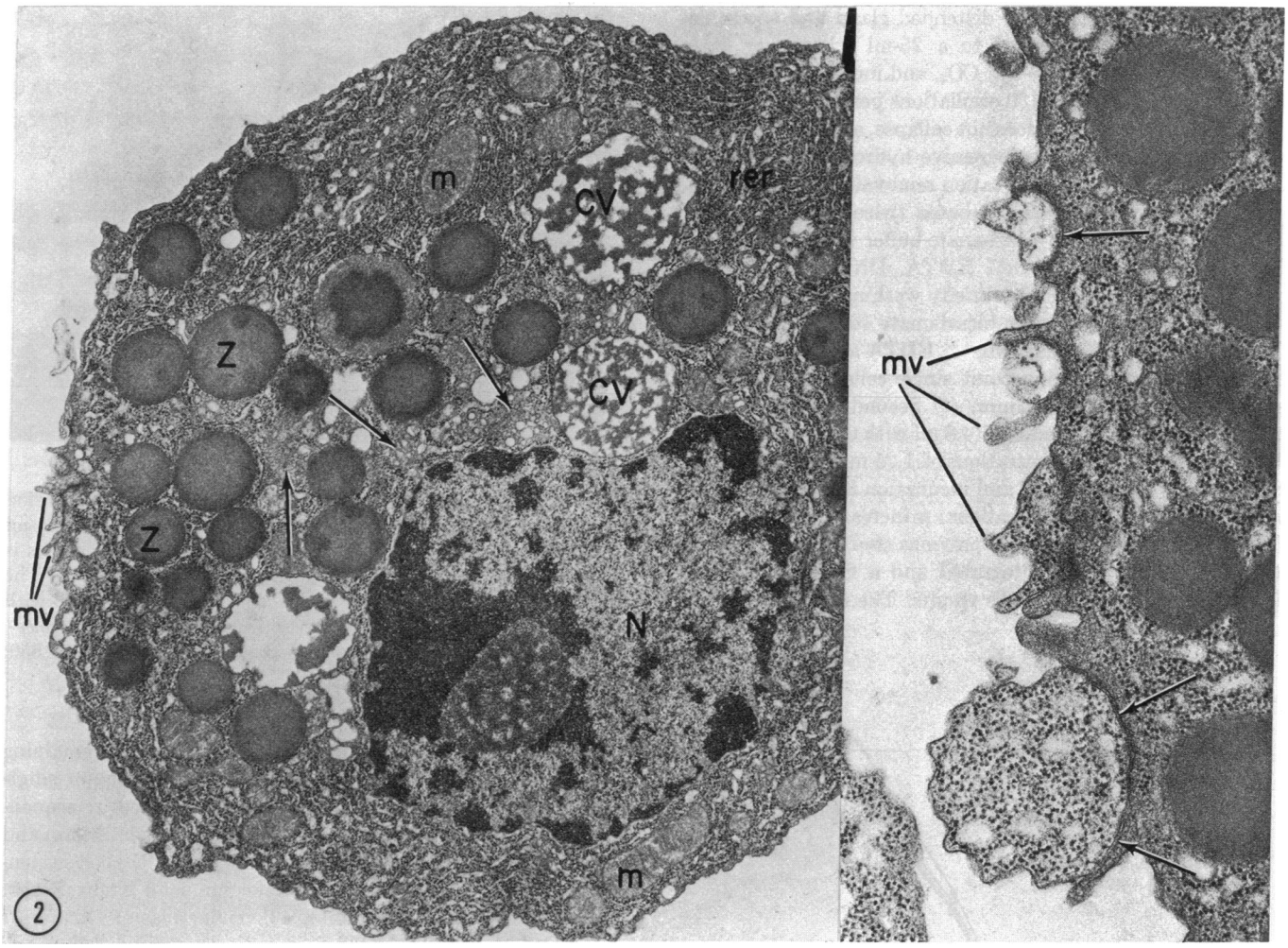

FIG. 2. Electron micrograph of a thin section of a pellet of isolated cells. The former apical pole of the cell is identifiable by a patch of plasmalemma provided with microvilli (*mv*). *N*, nucleus; *m*, mitochondria; *rer*, cisternae of the rough endoplasmic reticulum; *Z*, zymogen granules; *CV*, condensing vacuoles. *Arrows* denote elements of the Golgi complex. *Insert:* Higher-magnification view of the former luminal plasmalemma. Note microvilli (*mv*) and remnants of tight junctions associated with cytoplasmic blebs (*arrows*). ×10,000; *insert* ×26,000.

for 15 min to remove particulate matter. Pure collagenase, hyaluronidase, and soybean trypsin inhibitor were added as salt-free powders. Media used for incubation of cells after dissociation contained 100 U of potassium penicillin G per ml and 50 μg of streptomycin sulfate per ml. All glassware that the tissue and cells contacted was siliconized.

Analytical Procedures. Cell suspensions, cell pellets, or supernatants were precipitated and washed three times with 0.5 N $HClO_4$ at 4°. The precipitates were dissolved in 0.4 ml of 88% formic acid before liquid scintillation counting as in ref. 1. ^3H and ^{14}C counting rates were corrected for quenching by use of an external standard. DNA (8), amylase activity (9, 10), and general protease activity against Azocoll (11) and casein (12) were determined by the indicated procedures.

Microscopy. Cells were fixed in suspension for light and electron microscopy with 1% OsO_4 in 0.1 M Na cacodylate (pH 7.4) containing 2.5 mM $CaCl_2$ and 1.2 mM $MgCl_2$. Before embedment in Epon, cells were stained with 0.5% Mg uranyl acetate in 0.9% NaCl. Specimens for autoradiography were not treated with uranyl acetate. For electron microscopy, the sections were doubly stained with uranyl acetate and lead citrate; for light microscopy, 0.5-μm sections were stained with 1% methylene blue in 1% borate. Light microscopy autoradiograms were prepared as described in ref. 13.

RESULTS

Dissociation technique

Dissociation of the tissue involves two sequential digestions with collagenase–hyaluronidase, with an interposed chelation of divalent cations by EDTA, and is completed by application of mild shearing forces produced by gentle pipetting. The progress of the dissociation is monitored systematically by phase- and differential interference-contrast microscopy. The steps in the procedure are as follows: (*a*) First digestion: The pancreas is trimmed free of fat and mesentery, and 4.8 ml of the enzyme mixture (0.75 mg of crude collagenase per ml and 1.5 mg of hyaluronidase per ml in Krebs–Ringer bicarbonate solution containing 0.1 mM Ca^{++} and 1.2 mM Mg^{++}) is inoculated at room temperature (23°) into the interstitium of 0.8 g of tissue so as to distend the gland and rapidly expose the majority of its

lobules to the enzymes. The distended gland and excess enzyme solution is transferred to a 25-ml erlenmeyer flask, equilibrated with 95% O_2–5% CO_2, and incubated at 37° for 15 min with agitation at 130 oscillations per min. During this time, the tissue structure does not collapse, although the gland loosens, probably due to progressive hydrolysis of the intercellular matrix. (*b*) Divalent cation removal and replacement: the loosened tissue is next incubated twice for 5 min at 37° in 8 ml of Krebs–Ringer bicarbonate buffer without Ca^{++} and Mg^{++} and containing 2 mM EDTA. Divalent cations are subsequently added back by briefly washing the tissue twice with 8 ml of Krebs–Ringer bicarbonate solution containing 0.1 mM Ca^{++} and 1.2 mM Mg^{++}. EDTA appears to initiate dissociation, since at this point single cells begin to appear spontaneously in the medium. (*c*) Second digestion: Fresh collagenase and hyaluronidase (4.8 ml with 0.1 mM Ca^{++} and 1.2 mM Mg^{++}) at concentrations of 1.25 mg/ml and 2.0 mg/ml, respectively, are added and incubation is continued 45–55 min at 37° (enzyme concentrations are increased to compensate for fluid carried over from the previous step). At this time the tissue structure has disintegrated and a fine suspension of lobules and many single cells results. The end point is indicated by the free movement of cells within the remaining tissue pieces. (*d*) Final dissociation and harvesting of single cells: after step *c*, single cells are readily liberated by sequential passage through pipets with tip diameters of 1.3 mm and 0.9 mm (5 times in each). Duplicate aliquots of the cell suspension are then layered over two 8-ml columns of Krebs–Ringer bicarbonate solution containing 4% bovine-plasma albumin, 1 mM Ca^{++}, and 1.2 mM Mg^{++} in conical centrifuge tubes, and centrifuged at 50 × *g* for 5 min to form pellets of packed cells. This step rapidly and quantitatively separates the cells from the enzyme mix and cell debris (which remain in the supernatant) and places them in a protective environment. After two further washes of the pooled cells in 8 ml of the above solution, they are suspended in Krebs–Ringer bicarbonate buffer containing 1% bovine-plasma albumin*, 2.5 mM Ca^{++}, and 1.2 mM Mg^{++}. The effectiveness of the wash in removal of collagenase is indicated by the absence of activity against Azocoll in the final cell suspension. All subsequent incubations are performed at 37° with agitation at 60 oscillations per min.

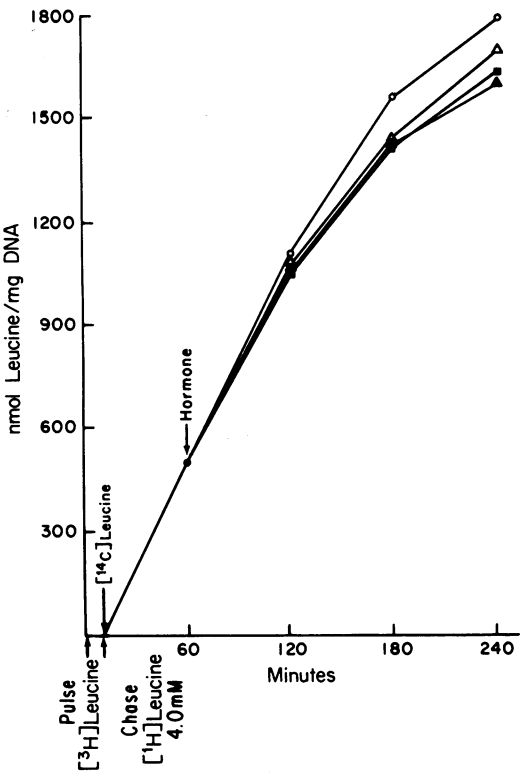

FIG. 3. Incorporation rates of isolated cells. After pulse labeling with [^{3}H]leucine preparatory to monitoring hormone responsiveness (same experiment as in Fig. 5), cell suspensions were incubated for 4 hr in chase medium containing 0.3 μCi of [^{14}C]-leucine per ml and 4.0 mM unlabeled L-leucine. At specified times, aliquots of the suspensions were treated as in Fig. 5, and the incorporation of [^{14}C]leucine into acid-precipitable proteins of cells plus proteins released to the medium was measured and related to cell DNA. Hormone addition at 60 min pertains to the discharge assay shown in Fig. 5. Representative of four identical experiments. O——O, control; Δ——Δ, 10 μM carbamylcholine Cl; ■——■, 0.1 μM pancreozymin; ▲——▲, 0.01 μM caerulein.

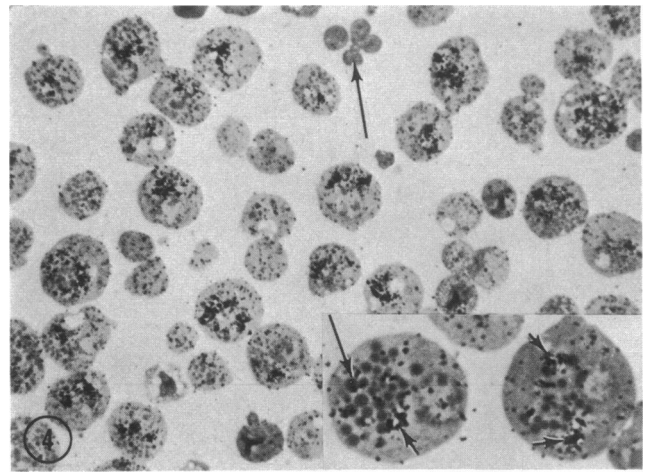

FIG. 4. Light-microscope autoradiogram of a cell suspension incubated continuously for 50 min with [^{3}H]leucine (40 μCi/ml plus 0.4 mM [^{1}H]leucine). The majority of the exocrine cells is labeled; nonexocrine cells (*arrow*) are very lightly labeled. The *insert* shows the generalized labeling of the cytoplasm with regional concentrations of label over the Golgi zone (*short arrows*). Some label appears over zymogen granules (*long arrow*). ×650; *insert* ×1300.

Yield and Morphology of Single Cells. The yield of washed cells is 50–60%, based on recovered DNA. Cell counts on a representative sample of the population in Epon thick sections (e.g., Fig. 2) showed that about 90% are exocrine cells, the remainder consisting primarily of endocrine cells and a few duct cells. Differential interference-contrast microscopy of living exocrine cells (Fig. 1) shows that although the cells are rounded up, they retain their *in situ* polarity as judged by the regional concentration of zymogen granules in one sector of the cytoplasm that is opposite to that occupied by the nucleus. While the isolated cells occasionally aggregate, the contact points are random on the cell circumference, i.e., no acini are reconstituted.

* Bovine-plasma albumin is essential in order to prevent membrane blebbing and adherence of cells to the glassware.

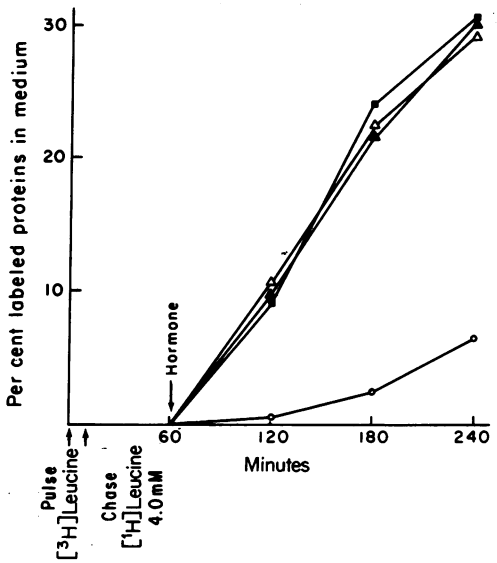

FIG. 5. Discharge rates of isolated cells. Cell suspensions obtained from 0.8 g of tissue (about 2.5 mg of cell DNA) were pulse labeled for 10 min at 37° in 5 ml Krebs–Ringer bicarbonate solution containing 40 μCi of [^{3}H]leucine per ml and 0.4 mM [^{1}H]-leucine. Chase incubation was initiated by dilution of the cells with 6 volumes of Krebs–Ringer bicarbonate solution containing 4.0 mM [^{1}H]leucine [and [^{14}C]leucine (see Fig. 3)]. After 50 min of chase, samples of cells were distributed equally into 12 flasks, some of which contained hormones. At the indicated times, 0.7-ml aliquots were removed, cells and media were rapidly separated by centrifugation (50 × *g* for 5 min), and the medium was collected with a Pasteur pipet. Media and cells were precipitated and washed with cold $HClO_4$ and the percent of ^{3}H-labeled proteins released to the medium was measurd [% secretion = (labeled proteins in medium per sum labeled proteins in medium plus cells) × 100]. Discharge data were corrected for labeled proteins released during the prestimulation chase period (about 2%). ■——■, 0.1 μM pancreozymin; ▲——▲, 0.01 μM caerulein; △——△, 100 μM carbamylcholine; ○——○, control. Representative of four identical experiments.

Electron microscopy of thin sections of fixed and embedded pellets of cell suspensions shows that the limiting plasmalemma retains its *in situ* specialization and polarization. The former luminal plasmalemma, which is identifiable by its microvilli, overlies the cytoplasmic zone containing the granule population (Fig. 2). This portion of the cell surface is frequently delineated laterally by remnants of tight junctions, some of which are attached to cytoplasmic blebs derived from neighboring cells. The remainder of the cell periphery is bounded by a unit membrane devoid of adherent basement membrane. Other cell organelles characteristic of exocrine cells also retain their *in situ* polarity and are well preserved, but an apparent increase in the number of condensing vacuoles is frequently detected.

Functional Studies. Integrity of function of the isolated cells was assessed by measurement of the rate of incorporation of labeled amino acids into protein and by testing for secretogogue responsiveness. For this purpose, double-label experiments of the type shown in Figs. 3 and 5 were performed. Cell suspensions were first pulse-labeled with [^{3}H]leucine for 10 min, following which they were diluted into medium containing [^{14}C]leucine and a 10-fold excess of [^{1}H]leucine (4.0 mM) to inhibit further incorporation of ^{3}H (chase medium). The suspension was then incubated for a further 50 min in the chase medium to allow intracellular transport of ^{3}H-labeled proteins to proceed. At this time, secretogogues were added to samples of cells, and incubation was continued for 3 hr. At selected times, cells and medium were separated by centrifugation and the % of [^{3}H]proteins discharged to the medium was measured. [^{14}C]Leucine in the chase medium made possible the monitoring of protein synthesis in the same samples over the 4-hr period. As can be seen (Fig. 4), the majority of the label incorporated is localized to exocrine cells. Consequently, the radioactivity data pertain mainly to this cell type.

As shown in Fig. 3, the isolated cells incorporate [^{14}C]leucine into proteins at a linear rate for the first 3 hr, the rate diminishing somewhat over the last hour. The amount of leucine incorporated is about double that previously obtained with guinea-pig pancreatic slices (10). Maximal doses of carbamylcholine and pancreozymin slightly inhibit incorporation, as already noted with the pancreatic slices.

Separate autoradiographic experiments in which cell suspensions were labeled continuously with [^{3}H]leucine for 50 min showed that about 95% of the exocrine cells are marked by high concentrations of autoradiographic grains and, hence, are active in protein synthesis (Fig. 4). Although the grains are, as expected, distributed over the cytoplasmic area occupied by the endoplasmic reticulum, the majority is localized to the Golgi region, with some over zymogen granules, indicating that the isolated exocrine cells are capable of transporting and concentrating secretory proteins. Nonexocrine cells incorporate little label.

The discharge pattern if the isolated cells is shown in Fig. 5. With optimal concentrations of pancreozymin (0.1 μM), caerulein (0.01 μM), and carbamylcholine (100 μM), dis-

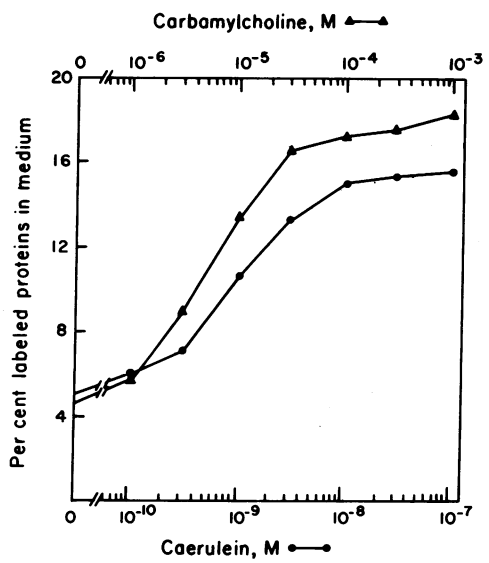

FIG. 6. Dose response of isolated cells to carbamylcholine and caerulein. Discharge assay as in Fig. 5. The data represent percent labeled proteins in the medium after 3 hr of stimulation. Separate batches of cells, obtained with the same batch of crude collagenase, were used for each curve.

charge is approximately linear over 3 hr, at which time about 30% of the pulse-labeled proteins appear in the medium†. Over the same time, about 5% of the label from controls is discharged. Discharge of amylase parallels that of radioactive proteins.

Caerulein and carbamylcholine both produce sigmoidal log-dose–response curves (Fig. 6). The concentrations of secretogogues eliciting optimal responses in the single cells are, however, about 10 times greater than those required for maximal responses in pancreatic slices.

Collagenase batches with higher protease activity (up to twice) than usual gave cells with proportionately lower responses to secretogogues (down to 65%), without affecting cell yields and rates of protein synthesis. The point is exemplified by a comparison of Figs. 5 and 6. The maximal discharge responses shown in Fig. 6 are about 40% lower than those obtained in Fig. 5; this is most likely due to the 20% higher protease (i.e., caseinase) activity in the collagenase batch used to prepare the cells.

DISCUSSION

Although our procedure for dissociation of a pancreas into single cells was developed empirically, certain aspects of it are well defined. Successful dissociation requires a minimal level of proteolytic activity supplied by the proteases present in crude collagenase, since pure collagenase and hyaluronidase alone, even at double activities, are not effective. Dissociation is, however, possible if a protease such as chymotrypsin is used in conjunction with pure collagenase and hyaluronidase (unpublished observations). That the proteolytic activity in our procedure is supplied only by crude collagenase is indicated by the fact that no increases in activity against Azocoll above that present in the initial enzyme mixture can be detected over the course of dissociation; this also demonstrates that soybean trypsin inhibitor prevents activation of endogenous proproteases liberated from damaged cells. Omission of soybean trypsin inhibitor results in substantial increases in total proteolytic activity by the end of the procedure. Finally, decreasing the time of incubation with the standard enzyme concentrations leads to inefficient dissociation, while increasing the concentration of crude collagenase 5-fold results in uncontrollable dissociation and eventual cell death.

Although neither hyaluronidase nor EDTA are obligatory for our dissociation procedure, both facilitate it. Thus, hyaluronidase reduces the pipetting forces required at the last step and leads to a less cohesive cell suspension (14). For economy, crude hyaluronidase was used throughout, although pure hyaluronidase is also effective. Both types of hyaluronidase are devoid of detectable proteolytic activity. If EDTA is reduced to 0.2 mM or omitted, longer digestion and stronger shearing forces are needed to achieve dissociation, which is obtained at the price of reduced yield. Introducing the chelator at the end of the procedure results in unsatisfactory cell preservation. The efficacy of EDTA in promoting dissociation is most likely related to the role of divalent cations in maintaining cell contact, although tight and gap junctions are unaffected by the chelator (e.g., Fig. 2) (7, 15).

The isolated exocrine cells, although rounded up, maintain the main structural features found in the intact tissue. Of interest is the retention of regional specialization of that part of the bounding plasmalemma that represents the former luminal surface *in situ*. Whereas in the intact acinus the luminal surface is separated physically from the rest of the plasmalemma by junctional complexes rimming the cell circumference and connecting neighboring cells, in the isolated cells these contact points are lost or reduced to remnants of tight junctions in which cytoplasmic fragments from adjacent cells are involved. Since this topical specialization is retained for several hours in the isolated cells, it is evident that the apical surface is separated physically from the remainder of the plasmalemma, for otherwise lateral diffusion and intermixing of membrane components should rapidly obliterate its morphologic identity.

The isolated cells maintain the main functional characteristics of the intact exocrine tissue *in vitro*. Aminoacid incorporation rates—in cells—exceed those of guinea-pig pancreatic slices (10), due in part to better accessibility to precursors. The secretory rate of the isolated cells, however, is only 30–50% that of the intact tissue *in vitro* (10). Although the reason for this difference is not clear, it may be related to destruction or decrease in affinity of hormone receptors that occurs during dissociation, as suggested by the lower secretory responses found in cells prepared with batches of collagenase containing higher protease activity. Other causes may include defects in intracellular transport and discharge, or inadequate substrate concentration to support these activities at optimal rates.

Finally, the data indicate that the receptors for the two main classes of pancreatic secretogogues (cholinergic agents and peptide hormones) are directly associated with the exocrine cells. Since the entire plasmalemma of the isolated cells is exposed and available for study, this system should lend itself to a detailed examination of the topographic distribution of hormone receptors on cell surfaces.

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† No enhancement of secretory response was obtained with combined optimal doses of carbamylcholine and caerulein.