

Stimulus–Secretion Coupling in the Developing Exocrine Pancreas: Secretory Responsiveness to Cholecystokinin

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Abstract. We have studied the onset of secretory responsiveness to cholecystokinin (CCK) during development of the rat exocrine pancreas. Although acinar cells of the fetal pancreas (1 d before birth) are filled with zymogen granules containing the secretory protein, α -amylase, the rate of amylase secretion from pancreatic lobules incubated *in vitro* was not increased in response to CCK. In contrast, the rate of CCK-stimulated amylase discharge from the neonatal pancreas (1 d after birth) was increased four- to eightfold above that of the fetal gland. The postnatal amplification of secretory responsiveness was not associated with an increase in the number or cell surface expression of ^{125}I -CCK binding sites. When ^{125}I -CCK-33 binding proteins were analyzed by affinity cross-linking, two proteins of M_r 210,000 and 100,000–160,000 were labeled specifically in both fetal and neonatal pancreas. To determine if cell surface receptors for CCK in the fetal pancreas are functional and able to generate a rise in the cytosolic $[\text{Ca}^{++}]$, we measured $^{45}\text{Ca}^{++}$ efflux from tracer-loaded lobules.

$^{45}\text{Ca}^{++}$ efflux from both fetal and neonatal pancreas was comparably increased by CCK, indicating CCK-induced Ca^{++} mobilization and elevated cytosolic $[\text{Ca}^{++}]$. The Ca^{++} ionophore A23187 also stimulated the rate of $^{45}\text{Ca}^{++}$ extrusion from pancreas of both ages. Increased amylase secretion occurred concurrently with A23187-stimulated $^{45}\text{Ca}^{++}$ efflux in neonatal pancreas, but not in the fetal gland. A23187 in combination with dibutyryl cAMP potentiated amylase release from the neonatal gland, but not from fetal pancreas. Similarly, the protein kinase C activator, phorbol dibutyrate, did not increase the rate of secretion from the fetal gland when added alone or in combination with A23187 or CCK. We suggest that CCK–receptor interaction in the fetal pancreas triggers intracellular Ca^{++} mobilization. However, one or more signal transduction events distal to Ca^{++} mobilization have not yet matured. The onset of secretory response to CCK that occurs postnatally may depend on amplification of these transduction events.

THE polypeptide cholecystokinin (CCK)¹ serves as a major physiologic secretagogue for the exocrine pancreas. This hormone has been shown to interact specifically with a sialoglycoprotein of M_r 85,000 that is localized on the basolateral plasma membrane of the acinar cell of the adult rat pancreas (41, 42). Consequent to CCK binding to its receptor, a cascade of molecular events occurs that culminates in the exocytotic release of secretory granule content. These signal transduction events are not well understood, but include increased degradation of phosphatidylinositol, generation of diacylglycerol (21, 36, 52), and a transient rise in the cytosolic Ca^{++} concentration ($[\text{Ca}^{++}]_c$) (35, 39, 49). The Ca^{++} mobilized during hormone stimulation appears to initially come from an intracellular store, i.e., the trigger pool, since the early phase of secretion can

occur in the absence of extracellular Ca^{++} (14, 48, 49). Net efflux of Ca^{++} from internal pools occurs during the initial phase of stimulated protein discharge; sustained secretion, however, is dependent on extracellular Ca^{++} , and net influx of Ca^{++} occurs during this period (14, 48). Recent evidence suggests that the sustained phase of stimulated secretion is mediated by the activity of the Ca^{++} /phospholipid-dependent protein kinase C (C-kinase) (33, 35, 40). We have studied the mechanism of stimulus–secretion coupling by analyzing the onset of CCK responsiveness during rat pancreatic development.

Morphogenesis and cytodifferentiation of the fetal pancreas are well-characterized processes. By day 19 of gestation in the rat (i.e., 3 d before birth), the acinar cells contain the mature complement of intracellular organelles (38). By day 21 of gestation (1 d before birth), a complete set of secretory proteins destined for export are synthesized, transported, and packaged into zymogen granules in a manner similar to that of the adult gland (Arvan, P., and A. Chang, manuscript in preparation; references 43 and 54). At this time, the acinar cell cytoplasm is filled with accumulated

1. *Abbreviations used in this paper:* $[\text{Ca}^{++}]_c$, cytosolic free calcium concentration; CCK, cholecystokinin; CCK-8, CCK COOH-terminal octapeptide; CCK-33, CCK triacontatriapeptide; C-kinase, protein kinase C; dbcAMP, dibutyryl cyclic AMP; KRH, Krebs Ringer's Hepes buffer; MBS, m-maleimidobenzoyl N-hydroxysuccinimide ester; MEM, Eagle's minimum essential medium; STI, soybean trypsin inhibitor.

zymogen granules (38), and secretory proteins are released basally *in vitro* (10, 55). However, the expression of a mature cell surface phenotype (as measured by lectin binding) does not begin to occur until day 21 of gestation (30). Furthermore, treatment of pancreatic organ cultures with the thymidine analog, 5-bromodeoxyuridine, dissociates cell surface glycoconjugate differentiation from cytodifferentiation (31). Such observations suggest that the maturation of plasma membrane proteins (perhaps including those involved in CCK action) may not be tightly coupled with the development of the secretory apparatus. Indeed, it has been observed previously that protein discharge in response to caerulein, a CCK analog, is low in the fetal pancreas (10); significant secretory response to stimulation occurs only after birth (28, 55).

In the present study we show that the cell surface expression of CCK binding proteins in the fetal pancreas is temporally distinct from the postnatal development of secretory responsiveness to CCK. One possible explanation for the increased secretory responsiveness of the neonatal pancreas to CCK compared with that of the fetal gland is a more efficient or effective coupling of hormone binding to the generation of the intracellular second messengers, $[Ca^{++}]_c$ and diacylglycerol. A similar mechanism for increased hormone sensitivity during development has been previously described in several systems in which the maturation of cellular responsiveness involves a more efficient or effective coupling of receptor occupancy to adenylate cyclase (and generation of the second messenger, cAMP) (24, 27). An alternative explanation for the amplified secretory response in the neonatal pancreas is the maturation of transduction events distal to the mobilization of Ca^{++} and/or C-kinase activation. To distinguish between these possibilities, we have examined Ca^{++} mobilization in response to CCK during pancreatic development. In addition, we investigated the sensitivity of the secretory machinery in developing pancreas to an elevation in $[Ca^{++}]_c$ produced by addition of a divalent cation ionophore, and/or to activation of C-kinase by phorbol dibutyrate.

Our data show that CCK binding to the fetal pancreas 1 d before birth results in Ca^{++} mobilization; however, the gland appears unresponsive to the second messenger generated by either the hormone, or the Ca^{++} ionophore A23187. Furthermore, combinations of A23187 with phorbol dibutyrate, or the cAMP derivative, dibutyryl cAMP (dbcAMP) did not stimulate the rate of amylase secretion in the fetal gland, in contrast to the effects observed in neonatal and adult pancreas.

Preliminary accounts of this work have been presented (7, 8).

Materials and Methods

Preparation of Tissue

Sprague-Dawley rats were obtained from Camm Research Lab Animals (Wayne, NJ) and allowed to feed or nurse freely. Pancreata were dissected from adult rats, defined as male animals weighing 125–150 g (~2-mo-old). Neonatal pancreata were dissected and pooled from one or more litters of rats ~1-d-old. Fetal rats at day 21 of gestation were removed from one or more mothers with timed pregnancies, and their pancreata dissected and pooled. The dissected glands were placed in cold, oxygenated Eagle's minimum essential medium (MEM) with Hank's salts, buffered with Hepes (25

mM), pH 7.4, and trimmed of connective tissue and/or mesenchyme under a binocular microscope.

Amylase Discharge Assay

Pancreatic lobules were isolated by dissection with fine scissors. In order to wash away debris spilled into the medium from cells damaged during lobule preparation, the tissue was preincubated in ~10 ml oxygenated MEM containing 0.01% (wt/vol) soybean trypsin inhibitor (STI), 0.1% wt/vol BSA for ~30 min at 37°C, and several changes of the medium were made. To initiate the secretion assay, 2 ml oxygenated MEM was added to six lobules (pooled from several fetal or neonatal glands, or dissected from random regions of the adult pancreas) in the presence or absence of a range of CCK COOH-terminal octapeptide (CCK-8) concentrations. All tubes were incubated for 2 h in a 37°C water bath shaking at 120 oscillations/min. During the course of the experiment, the samples were gassed with 100% O_2 at 30-min intervals. Aliquots of medium (100 μ l) were removed every 30 min and replaced with an equal volume of fresh medium containing the appropriate dose of hormone. At the end of 2 h, lobules were rinsed rapidly with MEM before being either sonicated or homogenized in 2 ml of 0.02% vol/vol Triton X-100, 20 mM NaCl, 10 mM Na phosphate, pH 6.9. Samples were frozen at -20°C before assaying for amylase activity according to the method of Bernfeld (2). Using linear regression analysis, we calculated the rates of amylase discharge (percent of total amylase released per minute) for each experiment.

Radioiodination of CCK

CCK-8, the most potent form of the hormone in stimulating pancreatic secretion (23), was used in the amylase discharge and $^{45}Ca^{++}$ efflux experiments, and ^{125}I -CCK-8 was used in the binding experiments. Since at least one free amino group on radiolabeled CCK triacontatriptide (CCK-33) is available for reaction with NH_2 -reactive cross-linking reagents, ^{125}I -CCK-33 was used for the affinity labeling and autoradiography studies. CCK-33 was acylated with ^{125}I -labeled Bolton-Hunter reagent, as described previously (41). ^{125}I -CCK-33 prepared by this method has a specific activity of ~500 Ci/mmol, and has been shown to retain full biologic activity (44).

Preparation of Pancreatic Membranes

Total membranes were prepared by homogenizing the pancreas using 10 up-down strokes with a Brendler Teflon pestle homogenizer (0.08–0.15 mm clearance) driven at 2,200 rpm in 10 times volume per tissue wet weight of Krebs-Ringer Hepes (KRH) buffer diluted fourfold with distilled H_2O . (KRH: 103 mM NaCl, 4.78 mM KCl, 1.16 mM KH_2PO_4 , 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.2% wt/vol BSA, 25 mM Hepes, pH 7.4.) Diluted KRH buffer (final Mg^{++} concentration, 0.25 mM) was used as the homogenization medium to minimize the aggregation of membranes that occurs in the presence of a higher Mg^{++} concentration (32). Included in the buffer were the protease inhibitors, aprotinin (10 U/ml), leupeptin (10 μ g/ml), pepstatin (4 μ g/ml), bacitracin (100 μ g/ml), and 0.01% STI. All manipulations were done at 4°C. Homogenates were centrifuged at 600 rpm for 5 min in an IEC PR6000 (International Equipment Company, Needham Heights, MA) to pellet any unbroken cells. The supernatant was collected, and the pellet was rehomogenized in 1 ml of buffer using five up-down strokes with the Brendler homogenizer. Supernatant and second homogenate were combined, filtered through two layers of gauze, and aliquots were removed for DNA assays by the Burton method (4). DNA in the remaining homogenates was then digested by incubation with 2 mg purified DNase per 4 g wet tissue weight for 60 min at 4°C. Total membranes from homogenates containing a known quantity of DNA were then pelleted by centrifugation for 5 min at 16,000 g in an Eppendorf microfuge (Brinkmann Instruments Co., Westbury, NY). Membranes were stored in KRH buffer under liquid N_2 before binding assays.

For affinity labeling of CCK binding proteins, a smooth pancreatic membrane preparation was made to reduce the amount of nonspecific labeling. Pancreata were homogenized in 10 vol/tissue wet weight of 0.3 M sucrose (containing the battery of protease inhibitors listed above) in a Dounce homogenizer using six strokes with a tight fitting pestle. The homogenate was filtered through two layers of gauze, and brought to 1.3 M sucrose by the addition of a 2-M sucrose stock solution. The volume of the 1.3 M homogenate was four times that of the 0.3-M sucrose homogenate volume (i.e., 40 times volume per tissue wet weight). The 1.3 M homogenate was loaded into centrifuge tubes, overlaid with 0.3 M sucrose, and these discontinuous

gradients were centrifuged in a rotor (SW 41; Beckman Instruments, Inc., Fullerton, CA) for 90 min (150,000 g_{av}). Material banding at the 0.3–1.3-M sucrose interface was collected, diluted to a final sucrose concentration of 0.06 M with distilled H₂O, and pelleted by centrifugation at 16,000 g for 5 min. The membrane pellet was resuspended in either KRH or distilled H₂O, stored in aliquots under liquid N₂, and used immediately upon thawing.

CCK Binding and Affinity Labeling

Total membranes representing 5- μ g equivalents of homogenate DNA were washed once by suspension and centrifugation in an Eppendorf microfuge at 16,000 g for 5 min in 1 M NaCl to remove adsorbed soluble proteins (47), and then washed again in KRH buffer (containing the battery of protease inhibitors listed above). To measure ¹²⁵I-CCK-8 binding as a function of radiolabel concentration, membrane pellets were resuspended in 50 μ l of KRH buffer and mixed with 50 μ l of ¹²⁵I-CCK-8 (sp act \sim 2,000 Ci/mmol) of varying concentrations in the presence or absence of unlabeled hormone in KRH buffer. After a 30-min incubation at room temperature, a time sufficient to reach steady-state binding (data not shown), the reaction was terminated by the addition of 1 ml ice-cold KRH buffer. Free ligand was separated from that bound to membranes by centrifugation (16,000 g for 5 min). The pellets were resuspended in an additional 1 ml of buffer and centrifuged again, as described above. Radioactivity associated with membrane pellets was measured in a gamma counter (Beckman Instruments, Inc.). Nonspecific binding was determined in the presence of 2 μ M unlabeled CCK-8. The absolute amount of nonspecific radioactivity bound was the same for membranes from pancreas of each age; this represented \sim 15% of the total counts bound to membranes from adult, and \sim 50% of total radioactivity bound in neonatal, and fetal pancreas, respectively. The low number of CCK binding sites relative to protein content in membranes from fetal and neonatal pancreas may contribute to the high percentage of nonspecific binding observed. In addition, our autoradiographic data (Fig. 3) suggest that ¹²⁵I-CCK was nonspecifically associated with mesenchymal matrix, which may contaminate membrane preparations from developing pancreas; nevertheless, nonspecifically bound radioactivity was not cross-linked (Fig. 4).

For affinity cross-linking studies, smooth membranes washed with NaCl were first treated with 10 mM dithiothreitol for 5 min on ice, since reduction of membrane proteins before binding dramatically improves the efficiency of cross-linking with ¹²⁵I-CCK-33 and m-maleimidobenzoyl N-hydroxy-succinimide ester (MBS) (29). Washed membranes (\sim 75 μ g protein determined by the fluorescamine method; reference 53) were incubated in KRH buffer with \sim 3 nM ¹²⁵I-CCK-33 in a final volume of 200 μ l. After a 15-min incubation at 23°C, 1 ml of ice-cold KRH buffer was added to all tubes. All subsequent manipulations were done at 4°C to decrease the rate of dissociation of radioligand from the membranes (23). Membranes were pelleted and washed a second time in 1 ml of KRH buffer without BSA. Cross-linking was initiated by resuspending labeled membrane pellets in 98 μ l of KRH buffer, and adding 2 μ l of a 2.5-mM MBS solution dissolved in DMSO (final MBS concentration, 50 μ M). The reaction was allowed to proceed for 5 min on ice before being quenched by the addition of 20 mM Tris buffer, pH 7.4. Cross-linked membranes were pelleted again, and solubilized in sample buffer (0.12 M Tris, pH 6.7, 4% wt/vol SDS, 2 mM EDTA, 20% vol/vol glycerol, 0.01% wt/vol bromophenol blue, 0.1 M dithiothreitol) before SDS gel electrophoresis (7% vol/vol polyacrylamide gels), according to the method of Laemmli (26). Samples were not boiled before electrophoresis since aggregation of CCK binding proteins was increased upon boiling. After electrophoresis, gels were stained with 0.2% wt/vol Coomassie Blue in 50% vol/vol methanol/7% vol/vol acetic acid, dried, and exposed at -70°C to x-ray film (Kodak XAR-5) with a Cronex intensifying screen (DuPont Co., Wilmington, DE).

Light Microscopic Autoradiography

Pancreatic lobules from fetal and neonatal rats were preincubated in \sim 10 ml KRH buffer (containing 0.2% wt/vol BSA, 0.01% wt/vol STI) for \sim 30 min with several changes of medium. Lobules were then incubated in 0.5 ml KRH buffer containing \sim 10 nM ¹²⁵I-CCK-33 for 5 min at 23°C in the presence or absence of 2 μ M unlabeled CCK-8. Lobules were rinsed twice in 50 ml ice-cold KRH buffer for \sim 1 min before being fixed overnight at 4°C with 2% vol/vol glutaraldehyde in 0.1 M Na cacodylate, pH 7.4. After the tissues were osmicated (1% OsO₄), and embedded in Epon, 0.5- μ m sections were cut and coated with emulsion (Ilford K5; Polysciences, Inc., Warrington, PA) according to standard methods (41). Autoradiographs were

exposed for \sim 4 wk, developed in Kodak D-19, and stained with 1% wt/vol methylene blue per 1% wt/vol azure II in 1% wt/vol Na borate. Photographs were taken on a Zeiss Photomicroscope II (Carl Zeiss, Inc., Thornwood, NY).

⁴⁵Ca⁺⁺ Efflux

Pancreatic lobules were prepared and preincubated in oxygenated MEM, as described above. The ⁴⁵Ca⁺⁺ efflux experiments were done with minor modifications of the procedure of Case and Clausen (5). Usually 12 pancreatic lobules were loaded for 60 min with radioactive tracer (20 μ Ci ⁴⁵CaCl₂) in 2 ml medium.

By 60 min of loading, a steady state of ⁴⁵Ca⁺⁺ uptake was approached in pancreatic lobules from adult, neonatal, and fetal rats. At this time, accumulated ⁴⁵Ca⁺⁺ normalized to DNA content was \sim 1,600, 2,900, and 3,000 cpm/ μ g DNA, in pancreatic lobules of adult, neonatal, and fetal rats, respectively. After loading, the pancreatic lobules were rinsed with MEM and blotted on filter paper. Usually six lobules were transferred to prewarmed MEM (2 ml) that contained CaCl₂ (0.95 mM), but that was tracer-free. ⁴⁵Ca⁺⁺ was permitted to wash out of the cells for 1 h before the start of the experiment to effectively drain trapped radioactivity from the extracellular space (5). Every 10 min during this period, 1 ml of medium was removed and replaced with an equal volume of fresh medium to reduce the reuptake of tracer by the tissue.

To start the experiment after 60 min of tracer washout, the medium was completely removed and replaced with fresh medium. Basal ⁴⁵Ca⁺⁺ efflux and amylase release were measured every 10 min for 40 min. Duplicate 0.5-ml aliquots of medium were collected and replaced with 1 ml of fresh medium. After 40 min, 1 ml of medium was removed, and replaced with MEM containing secretagogue. Incubation continued in the presence of secretagogue, and aliquots were taken at 2- and 3-min intervals initially, and 5–10-min intervals thereafter (for a total of 40–60 min). At the end of the experiment, lobules were blotted on filter paper, and homogenized as described above. Homogenate and medium aliquots were analyzed for amylase activity and lactate dehydrogenase activity (45), and counted in 5 ml Optifluor (Packard Instruments, Inc., Downers Grove, IL) with a liquid scintillation counter (Beckman Instruments, Inc.).

A23187 and phorbol dibutyrate were prepared as 5- and 1-mM stock solutions, respectively, in DMSO. A DMSO concentration of 0.1% vol/vol (the highest concentration present in any incubation) did not have any effect on ⁴⁵Ca⁺⁺ efflux or basal amylase release from pancreatic lobules. Doses of A23187 (5 μ M), dbcAMP (1 mM), and phorbol dibutyrate (1 μ M) used in this study have been reported by others (11, 18, 33) to cause maximal amylase secretion from pancreatic lobules or acinar preparations from adult rat pancreas.

Calculations

Cumulative ⁴⁵Ca⁺⁺ efflux, amylase release, and lactate dehydrogenase leakage into the medium are expressed as a percent of total tissue activity (i.e., the sum of the activities present in all samples of medium plus the activity remaining in the tissue homogenate at the end of the experiment). Fractional ⁴⁵Ca⁺⁺ efflux is the percent of total radioactivity appearing in the medium per minute, as described by Borle (3). Change in fractional efflux is defined as the maximum fractional efflux observed in the presence of the stimulus minus the basal fractional efflux measured immediately before stimulus addition. Since basal fractional ⁴⁵Ca⁺⁺ efflux varied from sample to sample ($<0.5\%$ /min), the data in some figures are expressed as relative fractional efflux for clarity, i.e., the curves are frame-shifted so that the basal fractional efflux measured immediately before the addition of stimulus equals 1%/min.

Materials

MEM with Hank's salts was purchased from Flow Laboratories, Inc. (McLean, VA). BSA (fraction V, RIA grade) was obtained from Armour Pharmaceutical Co. (Kankakee, IL). STI and purified DNase were purchased from Cooper Biomedical, Inc. (Malvern, PA). All other protease inhibitors (bacitracin, leupeptin, pepstatin, aprotinin), phorbol 12,13-dibutyrate, dbcAMP, and reagents for the lactate dehydrogenase assay (NADH and Na pyruvate) were obtained from Sigma Chemical Co. (St. Louis, MO). CCK-8, ¹²⁵I-labeled with Bolton-Hunter reagent, with a specific activity of \sim 2,000 Ci/mmol was purchased from Amersham Corp. (Arlington Heights, IL). Monoiodinated Bolton-Hunter reagent (sp act $>2,000$ Ci/mmol) was obtained from New England Nuclear (Boston, MA).

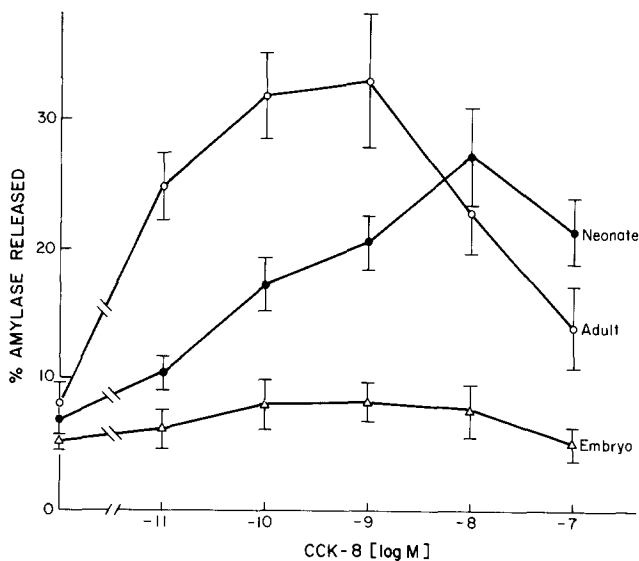


Figure 1. Dose-response curve of CCK-stimulated amylase release from pancreatic lobules. Pancreatic lobules were incubated in the presence or absence of the indicated concentrations of CCK-8 for 2 h at 37°C. Amylase activity discharged into the medium (expressed as a percentage of the total amylase activity present in the medium plus that remaining in the lobules after 2 h) is plotted as a function of the log molar CCK concentration. (Open circle) Adult pancreas; (solid circle) neonatal pancreas; (open triangle) fetal pancreas. Results are the mean of three or more experiments. Vertical bars represent SEM. The mean rates of amylase release (percent of total amylase activity appearing in the medium per minute) were determined for each experimental condition as described in Materials and Methods. The basal rates of amylase discharge from adult ($0.06 \pm 0.01\%/min$ [mean \pm SEM], measured in 12 separate incubations), neonatal ($0.06 \pm 0.01\%/min$ [mean \pm SEM], determined in 15 separate incubations), and embryonic ($0.05 \pm 0.01\%/min$ [mean \pm SEM], determined in 8 separate incubations) pancreas were not significantly different by Student's paired *t* test, $P > 0.2$. There was no significant difference (Student's paired *t* test, $P > 0.6$) between stimulated rates of discharge from adult pancreas at 1 nM CCK-8, and neonatal pancreas at 10 nM CCK-8.

Dithiothreitol and molecular mass standards were from Bio-Rad Laboratories (Richmond, CA). MBS was purchased from Pierce Chemical Co. (Rockford, IL). Synthetic CCK-8 was a gift from Dr. S. J. Lucania (Squibb Institute for Medical Research, Princeton, NJ). Natural porcine CCK-33 was obtained from Dr. Viktor Mutt (Gastrointestinal Hormone Research Laboratory, Karolinska Institutet, Stockholm, Sweden). A23187 was purchased from Calbiochem-Behring Corp. (La Jolla, CA).

Results

CCK-stimulated Discharge of α -Amylase from Adult and Developing Pancreas

Secretory response to CCK octapeptide (CCK-8) was measured by the extent of discharge of the granule content marker, α -amylase, from pancreatic lobules. In Fig. 1, the CCK dose-response relationship in the fetal pancreas was compared with those of the neonatal and adult glands. As in previous reports (12, 16, 46), 1 nM CCK was the most effective dose in triggering amylase release from adult pancreas; the level of amylase released into the medium after 2 h in the presence of 1 nM CCK-8 was 32.9% of total cellular amylase \pm

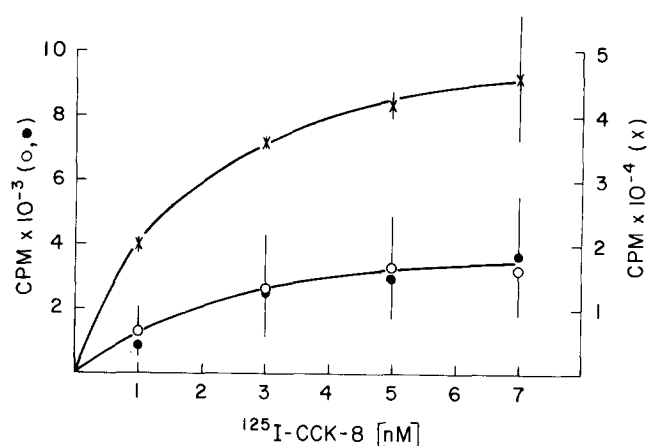


Figure 2. $^{125}\text{I-CCK}$ binding to pancreatic membranes. $^{125}\text{I-CCK-8}$ was incubated for 30 min at room temperature with total membranes representing 5- μg equivalents of homogenate DNA from adult (x), neonatal (open circle), and fetal (solid circle) pancreas. Specifically bound radioactivity is plotted as a function of $^{125}\text{I-CCK-8}$ concentration. Nonspecific binding, determined in the presence of 2 μM unlabeled CCK-8, has been subtracted (see Materials and Methods). CCK binding curves are averages of two to three experiments on pancreatic membranes of each age. Vertical bars represent SEM. Note that specific radioactivity bound to adult pancreas is greater by approximately an order of magnitude than that bound to an equivalent cell number in neonatal and fetal pancreas.

5.0% (mean \pm SEM) (Fig. 1). Amylase discharge from the neonatal pancreas was maximal at a CCK dose of 10 nM ($27.1 \pm 3.8\%$ [mean \pm SEM]), i.e., the dose-response curve was shifted to the right compared with that of the adult. In both adult and neonatal pancreas, supraoptimal doses of hormone induced submaximal release of amylase. In contrast to the neonatal and adult glands, the level of amylase discharge from the fetal pancreas remained low in the presence of all CCK doses tested (Fig. 1). The maximal level of amylase release from the fetal gland ($8.2 \pm 1.5\%$ [mean \pm SEM]) in the presence of 1 nM CCK-8 was not significantly greater than the level of basal discharge ($5.4 \pm 0.7\%$ [mean \pm SEM]; $P = 0.1$ by Student's *t* test). Thus it appears that the fetal pancreas at 1 d before birth shows an insignificant secretory response to CCK.

A time course of amylase release revealed that secretion was approximately linear with time for up to 120 min in developing as well as in adult glands, and the basal rates of secretion did not differ significantly from each other (see legend to Fig. 1). Optimal CCK doses of 1 and 10 nM induced comparable rates of amylase secretion from adult and neonatal pancreas, respectively. The rate of stimulated amylase discharge at the optimal CCK dose (minus basal discharge) was $0.26 \pm 0.04\%/min$ (mean \pm SEM; determined in 5 separate incubations) in adult pancreas versus $0.23 \pm 0.03\%/min$ (mean \pm SEM; measured in 13 separate incubations) in neonatal pancreas. Strikingly, the rate of CCK-induced amylase release from the neonatal pancreas at 1 nM ($0.12 \pm 0.1\%/min$ [mean \pm SEM]; measured in 15 separate incubations) and 10 nM CCK from the neonatal pancreas was four- and eightfold greater, respectively, than that of the fetal gland ($0.03 \pm 0.01\%/min$ [mean \pm SEM]; determined in 7 separate incubations).

Binding of CCK to Membranes of Adult and Developing Pancreas

It is generally accepted that the interaction of polypeptide hormones with their receptors at the plasma membrane is a prerequisite for eliciting cellular responses (13). A possible explanation for the insensitivity of the fetal pancreas to CCK is that hormone binding sites are not yet expressed at the cell surface. To ascertain whether the fetal gland expresses CCK binding sites, we tested for ^{125}I -CCK-8 binding to total membrane preparations from fetal and neonatal as well as adult glands. Fig. 2 shows the specific ^{125}I -CCK binding to membranes from pancreas of each age plotted as a function of hormone concentration. In all cases, binding appeared to approach saturation asymptotically at 7 nM ^{125}I -CCK-8, and no further increase in specific binding was observed at 10 nM hormone (data not shown). At 7 nM ^{125}I -CCK-8, the membranes derived from equal numbers of fetal and neonatal pancreatic cells specifically bound equal amounts of ^{125}I -CCK (see Materials and Methods); however, this binding represented only $\sim 10\%$ of that observed in adult pancreatic membranes. A difference in the number rather than the affinity of CCK binding sites may account for the difference in the binding capacity of membranes from adult pancreas compared with that of the fetal and neonatal glands. As in previously published data (22, 23, 51), we approximate an apparent K_d of ~ 1.5 nM, based on the concentration of ^{125}I -CCK-8 at which binding was half-maximal to membranes from adult, neonatal, and fetal pancreas.

Autoradiographic Localization of CCK Binding Sites in Adult and Developing Pancreas

To determine whether the CCK receptors of the fetal pancreas are present on the cell surface, we visualized ^{125}I -CCK-33 binding to pancreatic lobules by light microscopic autoradiography. Pancreatic lobules from both fetal and neonatal rats were incubated at 23°C for 5 min with 10 nM ^{125}I -CCK-33, before fixation with glutaraldehyde. As shown in Fig. 3, *a* and *c*, ^{125}I -CCK labeling of fetal pancreas was indistinguishable from that observed in the neonatal gland. Autoradiographic grains were predominantly localized around the periphery of acinar cells in pancreas of both ages, although they were sparse in comparison to that observed in adult pancreas (41; and data not shown). Some autoradiographic grains were also observed in the interior of acinar cells. ^{125}I -CCK labeling was more dense around acini at the periphery of each lobule; slow diffusion of radiolabeled hormone into the interior of the tissue probably accounts for this restricted labeling. The labeling of the acinar cells was specific; in the presence of an excess of unlabeled CCK-8, a very low level of nonspecific radioactivity was randomly associated with the cells (Fig. 3, *b* and *d*). Nonspecific autoradiographic grains that were not competed by 2 μM unlabeled CCK-8 also appeared homogeneously distributed over the mesenchymal matrix.

Although the cell surface expression of CCK binding sites appears similar in fetal and neonatal pancreas, the morphology of the acinar cells in each was strikingly distinct. Many large zymogen granules filled the acinar cells of the fetal pancreas, while the neonatal pancreas contained fewer and smaller secretory granules restricted to the apical region of acinar cells (Fig. 3, *b* and *d*; reference 40).

Affinity Labeling of CCK Binding Proteins in Membranes from Adult and Developing Pancreas

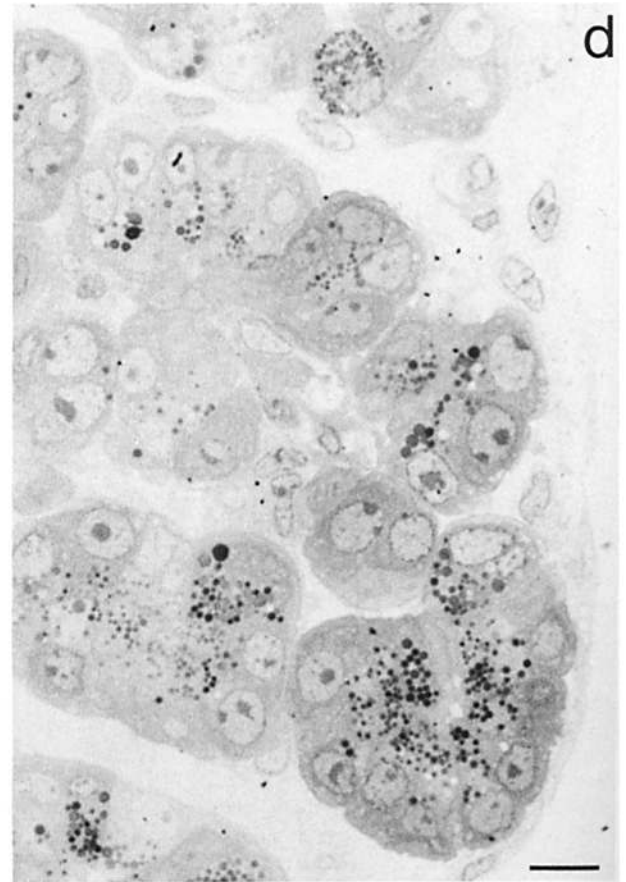
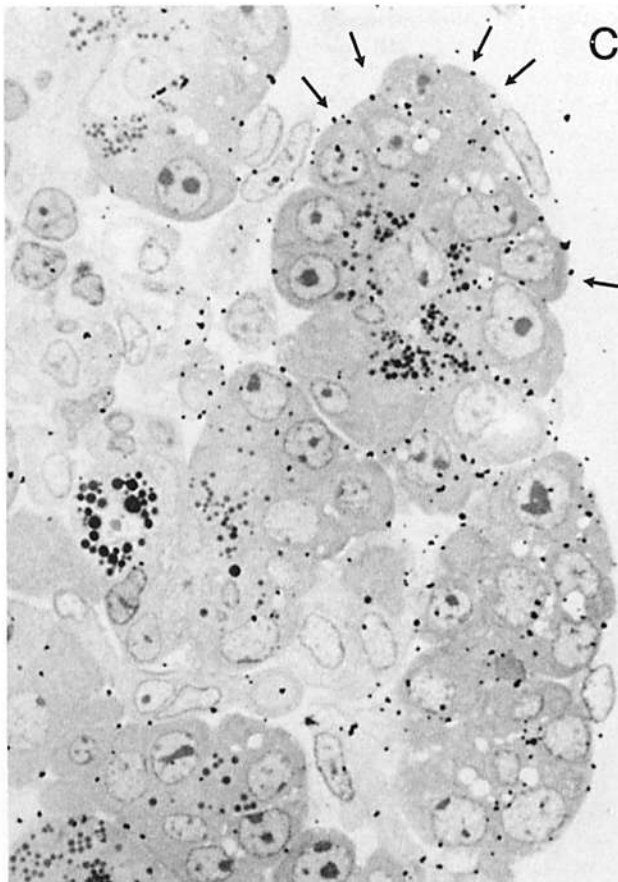
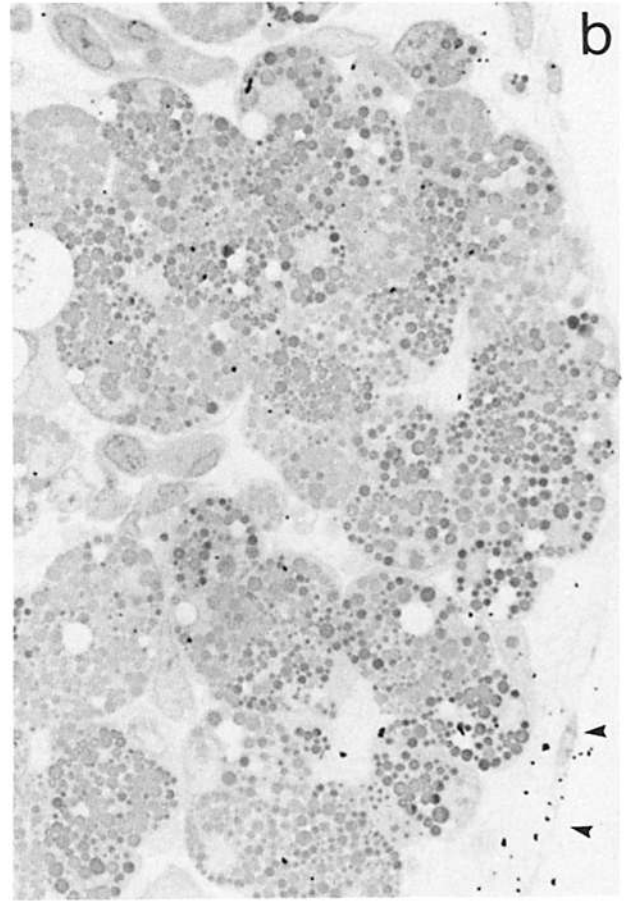
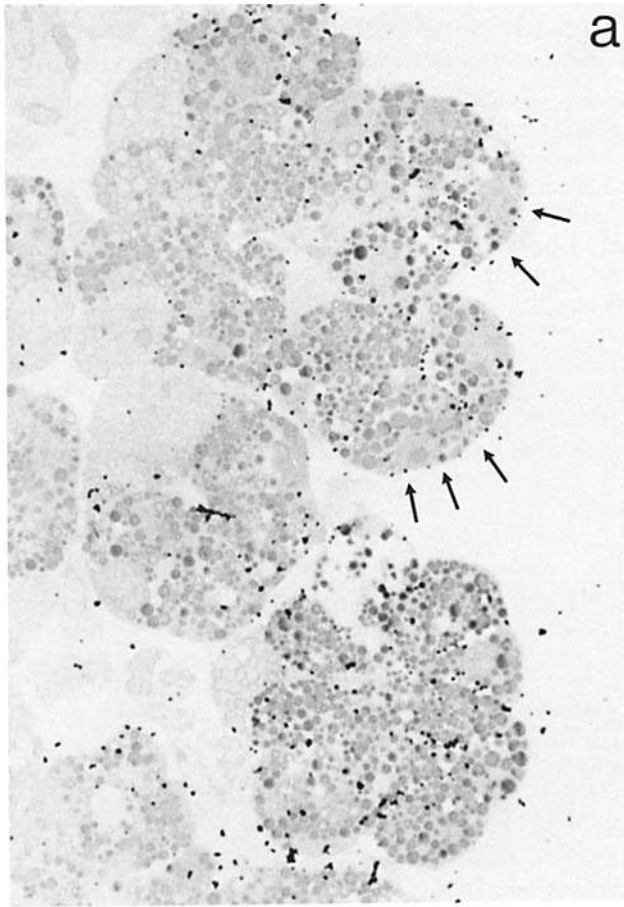
To test whether maturation of secretory responsiveness is related to structural changes in CCK binding proteins during development, these proteins were analyzed in fetal, neonatal, and adult pancreas by affinity cross-linking. The (SH, NH_2) heterobifunctional cross-linker MBS has been used to affinity label CCK binding proteins of pancreatic membranes from adult rats (29). Since MBS has a high cross-linking efficiency compared with several other cross-linking reagents (29), it was used to identify CCK binding proteins of developing as well as adult pancreas. When membranes from adult pancreas were cross-linked with ^{125}I -CCK-33 and MBS, and analyzed by SDS PAGE, proteins of M_r 85,000, 130,000, and 190,000 were labeled (Fig. 4 *d*), as in Madison et al. (29). Affinity labeling was specific since it was abolished in the presence of 2 μM unlabeled CCK-8 (Fig. 4). The M_r 85,000 protein is postulated to constitute a part of the CCK receptor, since it has been identified by a variety of affinity cross-linking techniques (41). The relationship of the other affinity-labeled membrane constituents with respect to CCK receptor structure is not clear at present; they may represent subunits of the receptor or neighboring proteins (29, 41). Nevertheless, we will refer to all affinity-labeled membrane constituents as CCK binding proteins.

The electrophoretic mobilities on SDS polyacrylamide gels of affinity-labeled CCK binding proteins of fetal pancreas (Fig. 4, lane *a*) appeared similar to those of neonatal pancreas (lane *b*); a band of apparent M_r of 210,000, and a broad region ranging from M_r 100,000–160,000 were labeled in both tissues. To establish a possible relationship between the affinity-labeled proteins of developing pancreas and those of the adult gland, CCK binding proteins of 3-wk postpartum pancreas were examined (Fig. 4, lane *c*). These labeled proteins had apparent M_r s of 195,000, 145,000, and 95,000 that were intermediate between those of the neonatal (lane *b*) and adult pancreas (lane *d*). This result suggested to us that the CCK binding proteins of fetal, neonatal, and adult pancreas are structurally related.

Simultaneous Measurement of $^{45}\text{Ca}^{++}$ Efflux and Amylase Discharge from Pancreatic Lobules: Effect of CCK

Since it appeared that the fetal pancreas expressed cell surface binding sites for CCK, we tested whether these CCK binding proteins were functional, i.e., were able to translate hormone binding into an intracellular response. It has been shown that CCK binding stimulates an increase in the $^{45}\text{Ca}^{++}$ efflux from preloaded pancreatic lobules of adult rats incubated in a calcium-containing, but tracer-free medium (5). The increased $^{45}\text{Ca}^{++}$ efflux is a reflection of CCK-induced Ca^{++} mobilization and redistribution (although it does not indicate net Ca^{++} movement; references 5 and 49).

The fractional $^{45}\text{Ca}^{++}$ efflux (cumulative $^{45}\text{Ca}^{++}$ present in the medium expressed as a percent of total cellular $^{45}\text{Ca}^{++}$ per minute; reference 3) from preloaded lobules of adult pancreas is shown as a function of time in Fig. 5 *a*. An increase in fractional $^{45}\text{Ca}^{++}$ efflux in the presence of CCK (1 nM) was observed within 2 min after hormone addition. The rate of $^{45}\text{Ca}^{++}$ appearance in the medium was maximal on average at ~ 12 min (ranging from 7–20 min over 11 experiments)



after CCK addition, and subsequently declined (Fig. 5 *a*). A quantitative measure of the CCK-induced change in fractional $^{45}\text{Ca}^{++}$ efflux was obtained by subtracting the fractional efflux observed immediately before the addition of hormone from the maximum fractional efflux observed in its presence. In adult pancreas, the CCK-stimulated change in fractional efflux was $0.63 \pm 0.06\%/ \text{min}$ cellular $^{45}\text{Ca}^{++}$ (mean \pm SEM) (Fig. 6).

Cumulative amylase release from adult pancreas, assayed simultaneously with $^{45}\text{Ca}^{++}$ efflux, is also shown in Fig. 5 *a*. Although the basal rate of amylase discharge varied slightly among separate incubation flasks, it was always low and linear. Addition of 1 nM CCK (the dose causing maximal amylase secretion from adult pancreas) dramatically increased the rate of amylase release, and this effect continued for the duration of the experiment.

In the neonatal pancreas, the change in fractional $^{45}\text{Ca}^{++}$ efflux induced by either 1 or 10 nM CCK ($0.09 \pm 0.02\%/ \text{min}$ [mean \pm SEM]; and $0.11 \pm 0.04\%/ \text{min}$ [mean \pm SEM], respectively) was substantially and consistently less than that observed in the adult gland (Fig. 5 *b* and 6). Furthermore, maximum $^{45}\text{Ca}^{++}$ efflux from neonatal pancreas occurred at a slightly earlier time after CCK addition (mean time over 13 experiments, ~ 7 min; range, 5–10 min) compared with the adult pancreas (compare Fig. 5, *b* and *a*). However, the rate of amylase secretion from the neonatal pancreas in the presence of an optimal CCK dose (10 nM) was comparable to maximally stimulated discharge from the adult gland, based on three individual experiments in which $^{45}\text{Ca}^{++}$ efflux and amylase release were assayed simultaneously (compare Fig. 5, *a* and *b*).

To determine whether CCK binding in the fetal pancreas is coupled to Ca^{++} mobilization from intracellular stores, $^{45}\text{Ca}^{++}$ efflux was monitored in the presence and absence of CCK. Fig. 5 *c* shows $^{45}\text{Ca}^{++}$ efflux and concomitant amylase discharge from fetal pancreas in response to 10 nM CCK. Although the rate of amylase secretion in the presence of CCK was low, the hormone induced an increase in $^{45}\text{Ca}^{++}$ efflux from the fetal pancreas; this change in fractional $^{45}\text{Ca}^{++}$ efflux ($0.09 \pm 0.02\%/ \text{min}$ [mean \pm SEM] at 1 nM CCK; and $0.10 \pm 0.04\%/ \text{min}$ [mean \pm SEM] at 10 nM CCK) was equal in magnitude to that seen in the neonatal gland in response to CCK (Fig. 6). The time after CCK addition at which maximal fractional efflux occurred (mean time over 10 experiments, ~ 7 min) was also similar to that of the neonatal pancreas. Although 10 nM CCK appeared to trigger a slightly greater increase in fractional $^{45}\text{Ca}^{++}$ efflux than did 1 nM CCK in pancreas of both ages, the differences were not statistically significant ($P > 0.5$ by Student's *t* test). The data indicate that CCK-induced Ca^{++} mobilization from fetal pancreas is equal to that from the neonatal gland.

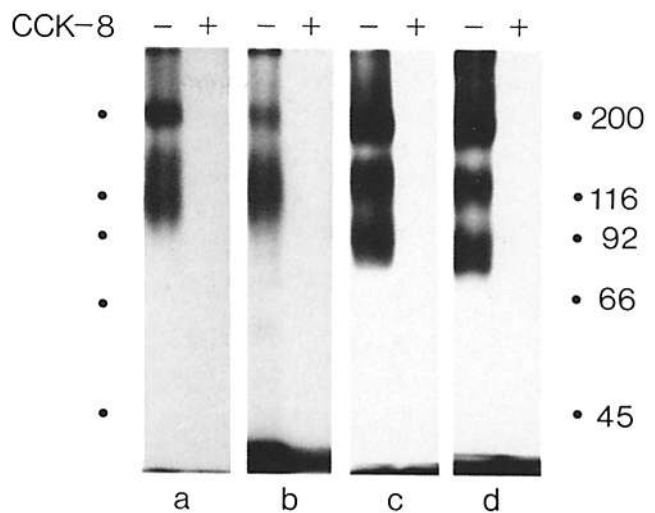


Figure 4. Affinity labeling of pancreatic membranes with ^{125}I -CCK-33 and MBS. Smooth membrane fractions isolated from fetal (lane *a*), neonatal (lane *b*), 3-wk postpartum (lane *c*), and adult (lane *d*) pancreata were incubated with ~ 3 nM ^{125}I -CCK-33 at 23°C in the presence or absence of $2 \mu\text{M}$ CCK-8, and cross-linked with $50 \mu\text{M}$ MBS (see Materials and Methods). Labeled CCK binding proteins were resolved by SDS gel electrophoresis (7% polyacrylamide gel) under reducing conditions, and the dried gel analyzed by fluorography. Molecular mass markers were: myosin (M_r 200,000), β -galactosidase (M_r 116,500), phosphorylase b (M_r 92,500), BSA (M_r 66,200), and ovalbumin (M_r 45,000). The amount of label incorporated into each band varied from experiment to experiment; however, the pattern of labeled proteins remained constant. Radioactivity appearing at the bottom of the gel represents labeled hormone that was not cross-linked to membrane proteins. The data presented are representative of three or more experiments.

Effect of A23187 Alone and in Combination with dbcAMP or Phorbol Dibutyrate on $^{45}\text{Ca}^{++}$ Efflux and Amylase Discharge in Pancreatic Lobules

Since CCK stimulated $^{45}\text{Ca}^{++}$ efflux without eliciting amylase secretion in the fetal pancreas, it is possible that one or more signal transduction events distal to Ca^{++} mobilization are uncoupled from the Ca^{++} signal. To test this proposition, we measured the effect of the Ca^{++} ionophore A23187 on amylase discharge and $^{45}\text{Ca}^{++}$ efflux. A23187 bypasses cell surface receptors to trigger protein secretion from adult pancreas, presumably by increasing the $[\text{Ca}^{++}]_i$ via mobilization of intracellular Ca^{++} stores and/or increasing Ca^{++} influx from the extracellular medium (6, 11, 15, 19, 35). Fig. 7 *a* shows the stimulatory effect of $5 \mu\text{M}$ A23187 on $^{45}\text{Ca}^{++}$ efflux from pancreatic lobules of fetal rats. For clarity and ease of comparison among different experimental condi-

Figure 3. Light microscopic autoradiography of pancreatic lobules labeled with ^{125}I -CCK-33. Pancreatic lobules were incubated in KRH buffer containing ~ 10 nM ^{125}I -CCK-33 for 5 min at 23° in the presence or absence of $2 \mu\text{M}$ unlabeled CCK-8, before being fixed and processed for light microscopic autoradiography (see Materials and Methods). (*a*) Autoradiogram of fetal pancreas labeled with ^{125}I -CCK-33; (*b*) control preparation of fetal pancreas incubated with ^{125}I -CCK-33 and $2 \mu\text{M}$ CCK-8; (*c*) autoradiogram of neonatal pancreas labeled with ^{125}I -CCK-33; (*d*) control preparation of neonatal pancreas incubated with radioligand in the presence of unlabeled hormone. The data presented are representative of three separate experiments. Arrows indicate autoradiographic grains localized around the periphery of acinar cells. Arrowheads in *b* indicate autoradiographic grains nonspecifically associated with mesenchymal tissue. Bar, $10 \mu\text{m}$.

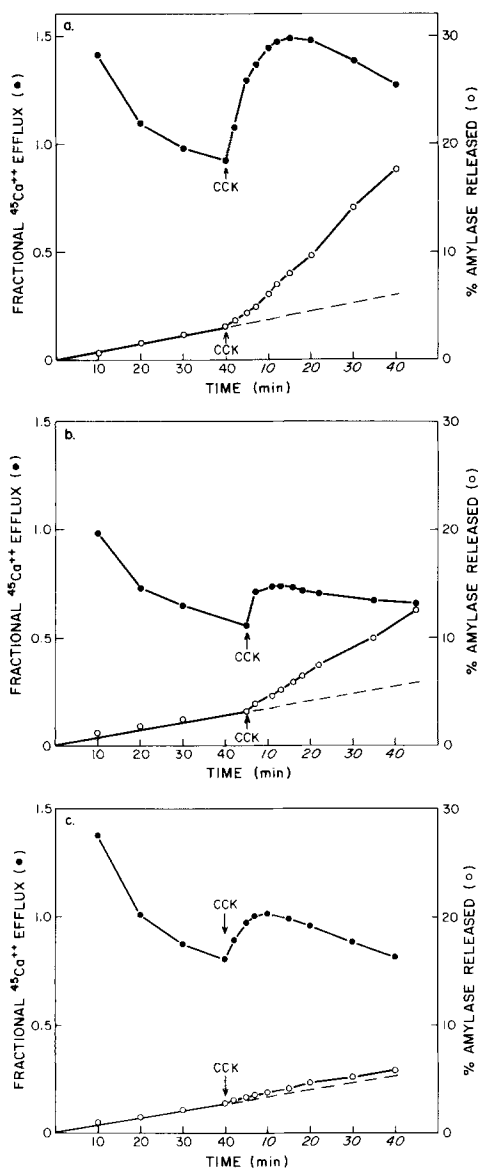


Figure 5. CCK stimulation of $^{45}\text{Ca}^{++}$ efflux and amylase release from pancreatic lobules. Pancreatic lobules from adult (a), neonatal (b), and fetal (c) rats were preloaded with $^{45}\text{Ca}^{++}$ as described in Materials and Methods, and were then incubated in medium that contained 0.95 mM calcium, but that was tracer-free. Aliquots of medium were collected at the indicated times and assayed for $^{45}\text{Ca}^{++}$, and were replaced with fresh medium. The efflux of $^{45}\text{Ca}^{++}$ is expressed as a rate coefficient (fractional efflux), i.e., the percent of total cell radioactivity appearing in the medium per minute. (Solid circle) Fractional $^{45}\text{Ca}^{++}$ efflux plotted as a function of time; (arrow) the addition (and continuous presence thereafter) of 1 nM CCK to adult pancreas, and 10 nM CCK to neonatal and fetal pancreas; italic numerals indicate the incubation period in the presence of hormone; (open circle) cumulative amylase release measured simultaneously with $^{45}\text{Ca}^{++}$ efflux and expressed as a percent of total activity; (dashed line) extrapolated basal secretion. The data are representative of at least three separate experiments on pancreas of each age.

tions, the data in Fig. 7 and subsequent figures are expressed as relative fractional $^{45}\text{Ca}^{++}$ efflux, i.e., the basal fractional efflux measured immediately before the addition of stimulus is arbitrarily set at 1%/min, and the fractional $^{45}\text{Ca}^{++}$ effluxes at all other time points are frame-shifted accord-

ingly. The stimulated changes in fractional $^{45}\text{Ca}^{++}$ efflux and the shapes of the curves remain the same.

As shown in Fig. 7, the increase in fractional $^{45}\text{Ca}^{++}$ efflux measured in the presence of A23187 ($0.24 \pm 0.09\%/min$ [mean \pm SEM] over five experiments) was higher than CCK-stimulated fractional $^{45}\text{Ca}^{++}$ efflux (Fig. 6). In addition, maximal A23187-induced $^{45}\text{Ca}^{++}$ efflux from the fetal pancreas (as well as neonatal and adult glands) occurred at a later time after ionophore addition than that observed in response to CCK. The differences in the shapes of the fractional $^{45}\text{Ca}^{++}$ efflux curves induced by the two agents support the idea that the mechanism of action of A23187 is different from that of CCK. Although the Ca^{++} ionophore stimulated fractional $^{45}\text{Ca}^{++}$ efflux from the fetal pancreas, the level of amylase release was not increased above basal secretion (shown in Fig. 7 as net amylase discharge minus basal discharge).

In an attempt to amplify the secretory response to A23187, dbcAMP, a cAMP analog, was added to pancreatic lobules in conjunction with the Ca^{++} ionophore. Agents that stimulate increased cAMP levels have been shown to act synergistically with agents that mobilize Ca^{++} , leading to increased enzyme secretion from the adult pancreas (18, 19). As shown in Fig. 7 a, A23187 (5 μM) in combination with dbcAMP (1 mM) increased $^{45}\text{Ca}^{++}$ efflux from fetal pancreas, but did not elevate the rate of amylase secretion. Neither fractional $^{45}\text{Ca}^{++}$ efflux nor amylase secretion were increased when dbcAMP (1 mM) was added alone. It appears that while the fetal pancreas mobilizes Ca^{++} in response to CCK, signal transduction events distal to Ca^{++} mobilization are not yet competent to respond to the second messengers.

A23187 increased the rate of amylase release from both neonatal (Fig. 7 b) and adult (Fig. 7 c) pancreas. In the neo-

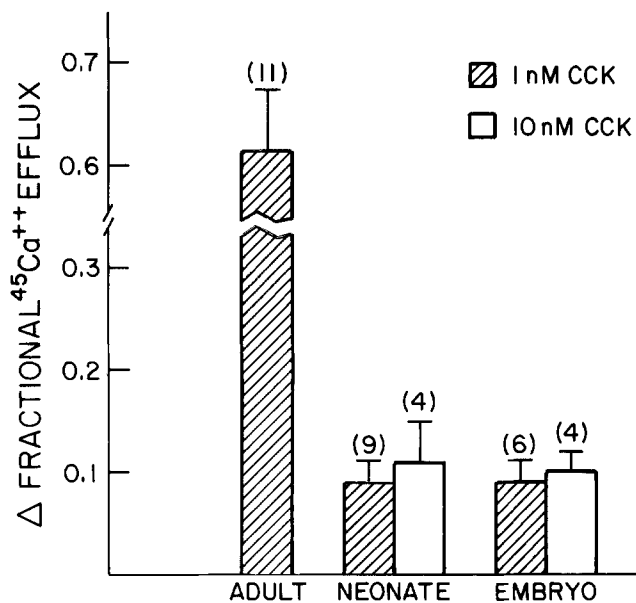


Figure 6. Increased fractional $^{45}\text{Ca}^{++}$ efflux in response to CCK. The mean values of the changes in fractional $^{45}\text{Ca}^{++}$ efflux (maximum fractional efflux measured in the presence of the hormone minus the basal fractional efflux measured immediately before its addition) stimulated by 1 nM (hatched bars) and 10 nM (open bars) CCK are shown for adult, neonatal, and fetal pancreas. The numbers in parentheses indicate the number of experiments performed. Vertical bars show SEM.

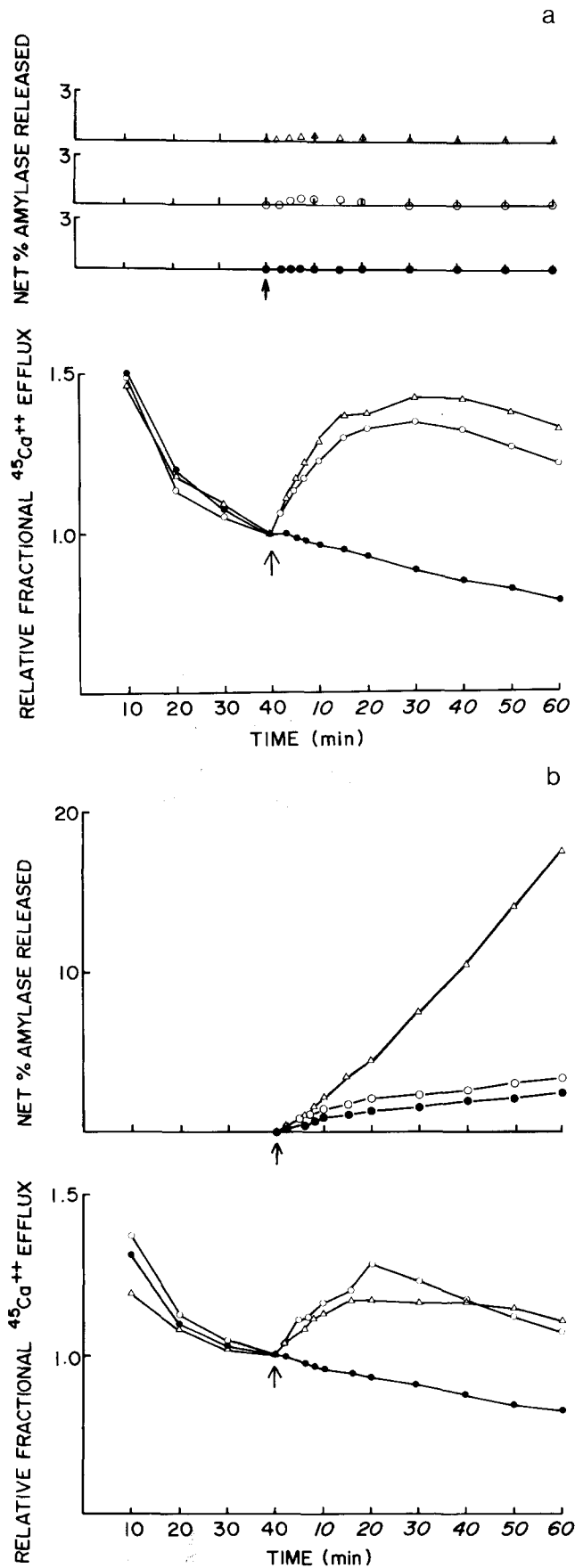


Figure 7. Effects of dbcAMP and A23187 on $^{45}\text{Ca}^{++}$ efflux and amylase release from fetal (a), neonatal (b), and adult (c) pancreas. These experiments were done as described in the legend to Fig. 5 and in Materials and Methods. In each panel net amylase released in response to each stimulus (minus extrapolated basal discharge) is shown at the top. At the bottom, the data are expressed as relative fractional $^{45}\text{Ca}^{++}$ efflux, i.e., the basal efflux measured immediately before CCK addition is set to a constant value of 1%/min. Arrows indicate the addition, and continued presence thereafter of 5 μM A23187 (open circle), 1 mM dbcAMP (solid circle), or A23187 in combination with dbcAMP (open triangle); italic numerals indicate the time period of incubation in the presence of stimulus. Basal $^{45}\text{Ca}^{++}$ efflux, the rate of basal amylase discharge, and the magnitude of response to each stimulus showed some variability in separate incubations. However, the depicted pattern of response is representative of at least three experiments. To minimize variation between samples, pancreatic lobules were pooled from several rat embryos or littermates.

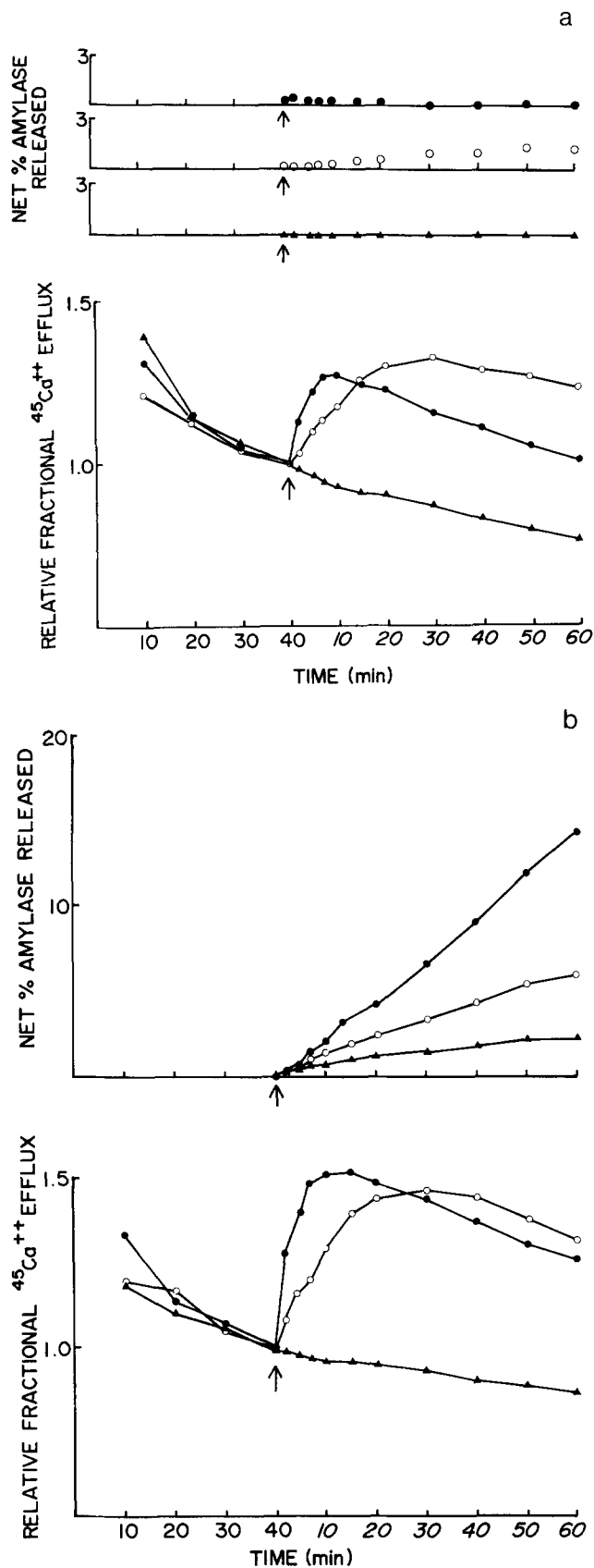


Figure 8. Effects of phorbol dibutyrate and A23187 on $^{45}\text{Ca}^{++}$ efflux and amylase discharge from fetal (a) and adult (b) pancreas, respectively. This experiment was done as described in the legend to Fig. 7 and in Materials and Methods. For clarity, the data are presented as a relative fractional $^{45}\text{Ca}^{++}$ efflux and net amylase re-

lease (minus the extrapolated basal discharge). Additions of phorbol dibutyrate (1 μM ; solid triangle), phorbol dibutyrate plus A23187 (5 μM ; open circle), and phorbol dibutyrate in combination with 10 nM CCK (solid circle) are indicated by arrows. The change in fractional $^{45}\text{Ca}^{++}$ efflux induced by phorbol dibutyrate and A23187 was 0.33%/min from fetal pancreas, and 0.47%/min from the adult gland. The changes in fractional $^{45}\text{Ca}^{++}$ efflux stimulated by a combination of phorbol dibutyrate and CCK were 0.28%/min and 0.52%/min from the fetal and adult pancreas, respectively. The responses shown here are representative of at least two experiments on pancreas of each age.

natal pancreas the A23187-induced change in fractional $^{45}\text{Ca}^{++}$ efflux ($0.19 \pm 0.04\%/min$ [mean \pm SEM], determined in four experiments) was greater than that effected by CCK, although the level of amylase secretion in response to A23187 was consistently less than hormone-stimulated discharge (compare Fig. 7 b with Fig. 5 b). In the adult pancreas, increased $^{45}\text{Ca}^{++}$ fractional efflux stimulated by A23187 ($0.49 \pm 0.09\%/min$ [mean \pm SEM], determined in six experiments) was less than that observed in the presence of CCK (Fig. 5 a), in parallel with the effect of these agents on amylase discharge.

DbcAMP potentiated the effect of A23187 on amylase release in both adult and neonatal pancreas, without dramatically enhancing the effect of the ionophore on $^{45}\text{Ca}^{++}$ efflux: $\sim 18\%$ of total amylase activity (minus basal secretion) was released in 60 min from pancreas of both ages. Alone, dbcAMP caused a slight increase in amylase discharge (net release in 60 min, $\sim 1\text{--}2\%$ in neonatal and adult pancreas), and had no effect on $^{45}\text{Ca}^{++}$ efflux (Fig. 7, b and c; reference 25). Since activation of C-kinase has been proposed to participate in the secretory response during secretagogue stimulation (33, 35, 40), it is possible that in the fetal pancreas CCK binding is not coupled to kinase activation. In an attempt to induce a secretory response while bypassing proximal transduction events, pancreatic lobules from fetal rats were incubated with phorbol dibutyrate (an activator of C-kinase) in conjunction with A23187 (Fig. 8 a). The rate of basal amylase release changed very little, if at all, in the presence of phorbol dibutyrate and A23187. Similarly, CCK in combination with phorbol dibutyrate had little or no effect on amylase secretion in the fetal gland. In contrast, secretion from the adult pancreas was increased by phorbol dibutyrate in combination with A23187; amylase release upon the addition of phorbol dibutyrate plus CCK appeared similar to that observed in the presence of hormone alone (Fig. 8 b). Added alone, phorbol dibutyrate slightly increased the secretion from the adult gland, but had no effect on amylase secretion from the fetal pancreas.

The magnitude and duration of $^{45}\text{Ca}^{++}$ efflux enhanced by A23187 and phorbol dibutyrate in both fetal and adult pancreas appeared similar to that observed with ionophore alone (compare Fig. 8, a and b with Fig. 7, a and c). Similarly, phorbol dibutyrate did not greatly affect the CCK-stimulated change in $^{45}\text{Ca}^{++}$ efflux in both fetal and adult glands. $^{45}\text{Ca}^{++}$ efflux from adult and fetal pancreas was not greatly affected by the addition of phorbol dibutyrate alone.

In pancreas of all ages, leakage of the cytosolic enzyme lactate dehydrogenase into the medium was always less than basal amylase release. Addition of A23187 and dbcAMP, or

the ionophore and phorbol dibutyrate did not increase the rate of lactate dehydrogenase leakage, indicating that these agents did not induce cell injury (data not shown).

Discussion

Our results show that by 1 d before birth, the pancreas of the fetal rat expressed specific CCK binding proteins (Fig. 2) localized at the acinar cell surface (Fig. 3). However, CCK binding to the fetal pancreas did not result in a stimulated secretory response (Fig. 1). These studies are consistent with previous reports that the CCK analog, caerulein, stimulates only a low level of amylase release in the fetal pancreas (10), and that secretion in the presence of CCK is not significantly greater than basal amylase discharge (28, 55). What is clear from these studies is the dramatically enhanced secretory response to CCK of the postpartum pancreas in comparison to that of the fetal gland. The CCK-stimulated amylase discharge over 120 min was $21 \pm 3.6\%$ (mean \pm SEM) from neonatal pancreas versus $3.7 \pm 1.1\%$ (mean \pm SEM) from fetal pancreas (Fig. 1). In the neonatal pancreas the four- to eightfold amplification in the rate of stimulated amylase discharge compared with the fetal gland was not correlated with a comparable change in receptor number or affinity.

^{125}I -CCK binding was normalized to pancreatic DNA content to obtain an estimate of the relative numbers of binding sites per acinar cell in fetal, neonatal, and adult pancreas (see Materials and Methods). Although both fetal and neonatal pancreas have slightly higher mitotic rates than the adult gland (37), the actual number of cells containing 4n DNA is small, and the total tissue DNA content remains a good approximation of cell number in the glands of each age. In addition, the fetal, neonatal, and adult pancreas each have the same proportion of acinar (CCK binding) cells to total pancreatic cells (38). Therefore, CCK binding capacity normalized to DNA content is an accurate reflection of the relative number of binding sites in pancreas of each age. We estimate that acinar cells of the fetal and neonatal pancreas express $\sim 10\%$ of the number of CCK binding sites per acinar cell in the adult gland (41), or ~ 500 – $1,000$ sites.

The CCK dose stimulating maximal amylase release from adult pancreas (1 nM) was approximately an order of magnitude less than the optimal dose in the neonatal gland (10 nM) (Fig. 1). As in a previous report (28), we find that the enhanced sensitivity of the adult pancreas correlates with an increase in the number of CCK binding sites. However, the presence of spare receptors, and desensitization at high CCK doses in adult pancreas (16), precludes establishing a quantitative relationship between receptor number and secretory response.

Light microscopic autoradiography of ^{125}I -CCK labeled lobules (Fig. 3) revealed grains that were predominantly localized on the periphery of the acinar cells of fetal and neonatal pancreas. The binding of ^{125}I -CCK to the cell surface of the acinar cells of both glands was specific, and abolished in the presence of unlabeled hormone. Thus, a lack of CCK binding proteins at the cell surface cannot account for the low secretory response to CCK observed in the fetal pancreas. Autoradiographic grains appeared to be distributed over all acinar cells within a specifically labeled acinus, suggesting that the expression of CCK binding sites is synchronized in acinar cells at day 21 of gestation.

Structural similarities in the CCK binding proteins of fetal and neonatal pancreas are suggested by the similar electrophoretic mobilities of the affinity-labeled proteins (Fig. 4). Nevertheless, we cannot exclude the possibility that there are subtle structural and/or charge differences between the CCK binding proteins of these two ages that are not detectable by the methods employed here. (Such purported differences do not affect the affinity of CCK binding or the capability of the hormone-receptor complex to stimulate $^{45}\text{Ca}^{++}$ efflux.) Preliminary experiments using endoglycosidase F to remove NH_2 -linked oligosaccharide chains from CCK binding proteins of adult and developing pancreas suggest that differential glycosylation of a common polypeptide backbone may account for the observed electrophoretic differences. In addition, we cannot rule out the possibility that other posttranslational modifications of CCK binding proteins occur during postnatal development.

The data on the structural similarity of CCK binding sites in fetal and neonatal pancreas is consistent with our observation that hormone binding to the fetal gland resulted in an increase in fractional $^{45}\text{Ca}^{++}$ efflux that was quantitatively identical to that observed in the neonatal gland (Fig. 6). It appears that the postnatal development of secretory responsiveness is independent of receptor properties per se, but involves the maturation of intracellular transduction events. This hypothesis is supported by the reports (10, 55) that secretory responsiveness to the cholinergic agonist, carbachol (known to stimulate Ca^{++} transients via a distinct receptor) is maximal only after birth.

Although the CCK-stimulated increase in $^{45}\text{Ca}^{++}$ efflux does not indicate net Ca^{++} movement and is not a direct measure of $[\text{Ca}^{++}]_c$ (5, 49), several independent lines of evidence suggest that it reflects a true elevation in $[\text{Ca}^{++}]_c$. First, the stimulated increases in fractional $^{45}\text{Ca}^{++}$ efflux of fetal, neonatal, and adult pancreas were not dependent on extracellular Ca^{++} , and were not affected by the presence of 0.5 mM EGTA in calcium-free medium (data not shown and reference 5). The observed change in $^{45}\text{Ca}^{++}$ efflux is therefore not likely to be a consequence of Ca^{++} uptake, nor solely a reflection of increased Ca^{++} cycling across the plasma membrane (39, 40). Instead, these data suggest that much of the tracer extruded from preloaded pancreatic lobules in the presence of CCK derives from intracellular pools. Since CCK-stimulated amylase secretion remained elevated after fractional $^{45}\text{Ca}^{++}$ efflux began to decline (Fig. 5), and since the secretory response, but not $^{45}\text{Ca}^{++}$ efflux, is inhibited by incubation in Ca^{++} -free, EGTA-containing medium (6), tracer extrusion is probably not merely a result of increased release of secretory granule content (containing $^{45}\text{Ca}^{++}$; reference 9). Lastly, our preliminary data using pancreatic lobules loaded with the Ca^{++} indicator, aequorin (50), directly show a transient rise in the $[\text{Ca}^{++}]_c$ of fetal, neonatal, and adult pancreas in response to CCK (Chang, A., and W. Apfeldorf, unpublished result).

The magnitude of CCK-stimulated change in fractional $^{45}\text{Ca}^{++}$ efflux from tracer-loaded adult pancreas is in agreement with the results of Case and Clausen (5). However, we observed that the time after CCK addition at which maximal $^{45}\text{Ca}^{++}$ efflux occurred was ~ 12 min compared with ~ 5 min previously reported by Case and Clausen (5). The fact that these authors measured $^{45}\text{Ca}^{++}$ efflux from the uncinata pancreas of ~ 4 -wk-old rats could account for the difference in

the shape of the CCK-induced $^{45}\text{Ca}^{++}$ efflux curve that we present here for the adult gland.

The change in $^{45}\text{Ca}^{++}$ efflux induced by CCK (as well as by A23187) in fetal and neonatal pancreas appeared smaller when compared with the response in the adult gland (Fig. 6). The quantitative result is based on an assumption that the specific activity of the tracer within the cell remains constant during stimulation. While it is not certain that stimulation does not alter $^{45}\text{Ca}^{++}$ exchange in intracellular pools, the simplest explanation for the smaller change in $^{45}\text{Ca}^{++}$ efflux is that it directly reflects a smaller rise in the $[\text{Ca}^{++}]_i$.

It is possible that a particular pool from which Ca^{++} is mobilized in response to CCK, i.e., the trigger pool (49), is smaller in developing pancreas. A total Ca^{++} pool that is smaller or that exchanges more slowly cannot account, however, for the smaller increase in CCK-stimulated $^{45}\text{Ca}^{++}$ efflux in developing pancreas, since the total $^{45}\text{Ca}^{++}$ loaded was greater in lobules from fetal and neonatal pancreas than in adult pancreas (see Materials and Methods). In addition, a faster reuptake of $^{45}\text{Ca}^{++}$ from the medium by fetal and neonatal pancreas is unlikely to account for the small stimulated change in fractional $^{45}\text{Ca}^{++}$ efflux; tracer appearing in the medium was quickly diluted into a large extracellular volume (relative to the intracellular volume), and removed after brief time intervals (see Materials and Methods). However, these considerations do not exclude the possibility that calcium metabolism in developing pancreas differs somehow from that of the adult gland. Such a possibility, including a diminished number or efficiency of Ca^{++} transporters responsible for extruding Ca^{++} from the cell, could conceivably account for the smaller change in fractional $^{45}\text{Ca}^{++}$ efflux in neonatal and fetal pancreas.

The smaller elevation in $[\text{Ca}^{++}]_i$ in the neonatal pancreas is apparently sufficient to induce a full secretory response. In the fetal pancreas CCK binding is coupled to a rise in $[\text{Ca}^{++}]_i$ of the same magnitude as that in the neonatal gland, but hormone binding does not result in increased secretion. Analogous uncoupling of Ca^{++} mobilization from secretory response has been observed in a rat mast cell line undergoing mitosis (20), or treated with metalloendoprotease inhibitors or Zn^{++} (1).

A23187 induced a level of amylase discharge from neonatal and adult pancreas that was substantially less than CCK-stimulated secretion. To augment the magnitude of the secretory response induced by A23187, we made use of the finding that an increased cAMP level potentiates the effect of elevated $[\text{Ca}^{++}]_i$ in stimulating secretion (18, 19). Although the mechanism underlying the synergistic effect of cAMP and Ca^{++} on protein secretion is not known, $^{45}\text{Ca}^{++}$ efflux in the presence of dbcAMP plus A23187 was not greater than that of the ionophore alone. These data suggest that dbcAMP does not dramatically influence the A23187-stimulated increase in $[\text{Ca}^{++}]_i$; however, experiments that directly measure the effect of Ca^{++} ionophore and dbcAMP on $[\text{Ca}^{++}]_i$ are necessary to determine definitively the effect of cAMP on the cytosolic Ca^{++} concentrations.

Our finding that amylase release from the fetal pancreas was not stimulated by either dbcAMP or A23187 (Fig. 7 a) stands in contrast to previously published reports from our laboratory (10) and others (28, 55). Although we are uncertain of the cause of these differences, the incubation conditions used in the present study are different from those of the

past. We believe that our present results are accurate since direct comparisons of the basal and stimulated rates of secretion were made on the same incubation. Furthermore, leakage of lactate dehydrogenase was monitored to exclude the possibility that treatment of pancreatic lobules with dbcAMP and A23187, or phorbol dibutyrate and A23187, increased cell injury.

Although the fetal pancreas did not respond to stimulation with an enhanced rate of secretion, it is nevertheless competent to discharge its secretory products constitutively (Figs. 1 and 5, references 10 and 55). Autoradiographic analysis indicates that this basal secretion derives from zymogen granule stores (Arvan, P., and A. Chang, manuscript in preparation). Thus, it appears that the exocytotic machinery of the fetal gland is functional, but stimulation is not coupled to a secretory response. The onset of stimulus-secretion coupling may involve maturation of one or more signal transduction events.

It has been suggested that some of the effects of Ca^{++} mobilization that occur during CCK stimulation in the pancreas are mediated by Ca^{++} /calmodulin-dependent protein kinases (40), as well as by C-kinase (34). We are currently investigating the possibility that there is specific postnatal amplification of the activity of one or more of these kinases (17). In this regard, the observation that the rate of amylase secretion from fetal pancreas was not increased in the presence of phorbol dibutyrate either alone or in combination with A23187 or CCK (Fig. 8 a) is consistent with the hypothesis that an activated C-kinase and increased $[\text{Ca}^{++}]_i$ are not sufficient to trigger a secretory response. It is our hope that an understanding of the signal transduction event(s) in the developing pancreas will increase our insight into the mechanism by which the secretory response is regulated.

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