INTRACELLULAR DIVALENT CATION RELEASE
IN PANCREATIC ACINAR CELLS
DURING STIMULUS-SECRETION COUPLING

II. Subcellular Localization of the Fluorescent
Probe Chlorotetracycline

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ABSTRACT
Subcellular distribution of the divalent cation-sensitive probe chlorotetracycline (CTC) was observed by fluorescence microscopy in isolated pancreatic acinar cells, dissociated hepatocytes, rod photoreceptors, and erythrocytes. In each cell type, areas containing membranes fluoresced intensely while areas containing no membranes (nuclei and zymogen granules) were not fluorescent. Cell compartments packed with rough endoplasmic reticulum or Golgi vesicles (acinar cells) or plasma membrane-derived membranes (rod outer segments) exhibited a uniform fluorescence. In contrast, cell compartments having large numbers of mitochondria (hepatocytes and the rod inner segment) exhibited a punctate fluorescence. Punctate fluorescence was prominent in the perinuclear and peri-granular areas of isolated acinar cells during CTC efflux, suggesting that under these conditions mitochondrial fluorescence may account for a large portion of acinar cell fluorescence.

Fluorometry of dissociated pancreatic acini, preloaded with CTC, showed that application of the mitochondrial inhibitors antimycin A, NaCN, rotenone, or CCCP, or of the divalent cation ionophore A23187 (all agents known to release mitochondrial calcium) rapidly decreased the fluorescence of acini. In the case of mitochondrial inhibitors, this response could be elicited before but not following the loss of CTC fluorescence induced by bethanechol stimulation. Removal of extracellular Ca$^{2+}$ and Mg$^{2+}$ or addition of EDTA also decreased fluorescence but did not prevent secretagogues or mitochondrial inhibitors from eliciting a further response. These data suggest that bethanechol acts to decrease CTC fluorescence at the same intracellular site as do mitochondrial inhibitors. This could be due to release of calcium from either mitochondria or another organelle that requires ATP to sequester calcium.
Although pancreatic enzyme secretion is a function of extracellular calcium (21-23, 35), complete removal of calcium does not abolish secretion (3, 23, 29-31, 35). This and the fact that stimulation of secretion by either cholinergic agonists or CCK-PZ is accompanied by a four- to fivefold increase in $^{45 \text{Ca}}$ efflux from slowly exchangeable pools (7, 20, 27, 31) have led a number of investigators to suggest that stimulus-secretion coupling in this tissue involves release of calcium that is intracellularly bound or sequestered (7, 13, 29, 31, 35). Although efflux studies have shown that stimulation-induced release of isotopic calcium is independent of extracellular calcium (7, 20, 25, 31, 33, 35), further characterization of the releasable pool has been limited because efflux is multiphasic and not easily divided into components as has been done for other tissues such as cultured kidney cells and HeLa cells (5, 6; however, see reference 25).

Therefore, when we found that release of calcium during stimulation could be monitored by the fluorescent probe chlorotetracycline (CTC), we anticipated that subcellular localization of CTC fluorescence might provide new information on the site of calcium release. CTC fluorescence should localize at sites that are rich in membrane-associated divalent cations, and this distribution should be detected by either pharmacological or histological methods. Although CTC fluorescence does not provide direct information on the manner in which cations are sequestered at these sites, it does detect changes in the amount of membrane-associated $\text{Ca}^{2+}$ that results from $\text{Ca}^{2+}$ accumulation or release.

In this study, experiments with mitochondrial inhibitors indicate that a large portion of CTC fluorescence in acinar cells comes from mitochondria or an organelle requiring ATP for divalent cation sequestration and that this pool is probably the one undergoing stimulation-induced fluorescence changes. Fluorescence microscopy confirmed the presence of small, punctate sources of intense CTC emission in acinar cells during CTC efflux, a pattern resembling that for mitochondria in hepatocytes and in retinal photoreceptors. From these data, we suggest that stimulus-secretion coupling in the exocrine pancreas may be accompanied by redistribution of mitochondrial calcium stores.

MATERIALS AND METHODS

Preparation of Dissociated Pancreatic Acinar Cells and Other Cell Types

Isolated pancreatic acinar cells (used for fluorescence microscopy) were prepared by the method of Amsterdam and Jamieson (2) with minor modifications (34). Dissociated pancreatic acini (used for fluorometry) were prepared as described in the preceding paper (15). After preparation, cells or acini were preincubated for 60 min at 37°C in 25-ml Erlenmeyer flasks in a water bath shaken at 60 cycles per min. Both preincubation and experiments were carried out in a Tris-buffered Ringer's (TR) previously described (15) containing 1.28 mM Ca$^{2+}$ and 0.56 mM Mg$^{2+}$. However, in some experiments (Figs. 4-6 and 8) TR containing 2.56 mM Ca$^{2+}$ and 1.13 mM Mg$^{2+}$ was used and will be referred to as TR$^*$. Isolated hepatocytes were prepared by the method of Seglen (32) from livers of male Sprague-Dawley rats weighing 130-160 g.

Human blood was collected into heparinized tubes and used the same day. Whole blood was diluted 1:30 with TR$^*$ and preincubated as described above for acinar cells.

Pieces of retina were obtained from dark-adapted leopard frogs (Rana pipiens) and incubated at room temperature in the dark in diluted TR$^*$ (prepared by adding 2 vol of distilled water to 7 vol of TR$^*$ and equilibrating with 100% O$_2$). The incubated tissue was used directly or sliced into 100-μm-thick sections with a Sorvall TC-2 tissue sectioner (DuPont Instruments, Sorvall Operations, Newtown, Conn.) before microscopy. In one experiment, retina pieces were incubated for 1 h in buffer containing no calcium and 1.3 mg/ml crude collagenase (Worthington Biochemical Corp., Freehold, N. J.) to free individual photoreceptors.

Fluorometry

Dissociated acini were preincubated for 60 min in TR containing 100 μM CTC and 1% BSA, washed and resuspended in TR containing no CTC or BSA, then stirred at 37°C in a fluorometer cuvette, and fluorescence was monitored as described previously (15). As indicated, the TR in a few experiments (Fig. 2) contained no added Ca$^{2+}$ or Mg$^{2+}$. The mitochondrial inhibitors CICCP (carbonyl cyanide m-chlorophenylhydrazone, Calbiochem, San Diego, Calif.), Antimycin A and rotenone (both from Sigma Chemical Co., St. Louis, Mo.), as well as the ionophore A23187 (a gift from Dr. Robert Hamill, Eli Lilly and Co., Indianapolis, Ind.), were added to incubation media in small volumes of ethanol such that the final ethanol concentration was.
This concentration had no effect on CTC fluorescence or amylase release from acini.

**Microscopy**

Fluorescence, phase-contrast, and dark-field microscopy of isolated cells were carried out with a Zeiss photomicroscope. Fluorescence microscopy utilized a 200-watt mercury vapor lamp, a BG12 exciter filter, and 500-nm cutoff filter. Dark-field and phase-contrast microscopy used a tungsten lamp without filters. In most cases, incubated cells and medium (TR* containing 100 µM CTC) were applied to a glass slide and covered with a glass slip. To observe CTC emission during efflux (Fig. 7), cells were washed and resuspended in TR containing no CTC, and this suspension was applied to the slide. Cells adhered to the slide and flattened out within 5-10 min, which allowed clearer photography. Cells were photographed using films with ASA ratings of 125 (Plus-X) or 400 (Tri-X, both Kodak) and an exposure time for the fluorescence image of 0.5-6 min. Initial film magnification was 160 or 320.

**RESULTS**

**Effect of Metabolic Inhibitors on CTC Fluorescence from Dissociated Acini**

Because CTC is known to accumulate in mitochondria of living cells (19) and is able to monitor calcium accumulation and release from isolated mitochondria (8, 9, 26), we looked for a mitochondrial component of the CTC signal from acini by using specific mitochondrial inhibitors. As can be seen in Fig. 1 A (upper trace), application of C1CCCP, an uncoupler of oxidative phosphorylation, to CTC-loaded acini resulted in a rapid loss of CTC fluorescence. Fluorescence was decreased abruptly by a small amount, then continued to decrease for 10 min at a rate much faster than that observed before C1CCCP addition. In contrast, if the acini were stimulated with bethanechol and if the stimulation-induced fluorescence changes were allowed to run their course, addition of C1CCCP had no effect except for the small, abrupt intensity decrease. (This abrupt decrease is thought to be artifactual since C1CCCP in the same concentration range reduced fluorescence of CTC incorporated into erythrocyte membranes by 10%. Because this effect is a function of C1CCCP concentration and because C1CCCP exhibits an absorption maximum at 380 nm, it is probably due to the inhibitor absorbing incident light.) Similar results were obtained using the mitochondrial electron-transport inhibitors rotenone, antimycin A, and NaCN. Experiments in which the latter two inhibitors were used are depicted in Fig. 1B and C. Addition of either inhibitor to CTC-loaded acini produced a rapid loss of fluorescence; but again, if acini were first stimulated with bethanechol, these inhibitors were without effect. This strongly suggested that be-
thanechol and mitochondrial inhibitors decrease CTC fluorescence at the same intracellular site. On the basis of evidence in the preceding paper (15), we can presume that the inhibitors all do so by causing release of calcium from that site. The site could be mitochondria themselves, or it could be some other compartment that requires mitochondrial ATP to sequester calcium.

Effect of Extracellular Divalent Cations on CTC Fluorescence from Dissociated Acini

A portion of the fluorescence from CTC-loaded acini was found to be sensitive to changes in divalent cations. Adding 4.4 mM EDTA to acini incubated in TR caused a rapid drop in their fluorescence to 80-85% of the initial value within 2 min (third trace, Fig. 2A); thereafter, their fluorescence continued to drop at a rate faster than usual. In contrast, adding EDTA to acini incubated in TR that had no Ca^{2+} or Mg^{2+} to start with did not affect the rate at which they lost fluorescence (first traces, Fig. 2A and B). Presumably, this means that there is a pool of CTC that probes superficial divalent cations, which can be depleted quickly by simply removing divalent cations from the extracellular medium. In addition, because CTC fluorescence continued to drop at an increased rate even 5-10 min after EDTA addition, it would appear that chelation of extracellular divalent cations may also deplete calcium and magnesium from intracellular sites that are more slowly exchangeable.

The most important experiment was the following: when either bethanechol (Fig. 2A) or caerulein (Fig. 2B) was added to CTC-loaded acini in media lacking calcium and magnesium (regardless of whether EDTA was present or absent), a rapid loss of fluorescence was observed very similar to that seen normally when extracellular divalent cations were present. It is unlikely that this was due to displacement of some superficial pool of cations. Nearly all cells in this preparation of acini are directly exposed to the bulk medium, and in these experiments (first traces, Fig. 2A and B) the medium had lacked calcium and magnesium for 15 min before stimulation. However, the fluorescence loss produced by either caerulein or bethanechol in the absence of divalent cations was somewhat smaller than that seen when extracellular divalent cations were present (in the case of caerulein, this may be seen if the first and third traces of Fig. 2B are compared). We prefer to think that this means that the CTC-probed pool of calcium inside cells is slightly depleted beforehand by the incubation in calcium-free medium.

Effects of Ionophore A23187 on CTC Fluorescence from Dissociated Acini

The divalent cation ionophore A23187 is known to release calcium from mitochondria in whole cells (4) as well as from subcellular fractions such as isolated mitochondria (26) and sarcoplasmic reticulum vesicles (10, 11). It was of interest, then, to see whether divalent cation release induced pharmacologically by A23187 would be accompanied by appropriate changes in CTC fluorescence.

Application of 1 μM of A23187 to acini loaded with CTC and suspended in TR resulted in an obvious decrease in fluorescence after a lag which ranged from 1 to 4 min in five experiments (upper trace, Fig. 3). This concentration of ionophore...
Figure 3. Effects of the ionophore A23187 on fluorescence of CTC-loaded pancreatic acini. Acini were loaded with CTC and their fluorescence was monitored as described in Fig. 1. The resuspension medium was 3 ml of TR containing 1.28 mM Ca$^{2+}$ and/or 0.56 mM Mg$^{2+}$ as indicated at the beginning of each trace. Additions to the specified concentrations were: A23187, 1 $\mu$M; and EDTA, 4.4 mM. The results are representative of three similar experiments.

stimulated amylase release but did not damage the cells (14). Furthermore, if acini were incubated in TR and 4.4 mM EDTA were added to chelate extracellular calcium and magnesium (middle trace, Fig. 3), addition of A23187 resulted in an extremely rapid and extensive loss of CTC fluorescence; this action occurred with no lag. This indicates that A23187 releases divalent cations from an intracellular compartment and raises the possibility that the ionophore may trigger amylase release by releasing intracellular stores of calcium. Possibly, the action of A23187 is more rapid in the absence of extracellular calcium and magnesium because it is taken up extremely rapidly under these conditions (14).

In contrast, addition of A23187 to acini in calcium-free medium (with magnesium still present) produced no fluorescence changes (lower trace, Fig. 3). It is not clear in this case why the ionophore failed to release divalent cations, but it does correlate with the observation that even high concentrations of the ionophore do not release amylase under these conditions (14).

Subcellular Localization of CTC by Fluorescence Microscopy

One important reason for choosing CTC as a probe for divalent cations is that it can be localized by fluorescence microscopy. Isolated acinar cells proved to be a favorable preparation for this purpose because the subcellular distribution of fluorescence in single cells could be observed. Localization of CTC in the pancreatic acinar cell was complicated by the variety of organelles and the high density of membranes in this cell, as illustrated in the electron micrograph of Fig. 4. Isolated cells, although spherical, retained much of the regional organization seen in the pyramidal-shaped cell of intact pancreas. One or occasionally two nuclei were eccentrically placed, zymogen granules were confined to well-defined clusters usually abutting on the plasma membrane, and rough endoplasmic reticulum filled nongranular regions. Golgi membranes, characteristically found between nucleus and zymogen granules in intact tissue, appeared to be distributed throughout the zymogen granule cluster in isolated cells. Fig. 4 also demonstrates that acinar cells, incubated for 10 min in 500 or 100 $\mu$M, retained normal ultrastructure.

When visualized by fluorescence microscopy, CTC emission from acinar cells appeared yellow-green at low intensities and yellow at higher intensities. After 15–30 min of incubation in TR$^*$ (2.56 mM Ca$^{2+}$ and 1.13 mM Mg$^{2+}$) containing 100 $\mu$M CTC but no BSA, acinar cells varied in intensity, but by 60 min the large majority of cells exhibited an intense yellow fluorescence.

As shown in Fig. 5, nuclei appeared as nonfluorescent “holes” while areas containing zymogen granules (arrows) were clearly demarcated by their lower intensity (the inset shows that these areas were identified by intense scattering of incident light when the barrier filter was changed from a 500-nm cut-off to a 470–650-nm band pass filter). Other areas of these cells contained a uniform fluorescence, although superimposed on this was a punctate pattern of emission; note the stippled appearance of many cells in Fig. 5. We observed this punctate pattern in a majority of cells after incubation with CTC in TR containing 1% BSA. However, even under the best of conditions, this pattern required careful observation because of the intense uniform fluorescence also present, and, in fact, it could not be discerned in a portion of the cell population. In addition, the emission from the punctate pattern faded quickly. Fading is characteristic of CTC emission from mitochondria in other cells (1, 19).

The uniform pattern of fluorescence is most clearly shown in Fig. 6 depicting an isolated cell that was compressed between the slide and cover.
FIGURE 4 Ultrastructure of an isolated mouse pancreatic acinar cell incubated for 100 min in TR* containing 500 μM CTC. Cell components are similar to those of intact tissue, consisting of abundant zymogen granules, rough endoplasmic reticulum, and mitochondria. The region containing zymogen granules is well defined and interlaced with Golgi vesicles and condensing vacuoles. Bar, 2 μm. × 7,100.

glass after partial evaporation of the incubation medium. The phase-contrast image (Fig. 6 A) identifies the nucleus and zymogen granules. When compared with the fluorescence image (Fig. 6 B), it confirms that the nucleus did not fluoresce and that the granule-containing area was of lower intensity. In favorable photographs such as this, the position of individual zymogen granules (arrows in Fig. 6 A) could be matched to nonfluorescent “shadows” (arrows in Fig. 6 B). Fig. 6 C, showing incident light scattered from individual granules, not only confirms the position of previously located granules (arrows) but also provides evidence that incident light did not contribute to the fluorescence image of Fig. 6 B. CTC fluorescence appeared in all other areas of the acinar cell including areas of high intensity at the periphery of the cell and in the granule-containing region between granule shadows. It is not clear which (if any) of these highly fluorescent areas correspond to the punctate pattern seen in Fig. 5. Such a pattern is not obvious in this cell, possibly because of fading since it had been exposed to incident light for a number of minutes.
Fluorescence Microscopy of Acinar Cells during Efflux of CTC

Cells were incubated with CTC in the presence of BSA for 90 min, then washed and resuspended in TR (Ca\(^{2+}\) = 1.28 mM, Mg\(^{2+}\) = 0.56 mM) containing no CTC or BSA before microscopy; these conditions were comparable to those under which fluorometry was done. When cells were first placed on a slide, they exhibited a uniform yellow-green fluorescence with nonfluorescent nuclei and granule-containing regions as described for cells during CTC uptake. Superimposed upon this pattern in a majority of cells were small yellow dots (see Fig. 7 A). One could focus up and down through the cell and see that they were distributed throughout its volume; this was particularly apparent when focusing on spots above and below the nonfluorescent nucleus. Although the relative intensity of the punctate pattern in the cell of Fig. 7 A is greater than normal, most cells (in three experiments) exhibited such a pattern.

When cells had adhered to the slide and flattened out, it became clear that there were areas of intense fluorescence around the nucleus and at the edge of the granule mass. This distribution is clearly seen in the single cell of Fig. 7 B in which pointlike sources of light occur next to the nucleus, at the periphery of the zymogen granule region, and at the interface between the nucleus and granule cluster. A few spots occurred within the mass of granules itself. This can also be seen by comparing the fluorescence and phase-contrast images of another cell in Fig. 7 C and D. In this cell, all intense fluorescence is associated with the granule-containing area (whose limits are defined in the phase-contrast image) or is around the nucleus; again, the greatest fluorescence occurs at the interface between these two structures.

Although we have not yet been able to identify what structure is responsible for this intense fluorescence, one possibility is that these structures are mitochondria; this is suggested by the work of DuBuy and Showacre (19), who observed a pointlike localization of CTC fluorescence at mitochondria in cultured kidney cells and in liver cells. To evaluate this possibility, we studied CTC fluorescence from cells containing large numbers of mitochondria—isolated liver cells and rod photoreceptors.
**CTC Fluorescence from Dissociated Hepatocytes, Erythrocytes, and Photoreceptors**

Dissociated hepatocytes showed uniform emission from non-nuclear areas when incubated in 100 μM CTC for <1 h. Longer incubation produced a distinct punctate pattern of emission as shown in Fig. 8 A and B. In Fig. 8 B, two types of fluorescence can be distinguished: (a) a uniform emission of low intensity from non-nuclear areas, and (b) a punctate emission of high intensity distributed throughout the cell but with lower intensity over the nucleus. Nuclei were nonfluorescent as shown in Fig. 8 A. If one passed the plane of focus through a hepatocyte, well-focused punctate sources of light filled the cytosol at every plane. Although the punctate pattern varied in intensity from cell to cell, it was in all cases similar to that seen in acinar cells but much more distinct due to the low intensity of the uniform background fluorescence. Although we did not correlate these points of emission with the position of individual mitochondria, the fact that these isolated hepatocytes were filled with mitochondria suggests this identity (micrograph not shown). These observations confirm those of DuBuy and Showacre (19) on liver cells teased from intact tissue and suggest that mitochondria (because of their great number) may represent a large fraction of total cell intensity in hepatocytes.

Erythrocytes were used to observe CTC fluorescence from plasma membrane in the absence of mitochondrial or other intracellular membranes. After incubation in 500 μM CTC, these cells exhibited a uniform halo of low intensity requiring film exposure 5-10 times longer than that used for other cell types (see Fig. 8 C).

Photoreceptors in the retina provided the opportunity to compare CTC emission from a purely mitochondrial source with that from a nonmitochondrial membrane in the same cell. Electron microscopy has shown that the ellipsoid region of the rod inner segment is very tightly packed with mitochondria while the outer segment is exclusively filled with plasma membrane-derived disk

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*Figure 6: Phase-contrast, fluorescence, and dark-field images of an isolated mouse pancreatic acinar cell incubated in TR* containing 100 μM CTC. The nuclear area identified in phase contrast is not fluorescent. The position of zymogen granules in the phase-contrast and dark-field images (arrows in Fig. 6A and C, respectively) corresponds to nonfluorescent shadows (arrows in Fig. 6B). Bar, 5 μm. × 1,670.*
Fluorescence of isolated acinar cells during efflux of CTC. Cells were preincubated for 90 min in TR containing 100 μM CTC and 1% BSA, then washed and resuspended in TR containing no CTC or BSA. Micrographs were taken 20-40 min after resuspension. Bar, 10 μm. × 1,480. (A) Cell which is still rounded and displays a punctate pattern of fluorescence. (B and C) Isolated cells displaying punctate fluorescence around the nucleus and zymogen granule area (Z), respectively. (D) Phase-contrast image of the cell shown in (C).

membranes (28). Fig. 9 A depicts the CTC fluorescence of the retinal surface from above showing a regular array of photoreceptors. Outer segments are cylindrical and most easily seen at the horizon. The ellipsoid regions of the inner segments (arrows) appear at the base of the outer segments in a regular pattern. Both segments showed intense fluorescence after 30 min of incubation in 100 μM CTC. The emission from these regions is more easily assessed from Fig. 9 B and C showing a photoreceptor isolated by crude collagenase digestion of the retina. The phase-contrast image (Fig. 9 C) identifies the outer segment (OS), ellipsoidal region (E), and nucleus (N). Fluorescence from the outer segment is uniform whereas that from the ellipsoid region is intense and punctate. The punctate appearance is illustrated clearly in Fig. 9 E which shows a group of inner segments (with ellipsoid regions) whose outer segments have been sheared off. This demonstrates that
the CTC fluorescence from an identified area rich in mitochondria is intense and punctate and suggests that mitochondria could be the source of punctate fluorescence in acinar cells.

DISCUSSION
In the preceding paper (15), we presented evidence that CTC monitors release of calcium from an intracellular site during stimulation of pancreatic enzyme secretion. This report presents pharmacologic and histological data suggesting that this site is mitochondria.

The strongest evidence for this suggestion comes from experiments with metabolic inhibitors. FCCP (an uncoupler similar to CICCP), antimycin A, and cyanide are all known to release calcium from isolated mitochondria and to rapidly decrease CTC fluorescence from these organelles (8, 26). Thus, if CTC is present in mitochondria of acinar cells (which is highly likely on the basis of the intense CTC fluorescence from mitochondria in other cells; see Figs. 8 and 9 and reference 19), each of these inhibitors should decrease CTC fluorescence of acini, as was observed. Stimulation with bethanechol prevented these inhibitors from having their usual effect on CTC fluorescence,
presumably because the agonist had already triggered Ca\(^{2+}\) and CTC release from this site. An alternative explanation is that metabolic inhibitors, by blocking mitochondrial ATP synthesis, cause calcium to be released from a nonmitochondrial site which binds or sequesters calcium by an ATP-dependent mechanism. However, it is difficult to conceive of how bethanechol, acting at a nonmitochondrial site, could prevent an uncoupler or inhibitor of oxidative phosphorylation from releasing mitochondrial calcium and thereby affecting CTC fluorescence.

Experiments with A23187 (Fig. 3) offer further evidence that CTC fluorescence monitors divalent cation stores in an intracellular membrane-bounded compartment. A23187, in the presence of extracellular calcium, is known to increase Ca\(^{2+}\) uptake by acinar cells (16, 24, 36) and is
thought to increase cytoplasmic calcium levels as reflected by an increased amylase release (14). Yet, Fig. 3 shows that A23187 produced a decrease in CTC fluorescence. This must mean that the ionophore released ions stored in a noncytoplasmic compartment. This is reasonable since A23187, at the low concentrations used in these experiments (1 μM), is known to rapidly deplete calcium and reduce CTC fluorescence of mitochondria in whole cells (4), of isolated mitochondria (26), and of sarcoplasmic reticulum vesicles (10). In fact, Babcock et al. (4) have demonstrated in sperm that the predominant action of 1 μM of A23187 is to release rapidly mitochondrial calcium before promoting net calcium influx. They also showed that CTC fluorescence monitors the mitochondrial calcium release but not the subsequent calcium uptake. This observation supports the conclusion that CTC in acinar cells is monitoring A23187-induced calcium release from an intracellular compartment, with the further implication that this compartment might be mitochondria.

Fluorescence microscopy provided corroborating data: a small, intracellular organelle was an intense source of CTC fluorescence in isolated acinar cells which had been preloaded with the probe. This punctate pattern, found adjacent to the nucleus and at the periphery and within the granule-containing region (Fig. 7), was remarkably similar to CTC emission from mitochondria in hepatocytes (Fig. 8) and photoreceptors (Fig. 9), suggesting that mitochondria could be the source of this emission in acinar cells. This conclusion is supported by observations of DuBuy and Showater (19) showing that CTC selectively localizes in the mitochondria of cultured kidney cells, resulting in a punctate pattern of emission. But again, we cannot rule out the alternative that this fluorescence in acinar cells comes from Golgi vesicles or other small, smooth-surfaced vesicles which fill the granule-containing region (see Fig. 4).

Evidence from previous studies also supports the hypothesis that an intracellular organelle releases calcium during stimulation. Shelby et al. (33) have detected a pool of isotopic calcium in isolated acinar cells that is relatively tightly bound or sequestered (operationally defined as that ⁴⁰Ca²⁺ which remained with cell membranes after lysis in cold 10 mM EDTA). The size of this "membrane bound" pool was shown to decrease 40% in the first 5 min of stimulation with either carbachol or CCK-PZ octapeptide. Ceccarelli et al. (12) and Clemente and Meldolesi (17, 18), using subcellular fractionation, have shown that most calcium in acinar cells is associated with the plasma membrane, zymogen granule membranes, smooth-microsomal membranes, and mitochondria. By fractionating pancreatic fragments which had been loaded with ⁴⁰Ca²⁺ and washed for 2 h in isotope-free media, they found that all of these membranes contain slowly exchangeable calcium (17, 18). However, when they applied caerulein as little as 5 min before fractionation, the ⁴⁰Ca²⁺ content of the mitochondrial fraction decreased by 50% while the isotope contained in all other fractions was unchanged. This suggested that pancreatic secretagogues trigger a rapid release of mitochondrial calcium and that this might account for the increased efflux of slowly exchangeable ⁴⁰Ca²⁺ from pancreatic fragments seen at the onset of stimulation.

There is considerably less information on whether other cell membranes contribute to calcium release during secretion. Plasma membranes and zymogen granule membranes do bind large amounts of calcium (12), but is unlikely that these membranes account for the stimulation-induced decrease in CTC fluorescence. Although it was possible to detect CTC fluorescence from the erythrocyte plasma membrane (Fig. 8C), this membrane probably contributes little to fluorescence of the acinar cell and could not account for the 40% loss of intensity that secretagogues trigger. Similarly, CTC fluorescence from the granule-containing regions is patchy or absent (Fig. 7B and C), and granule contents appear to be nonfluorescent (Fig. 6). This suggests that CTC associates with neither granule membranes nor contents to any large extent.

On the other hand, areas of the acinar cell that contain rough endoplasmic reticulum display considerable fluorescence (compare Figs. 4 and 7B), raising the possibility that changes in their fluorescence can affect total cell intensity. However, it seems unlikely that this is the source of the stimulation-induced fluorescence changes, since Clemente and Meldolesi (17, 18) have shown that rough microsomal fractions of pancreas contain exceedingly small amounts of slowly exchangeable calcium (as indicated by ⁴⁰Ca²⁺ retention) and that this small amount is not affected by stimulation.

In conclusion, we have used CTC as a divalent cation probe to provide pharmacological and microscopy evidence which suggests or is consistent...
with the hypothesis that calcium is released from mitochondria or possibly another ATP-dependent calcium-sequestering organelle. Although evidence was presented that the Ca\(^{2+}\) release process studied does not require extracellular calcium, it should be emphasized that none of the data limit or delineate the role of extracellular calcium in stimulus-secretion coupling. There is growing evidence that extracellular calcium either modulates (13, 35) enzyme release or, by an increased calcium influx (23–25), acts in concert with intracellular calcium release to trigger secretion. Thus, data in this study substantiate that intracellular calcium release is involved in stimulus-secretion coupling but do not require that this be the only way in which calcium is involved.

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