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

I. Use of Chlorotetracycline as Fluorescent Probe

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ABSTRACT

Stimulus-secretion coupling in pancreatic exocrine cells was studied using dissociated acini, prepared from mouse pancreas, and chlorotetracycline (CTC), a fluorescent probe which forms highly fluorescent complexes with Ca\(^{2+}\) and Mg\(^{2+}\) ions bound to membranes. Acini, preloaded by incubation with CTC (100 \(\mu\)M), displayed a fluorescence having spectral properties like that of CTC complexed to calcium (excitation and emission maxima at 398 and 527 nm, respectively). Stimulation with either bethanechol or caerulein resulted in a rapid loss of fluorescence intensity and an increase in outflux of CTC from the acini. After 5 min of stimulation, acini fluorescence had been reduced by 40% and appeared to be that of CTC complexed to Mg\(^{2+}\) (excitation and emission maxima at 393 and 521 nm, respectively). The fluorescence loss induced by bethanechol was blocked by atropine and was seen at all agonist concentrations that elicited amylase release. Maximal fluorescence loss, however, required a bethanechol concentration three times greater than that needed for maximal amylase release. In contrast, acini preloaded with ANS or oxytetracycline, probes that are relatively insensitive to membrane-bound divalent cations, displayed no secretagogue-induced fluorescence changes. These results are consistent with the hypothesis that CTC is able to probe some set of intracellular membranes which release calcium during secretory stimulation and that this release results in dissociation of Ca\(^{2+}\)-complexed CTC.

KEY WORDS exocrine pancreas · stimulus-secretion coupling · calcium · fluorescent probe · chlorotetracycline

Pancreatic acinar cells are thought to use calcium as a second messenger because the divalent cation ionophore, A23187, is able to increase enzyme release from these cells in the presence of extracellular calcium (10, 16, 32, 39). However, the manner in which physiological secretagogues initiate a rise in cytosol calcium is not known. Acetylcholine or CCK-PZ may stimulate influx of extracellular Ca\(^{2+}\) as suggested by Kondo and Schulz (20, 21) although this point is still controversial because of conflicting data (4, 19–21, 38). There is clear evidence, though, that these secretagogues initiate a rapid increase of Ca\(^{2+}\) efflux (4, 18, 26, 32) and stimulate enzyme release even in the absence of extracellular Ca\(^{2+}\) (2, 27, 31, 32, 37, 38). This suggests that release of...
intracellular calcium occurs in response to secretagogues and that this leads to a rise in cytosol Ca$^{2+}$ activity.

Since most calcium in acinar cells is bound to membranes or sequestered in mitochondria (9, 13, 14), it was anticipated that monitoring these Ca$^{2+}$ stores in living acinar cells might detect this release process. Chlorotetracycline (CTC) was chosen as a probe for this purpose because it easily incorporates into cell membranes and its fluorescence is sensitive to Ca$^{2+}$ and Mg$^{2+}$ in amphipathic environments. In the presence of erythrocyte and microsomal membranes (6, 17), detergent micelles (6), or alcohols (5), this probe forms chelation complexes with Ca$^{2+}$ and Mg$^{2+}$ which fluorescence with 50- to 200-fold greater intensity than does uncomplexed CTC in aqueous solution. This fluorescence enhancement is dependent on the amount and type of divalent cation bound by the micelle or membrane. In addition, the Ca$^{2+}$ and Mg$^{2+}$ complexes of CTC in a membrane environment can be distinguished by their fluorescence excitation and emission spectra (5). Fluorescence of CTC in mitochondria (5, 6, 24) and sarcoplasmic reticulum vesicles (7, 8) has shown to parallel Ca$^{2+}$ movements produced in these organelles by substrates and inhibitors. CTC has also been used as an approach to studying cell calcium in platelets (22) and in pancreatic islets (31).

In this report, dissociated pancreatic acini are preincubated with CTC, then suspended in a fluorometer cuvette to allow continuous recording of fluorescence from cell-associated CTC. A rapid decrease in CTC fluorescence is observed upon stimulation with either cholinergic or hormonal secretagogues, and this intensity change is accompanied by dissociation of CTC complexes to calcium.

MATERIALS AND METHODS

Buffers

Krebs-Henseleit bicarbonate buffer (KHB) contained per liter (in mM): NaCl, 118; KCl, 4.7; CaCl$_2$, 2.56; MgCl$_2$, 1.13; NaHCO$_3$, 25; Na$_2$HPO$_4$, 1.15; and was equilibrated with 95% O$_2$–5% CO$_2$ to give a pH of 7.35. Tris buffered Ringer's (TR) contained per liter (in mM): NaCl, 134; Tris HCl, 10; CaCl$_2$, 1.28; MgCl$_2$, 5.6; NaH$_2$PO$_4$, 1; KCl, 4.7; and was adjusted to a pH of 7.38 with NaOH and equilibrated with 100% O$_2$. Both KHB and TR contained 14 mM glucose, 1% bovine serum albumin (BSA, fraction V, Miles Laboratories Inc., Elkhart, Ind.), 0.1 mg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Mo., type 1-S), and Minimal Eagle's Medium amino acid supplement (Grand Island Biological Co., Grand Island, N. Y.).

Preparation of Dissociated Pancreatic Acini

Dissociated pancreatic acini were prepared by the method that Amsterdam and Jamieson (1) used to prepare isolated cells, but it was modified to exclude the divalent cation chelation step (36). This isolated acinar preparation consisted of groups of up to 20-30 cells surrounding one or two lumens with individual cells retaining the polarity that they possess in intact tissue (36).

Pancreatic tissue (0.7-1.0 g) was obtained from 8 to 12 Swiss white mice (18-24 g) which were fasted 16 h before use. Dissociation of the tissue involved two sequential digestions of 10 and 40 min with a KHB enzyme mixture containing per milliliter: 75 U of chromatographically pure collagenase, 0.05 mg of a-chymotrypsin (both from Worthington Biochemical Corp., Freehold, N. J.), and 1.8 mg of hyaluronidase. Dissociation was completed by application of shearing forces produced by pipetting through a restrictive orifice. After dissociation, acini were filtered through 150-gm mesh nylon cloth (Nytex) (Tobler, Ernst & Traber, Elmsford, N. Y.) and centrifuged through KHB containing 4% albumin to remove digestive enzymes and broken cells.

After preparation, acini were preincubated for 60 min in TR. Then, at the beginning of experiments, acini were washed once and resuspended at a density of 0.55 to 1.65 mg of protein per ml in TR with the designated modifications. Unless noted otherwise, preincubation and incubations for experiments were carried out in 25-ml siliconized glass flasks at 37°C in a water bath shaken at 60 cycles per min.

Measurements of Amylase or Fluorescent Probes Released into the Medium

Dissociated acini were incubated in 2-ml aliquots of TR with additions or deletions as noted. At the start of the incubation period, 1 ml of the cell suspension was centrifuged at 8000 g for 20 s in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, N. Y. no. 3200) and the supernate was assayed for amylase or for fluorescent probe content. The initial values (usually ~0.2 U/mg protein for amylase) were subtracted from values obtained after incubation to obtain the enzyme activity or the amount of probe released during incubation. The pellets were sonicated in isotonic saline, and the protein content was assayed by the Lowry method (23). Amylase activity was measured by the method of Rinderknecht et al. (30) and is expressed in international units based on the reported activity of the amylase standard (Sigma Chemical Co., type VI). Fluorescent probes were assayed as described below.

Uptake of CTC

Dissociated acini were washed, then incubated in 7
ml of TR containing 100 μM CTC but not BSA. At
designated times, 0.7-ml aliquots were withdrawn,
added to 8 ml of ice-cold, isotonic saline, and filtered
with suction through a Nuclepore Corp. (Pleasanton,
Calif.) filter (3-μm pore diam). The filters, with acini,
were placed in 2 ml of isotonic saline and sonicated for
10 s with a probe sonicator (as described below). Ali-
quoits of the sonicate were assayed for protein by the
method of Lowry et al. (23) and for CTC as described
below.

**Assay of CTC, Oxytetracycline (OTC), and
1-Anilino-8-Naphthalenesulfonate (ANS)**

To assay CTC or OTC, a 200-μl aliquot of either
sonicated acini or incubation medium was added to 800
μl of a solution containing 10 mM sodium dodecyl
sulfate (SDS) (Sigma Chemical Co., 95% pure) and
1.13 mM MgCl₂. Fluorescence was measured using
excitation at 380 nm and read at 520 nm for CTC and
510 nm for OTC. The amount of CTC or OTC present
in the assay was determined from standard curves which
were linear between 10⁻¹⁰ and 2 x 10⁻⁹ mol of CTC or
OTC. The presence of Ca²⁺ (1.28 mM) in the medium
samples lowered the reading by 6%. In addition to its
excellent sensitivity, this assay offered the advantage
that sonicated membranes had no effect on the assay (if
the fluorescence of a membrane blank was subtracted)
and were readily solubilized in the SDS reagent.

To assay ANS, a 200-μl aliquot of incubation medium
was added to 800 μl of ethanol (100%, Rossville Gold
Shield, IMC Chemical Group, Inc., Organic Chemicals
Div., Boston, Mass.) and fluorescence was excited at
370 nm and read at 470 nm. ANS contained in the
assay was determined from a standard curve which was
linear between 10⁻¹⁰ and 2 x 10⁻⁹ mol of ANS.

**Fluorescent Probe Addition**

CTC HC₁ and OTC HC₁ were obtained from the
ICN Nutritional Biochemical Div. (Cleveland, Ohio)
or from Sigma Chemical Co., and ANS from the East-
man Organic Chemicals Div., Eastman Kodak Co.
(Rochester, N. Y.); all were used without further purifi-
cation. They were dissolved directly in the preincubation
or incubation medium on the day of use. CTC was
added before addition of calcium or magnesium to the
buffer because this probe is only moderately soluble in
divalent cation-containing media. The methanol used as
a solvent for recording spectra was reagent grade from
Mallinkrodt Inc. (St. Louis, Mo.) and contained no
fluorescent impurities.

**Fluorometry**

Fluorometric experiments were carried out with an
Aminco-Bowman spectrophotofluorimeter (American Instrument
Co., Silver Spring, Md.) having a band pass of
15 nm. The instrument was modified to allow stirring
and temperature control of the cuvette contents. Uncon-
rected spectra were recorded on strip chart recorder
(Esterline Angus Instrument Corp., Indianapolis, Ind.)
with the wavelength scale marked manually via the
event marker. Quinine sulphate dihydrate (1 mg/liter
0.1 N H₂SO₄) was used to verify the wavelength settings
for the excitation monochromator at 254 and 352
nm.

To study fluorescence of acini during efflux of CTC,
acini were preincubated for 60-80 min in TR containing
100 μM CTC and 1% BSA. The CTC-loaded acini
were washed once and resuspended in 3 ml of medium
containing no CTC or BSA (calcium, magnesium, or
EDTA present as indicated), stirred in a cuvette main-
tained at 37°C, and their fluorescence was monitored
continuously on a strip chart recorder.

Uptake experiments were carried out in one of two
ways. Whole or sonicated acini were added as a concen-
trated suspension in a 0.15 or 0.3 ml volume, via
syringe and 16-gauge needle to a cuvette containing 3
ml of CTC-containing medium at 37°C and the fluores-
cence intensity of the suspension was monitored contin-
uously as described above. Alternatively, acini were
suspended in 3 ml of CTC-containing medium, incu-
inated in 25-ml siliconized flasks in a shaking water bath
at 37°C, and, at designated times, poured into a cuvette
and the fluorescence intensity was read.

The "concentrated" suspensions of acini added to
the cuvette were prepared as follows: whole acini (0.5-1.5
mg of protein) were washed once and resuspended in
0.15 ml of medium identical to that in the cuvette but
lacking CTC. Concentrates of sonicated acini were pre-
pared by suspending an equal amount of dissociated
acini in 0.15 ml of 7 mM EDTA (pH 7.4) and sonicating
for 10 s with a probe sonicator (Bronwill, Rochester,
N. Y., Biosonik IV, set at 15, low scale).

The suspension medium for all fluorometric experi-
ments was TR containing calcium and EDTA as indi-
cated but without albumin to minimize background
fluorescence. In most experiments, fluorescence was
excited at 400 nm and emission was read at 525 nm.

**Microscopy**

Fluorescence microscopy was carried out with a Zeiss
photomicroscope equipped with a 200-watt mercury
vapor lamp, BG 12 exciter filter, and a 500-nm cutoff
filter. Isolated pancreatic acinar cells (prepared
by the method of Amsterdam and Jamieson [1] as
modified by Williams et al. [37]) were suspended in TR
and applied to a glass slide and covered with a glass
cover slip.

**RESULTS**

**Fluorescence Changes in CTC-Loaded Acini during Stimulation**

Acini were preincubated for 60 min in TR with
100 μM CTC and 1% BSA, then washed and
Figure 1 Effect of bethanechol, caerulein, atropine, and KCl on fluorescence of CTC-loaded pancreatic acini. Acini were preincubated for 60-80 min in TR containing CTC (100 μM) and 1% BSA, then washed and resuspended in 3 ml of TR and stirred at 37°C in a fluorometer cuvette. Fluorescence was excited at 400 nm and read at 525 nm. Additions to the following concentrations were, bethanechol, 100 μM; caerulein, 10 ng/ml; atropine, 5 μM; and KCl, 58 mM.

resuspended in medium containing no CTC or BSA. These “CTC-loaded” acini, when stirred in a fluorometer cuvette, exhibited a fluorescence which decreased with time. As shown in Fig. 1 A, the intensity decreased rapidly in the first 5 min and more slowly but consistently thereafter. Addition of bethanechol (100 μM) during the slow phase (see Fig. 1 B) resulted in a marked loss of fluorescence during the first 6 min of secretagogue action. After this time, fluorescence intensity continued to decrease at a rate similar to that before bethanechol addition.

A similar decrease in fluorescence was seen upon application of caerulein, a 10 amino acid polypeptide containing the active site of CCK-PZ (Fig. 1 C). The drop in intensity was generally more abrupt with caerulein than with bethanechol even at supramaximal doses of bethanechol (300 μM). As shown in Fig. 1 D, atropine completely inhibited the bethanechol-induced fluorescence change but did not affect that resulting from caerulein stimulation. These effects parallel the ability of atropine to inhibit bethanechol-stimulated but not caerulein-stimulated amylase release and suggest that the fluorescence decrease, like secretion, is initiated by bethanechol acting at a muscarinic cholinergic receptor or by caerulein acting as another receptor that is not blocked by atropine. Because stimulation by either bethanechol or caerulein in intact pancreas is accompanied by depolarization, we tested whether depolarization itself could produce the fluorescence changes seen during stimulation. As shown in Fig. 1 E, an increase of extracellular K+ from 4.7 to 58 mM—a concentration known to substantially depolarize acinar cells (25)—had no effect on the fluorescence of CTC-loaded acini, while 4 min later bethanechol had its usual effect.

To study the relationship between amylase release and the decrease in CTC fluorescence produced by bethanechol, we compared the dose-response relation for each of these actions. As shown in Fig. 2 A, the ability of bethanechol to elicit a decrease in CTC fluorescence was dose-dependent. Raising the bethanechol concentration increased the extent of fluorescence loss and decreased the response time. Fig. 2 B (open circles) shows that the fluorescence decrease in the first 8 min after bethanechol addition is half-maximal at a bethanechol concentration of 20 μM and maximal at 100 μM and higher concentrations. Acini preincubated with CTC in an identical manner, then incubated for 10 min (although in a separate experiment), released amylase in response to somewhat lower concentrations of bethanechol; half-maximal and maximal stimulation occurred at 5 and 30 μM bethanechol, respectively). These doses are similar to those required to elicit amylase release from fragments of mouse pancreas (35), and the maximal stimulation achieved in acini, as high as 400% above unstimulated values.
Dose dependence of the fluorescence decrease and amylase release elicited by bethanechol from acini loaded with CTC. Acini were preincubated for 60 min in CTC (100 μM) and 1% BSA, then washed and resuspended in TR containing no CTC or BSA. (A) Acini were placed in a fluorometer cuvette and their fluorescence was monitored continuously as described in Fig. 1. Bethanechol, at the concentrations indicated, was added at the arrow in each trace. All traces used cells from the same preparation. These results are representative of four similar experiments. (B) Acini (2-ml aliquots) were incubated in 25-ml flasks in a shaking water bath for 10 min. Amylase released during incubation (filled circles) is expressed as the relative increase above control values, i.e., percent increase = (stimulated - control)/control x 100. Values are means ± SE of triplicate incubations in a single experiment. Results are representative of three similar experiments. Open circles indicate the relative decrease in fluorescence of CTC-loaded acini during the first 8 min of bethanechol stimulation. Values are means ± SE using data taken from traces like those in panel A, which were obtained in four independent experiments. The intensity decrease observed after application of 100 μM bethanechol is designated 100% and that in control traces is designated 0%.

in some experiments, is greater than the 150-250% increase usually obtained with fragments (35, 38). This provides evidence that CTC-loaded acini maintain an excellent secretory response. Although the dose-response curves for stimulation-induced fluorescence changes and amylase release are different, it is clear that bethanechol did elicit fluorescence changes at concentrations which stimulate amylase release.

Divalent Cation Complexation of CTC Incorporated into Acinar Cell Membranes

To demonstrate that the fluorescence of CTC incorporated into acinar cell membranes is dependent on the presence of divalent cations, we compared the effect of extracellular Ca²⁺ and Mg²⁺ on the fluorescence of CTC in the presence of whole and sonicated acini. Fig. 3 A shows that addition of acini to CTC in the presence of calcium resulted in a gradual increase in fluorescence, almost reaching a plateau in 20 min. Addi-
tion of acini to CTC in the absence of divalent cations (EDTA, Fig. 3 A) resulted in a fluorescence increase having a similar time-course and slightly lower magnitude. In contrast, if acini were broken by sonication to expose intracellular as well as plasma membrane to the extracellular medium and then added to CTC in EDTA-containing media, almost no increase in fluorescence was observed (see Fig. 3 B). The small increase seen was largely accounted for by light scattering of the added membranes. Upon addition of 1.33 mM Ca\(^{2+}\) to these membranes, CTC fluorescence was rapidly increased 60-fold, reaching a plateau within 5 min (note difference in time scale between Fig. 3 A and B). Complexation of this calcium by addition of 4.4 mM EDTA reduced the fluorescence intensity to the value observed before Ca\(^{2+}\) addition. Addition of 1.33 mM Mg\(^{2+}\) resulted in a considerably smaller intensity increase, half of which is accounted for by fluorescence changes in the medium while further addition of 1.33 mM Ca\(^{2+}\) increased fluorescence intensity to the value seen when Ca\(^{2+}\) alone was present.

These results indicate that fluorescence of CTC in acinar cell membranes is markedly dependent on divalent cations as observed previously in other membrane systems (5). The greater fluorescence enhancement with Ca\(^{2+}\) than Mg\(^{2+}\) is similar to the selectivity seen for CTC incorporated into erythrocyte membranes (17). The fluorescence increase seen when CTC is taken up by whole acini in the presence of EDTA is too large to be accounted for unless CTC is incorporated into intracellular membranes which bind divalent cations and are not exposed to EDTA. The slower uptake of CTC by whole cells in either Ca\(^{2+}\) or EDTA media is also consistent with CTC having to cross the plasma membrane to produce an enhancement in fluorescence.

Because the fluorescence spectra of the Ca\(^{2+}\) and Mg\(^{2+}\) complexes of CTC differ, it is possible to determine which ion is complexed to CTC incorporated into membranes. Alcohol-water mixtures have been commonly used as a model system for studying the spectra of divalent cation complexes of CTC because spectra obtained in these solvents are similar to those observed for CTC in biological membranes (6, 33). Alcohols have the advantage that formation of CTC complexes is not determined by the affinity and selectivity of membrane-binding sites for Ca\(^{2+}\) and Mg\(^{2+}\), but rather by the properties of CTC itself. In 90% methanol-10% H\(_2\)O containing 10 mM Tris HCl, pH 7.4, and 130 mM NaCl, the normalized spectra of Ca\(^{2+}\)-CTC, Mg\(^{2+}\)-CTC, and uncomplexed CTC differ substantially as shown in Fig. 4 (solid symbols). Excitation and emission maxima for free CTC are at 382 and 536 nm (Fig. 4, filled circles; emission spectrum not shown), while those for Mg\(^{2+}\)-CTC are at 385 and 519 and those for Ca\(^{2+}\)-CTC are at 398 and 529 nm.

![Figure 4](https://example.com/figure4.png)

**Figure 4** Excitation and emission spectra of CTC and its divalent cation complexes in methanol-Tris Ringer's and comparison of these to spectra from unstimulated acini loaded with CTC. To CTC (100 μM) in 90% methanol:10% 10 mM Tris HCl, pH 7.4, in 130 mM NaCl were added 1 mM Ca\(^{2+}\) (▲); 1 mM Mg\(^{2+}\) (●); or 0.33 mM EDTA (●), and excitation and emission spectra were recorded at 30°C. Unstimulated acini loaded with CTC (○) were in TR and stirred continuously at 37°C in a cuvette while spectra were recorded. All spectra were normalized, and those for whole acini (○) were corrected by subtracting the light scattering of acini not incubated with CTC. Relative peak intensities for excitation spectra of CTC in the presence of Mg\(^{2+}\), Ca\(^{2+}\), EDTA, and acini were 27.6, 18.8, 0.42, and 28.5, respectively. In all cases, fluorescence was excited at 400 nm and emission read at 525 nm.
Thus, the entire excitation and emission peaks for Mg$^{2+}$-CTC are shifted 10 nm to the blue compared to these peaks for Ca$^{2+}$-CTC. Furthermore, the peak intensities for CTC in the presence of 1 mM Ca$^{2+}$ and 1 mM Mg$^{2+}$ are 45- and 60-fold greater, respectively, than the intensity of CTC in the presence of EDTA, demonstrating that CTC fluorescence is very sensitive to divalent cation complexation in this solvent.

The excitation spectrum of pancreatic acini preincubated in CTC and then washed free of extracellular probe (Fig. 4, open symbols) is identical to that of the Ca$^{2+}$ complex of CTC in 90% methanol. It is unlike the spectrum of uncomplexed or the Mg$^{2+}$-complexed probe in 90% methanol and unlike the excitation spectra of either divalent cation complex in aqueous medium ($\lambda_{\text{max}} = 385$ and 383 nm for Mg$^{2+}$-CTC and Ca$^{2+}$-CTC, respectively, in TR). The emission spectrum of cell-associated CTC is not so conclusive, since it appears as a narrower peak that overlaps in part the spectrum of each complex. Yet, the spectrum is clearly not that of uncomplexed CTC ($\lambda_{\text{max}} = 536$ nm, spectrum not shown) and its maximum at 526 nm suggests that it is CTC complexed to a mixture of Mg$^{2+}$ and Ca$^{2+}$, mostly the latter. From these spectra, we conclude that fluorescence of CTC in acinar cells is emitted from probe molecules complexed to divalent cations, primarily Ca$^{2+}$, in a membrane environment. This conclusion is corroborated by fluorescence microscopy which shows that the distribution of CTC fluorescence in acinar cells is similar to that of intracellular membrane (see Fig. 8 and reference 12).

**Preferential Loss of Fluorescence from the Calcium Complex of CTC during Secretory Stimulation**

The spectral differences between the Ca$^{2+}$ and Mg$^{2+}$ complexes were further utilized in studying changes in divalent cation complexation to CTC in pancreatic acini during stimulation of secretion. As shown in the inset of Fig. 5, application of caerulein to CTC-loaded acini elicited a rapid intensity decrease. Spectra taken 2 min before stimulation (Fig. 5) were similar to those described above for CTC in unstimulated acini and had excitation and emission maxima at 398 and 527 nm, respectively. 5 min after stimulation, the peak fluorescence intensity had decreased 39% and excitation and emission maxima had shifted to 393 and 521 nm, respectively. In control experiments the spectra of CTC in acini not stimulated but stirred continuously in a cuvette for 40 min underwent no shifts although they did sustain a loss in intensity. This suggests that...

![Figure 5](image-url)

**Figure 5** Excitation and emission spectra of CTC-loaded acini before and after stimulation with caerulein. Acini were loaded with CTC as described in Fig. 1, then washed and resuspended in 3 ml of TR containing no CTC or BSA, and their fluorescence was monitored while they were being stirred at 37°C in a cuvette. Solid line spectra were taken 2 min before stimulation at (1) in the inset and dashed spectra taken 5 min after stimulation at (2) in the inset. Caerulein was added at the arrow to a concentration of 17 ng/ml. Light scattering of acini containing no CTC is also shown. Fluorescence was excited at 400 nm and emission read at 525 nm.
stimulation may induce loss of signal from a specific subcellular compartment rather than simply facilitating outflux by the normal routes.

If these spectra are normalized, as shown in Fig. 6 A, it can be seen that the entire excitation and emission peaks from unstimulated cells (open circles) were shifted 6 nm to shorter wavelengths after stimulation (filled circles). Cholinergic stimulation shifted these peaks in an identical manner, as reported previously (11).

As judged by the model studies in 90% methanol that were described above, the fluorescence of CTC in acini is from a mixture of its Ca$^{2+}$ and Mg$^{2+}$ complexes. However, before stimulation the major component appears to be Ca$^{2+}$-complexed CTC whereas after stimulation it is largely the Mg$^{2+}$-complexed probe. The following experiment supports this conclusion: Spectra of identical aliquots of CTC-loaded acini were resuspended in medium containing Ca$^{2+}$ or Mg$^{2+}$ but no CTC. The cells were then broken by sonication to expose CTC-laden intracellular membranes to the divalent cation contained in the extracellular medium, and the spectrum was recorded immediately. As shown in Fig. 6 B, CTC incorporated into acinar cell membranes and exposed to Ca$^{2+}$ (filled triangles) exhibited excitation and emission maxima almost identical to the maxima observed in unstimulated whole acini (open circles). In contrast, the excitation and emission peaks for CTC in acinar membranes exposed in Mg$^{2+}$ were shifted ~7 nm and exhibited maxima close to those observed for CTC in acini after stimulation.

We conclude from these data that there is a preferential loss of signal from Ca$^{2+}$-complexed CTC during stimulation.

**Control Experiments with Divalent Cation-Insensitive Probes**

Further evidence that the decrease in CTC fluorescence during stimulation resulted from dissociation of CTC-divalent cation complexes came from experiments on acini loaded with OTC or ANS. These probes are similar to CTC in that they undergo fluorescence enhancement when transferred from aqueous to membrane environments but are unlike CTC in that this enhancement is relatively insensitive to the amount of Ca$^{2+}$ and Mg$^{2+}$ bound to the membrane. Acini preincubated with either 200 μM ANS (Fig. 7 A) or 100 μM OTC (Fig. 7 B) displayed a gradual decline of fluorescence which was entirely unaffected by the addition of bethanechol to stimulate secretion, while acini preincubated with CTC (20 or 100 μM) showed the usual intensity decrease.

Preincubation of acini with OTC or ANS had no effect on their ability to secrete amylase under basal or stimulated conditions as shown in Table I.

One possibility was that the stimulated decrease of CTC fluorescence was the result of CTC partitioning out of intracellular membranes after dissociation of its divalent cation complexes. Consist-
FIGURE 7 Fluorescence of acini loaded with ANS or OTC is not affected by bethanechol stimulation. In the upper panel, acini were preincubated for 60 min with ANS (200 μM) or CTC (100 μM) in TR containing no BSA; in the lower panel, acini were preincubated for 60 min with OTC (100 μM) or CTC (20 μM) in TR containing 1% BSA. After preincubation, acini were washed and resuspended in TR containing no BSA or fluorescence probes, and stirred continuously at 37°C in a fluorometer cuvette. Fluorescence was excited and emission read at 380 and 470 nm, respectively, for ANS, at 380 and 510 nm for OTC, and at 400 and 525 nm for CTC. Bethanechol (100 μM) was added at the arrows.

ent with this possibility is that release of CTC into the extracellular medium was increased 113% during the first 10 min of stimulation (see Table I). In contrast, stimulation did not affect the efflux of ANS or OTC, probes which also displayed no intensity changes during stimulation.

These data indicate that both the decrease in CTC fluorescence and its increased release into the medium stem from its affinity for divalent cations in acinar cell membranes. Therefore, we conclude that the stimulation-induced loss of CTC fluorescence results from release of divalent cations that are bound or sequestered at membranes and that this release is followed by dissociation of CTC-cation complexes and partitioning of free CTC out of the membranes. The preferential loss of signal from the Ca²⁺-CTC complex suggests that the ion released is calcium.

The absence of any changes in ANS or OTC fluorescence during stimulation also gave assurance that those changes seen for CTC fluorescence did not result from optical effects. The excitation and emission maxima of OTC (380 and 510 nm, respectively) were nearly identical to those of CTC (400 and 520 nm, respectively). In addition, ANS, OTC, and CTC all displayed a similar pattern of emission from isolated acinar cells (see Fig. 8). The nuclei were nonfluorescent, and areas containing zymogen granules (arrows) had a lower fluorescence intensity. This pattern (as discussed in the following paper) is similar to the distribution of intracellular membranes and reflects the fluorescence enhancement that these probes display in a membrane environment. Thus, if fluorescence had been altered optically during stimulation, acini loaded with ANS and OTC should have displayed similar fluorescence changes.

Detection of an Intracellular Compartment whose Interaction with CTC is Stimulation-Dependent

We studied the loss of CTC from acini further by measuring the CTC content of acini and of the incubation medium during application of bethanechol. Fig. 9 A (open circles) shows that acini that were preincubated in 100 μM CTC for 60 min and then washed and resuspended in medium without CTC contained about 6.5 nmol of CTC per mg protein. This content decreased slowly (~1 nmol/mg protein over the next 40 min) and could be completely accounted for by CTC appearing in the medium (Fig. 9 B, open squares). Loss of CTC during stimulation (Fig. 9 A, filled circles) was much greater: 33% in the first 20 min of stimulation compared to a loss of only 10% from control acini during the same period. Again, this stimulation decrease of CTC content was reasonably well accounted for by increased amounts of CTC appearing in the incubation medium (Fig. 9 B, filled squares). Thus, increased release of CTC and of amylase (see Fig. 9 C) was
Table I

Amylase and Fluorescent Probe Release from Pancreatic Acini Preincubated with CTC, ANS, or OTC

<table>
<thead>
<tr>
<th>Probe in preincubation medium</th>
<th>Amylase release</th>
<th>Fluorescent probe release</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Bethanechol (100 μM)</td>
</tr>
<tr>
<td></td>
<td>U/10 min/mg protein</td>
<td>nmol/10 min/mg protein</td>
</tr>
<tr>
<td>Exp 1</td>
<td></td>
<td></td>
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<tr>
<td>CTC</td>
<td>0.32 ± 0.05</td>
<td>1.03 ± 0.03</td>
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<tr>
<td>ANS</td>
<td>0.51 ± 0.06</td>
<td>1.58 ± 0.25</td>
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<tr>
<td>OTC</td>
<td>0.49 ± 0.06</td>
<td>1.43 ± 0.06</td>
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<tr>
<td>None</td>
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<td>1.24 ± 0.12</td>
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<tr>
<td>Exp 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANS</td>
<td>0.51 ± 0.06</td>
<td>1.58 ± 0.25</td>
</tr>
<tr>
<td>OTC</td>
<td>0.49 ± 0.06</td>
<td>1.43 ± 0.06</td>
</tr>
<tr>
<td>None</td>
<td>0.47 ± 0.03</td>
<td>1.24 ± 0.12</td>
</tr>
</tbody>
</table>

Acini were preincubated for 60 min in TR containing either 100 μM CTC and 1% BSA, or 100 μM OTC and 1% BSA, or 200 μM ANS and no BSA, then washed and resuspended in TR containing no BSA or fluorescent probes. 2-ml aliquots of this suspension were incubated in 25-ml flasks for 10 min at 37°C and amylase and fluorescent probe released into the incubation medium were assayed. Values are means ± SE of triplicate or quadruplicate incubations. The results are representative of five experiments like no. 1 and two sets of experiments like no. 2.

Figure 8 Fluorescence images of isolated pancreatic acinar cells incubated in TR containing (A) ANS (200 μM), (B) OTC (100 μM), or (C) CTC (100 μM). Arrows indicate areas containing zymogen granules that fluoresce with a lower intensity. Bar, 10 μm. × 1,200.

Studies of CTC uptake also suggest that secretory stimulation alters the ability of some set of intracellular membranes to interact with the probe. We measured the CTC content of acini during incubation in 100 μM CTC and found that betahanechol stimulation did not affect CTC uptake during the first 20 min (see Fig. 10A). After this time, uptake was markedly reduced in stimulated acini (filled triangles). The lack of an effect at early times indicates that stimulation did not affect plasma membrane permeability to the probe. The reduced CTC content of stimulated acini at long times suggests that some cell compartment that usually took up CTC slowly was refractory to uptake.

The time-course for CTC uptake in stimulated and unstimulated acini corresponded well with that for fluorescence intensity changes observed during CTC uptake (see Fig. 10B). At times...
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Time (minutes)

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9 CTC content, CTC release, amylase release, and fluorescence of pancreatic acini during stimulation with bethanechol. Acini were preincubated for 60 min in TR containing 100 μM CTC and 1% BSA, then washed and resuspended in TR containing no CTC or BSA. In experiments which determined CTC content of acini (A), CTC released into the medium (B), and amylase released into the medium (C), 7 ml of the acini suspension was incubated in a 25-ml flask at 37°C with (filled symbols) or without (open symbols) addition of bethanechol (100 μM) at 17 min (dashed line). At the times designated, 0.5-ml aliquots were removed, centrifuged, and the supernate was assayed for CTC and amylase. Pellets were resuspended in 1 ml of TR, then sonicated and assayed for CTC and protein as described in Materials and Methods. Values are means ± SE of four to eight determinations on four cell preparations. In (A), 5- to 7-ml samples of acini were incubated at 37°C in a 25-ml flask; 0.7-ml aliquots were withdrawn at the designated times, and acini were separated by suction filtration and assayed for CTC content and protein as described in Materials and Methods. Values are means ± SE of four to eight determinations on four cell preparations. In (B), 3-ml samples of acini were incubated at 37°C in 25-ml flasks and at the designated time poured into a cuvette, and fluorescence was read. Values are means ± SE of triplicate incubations; the results are representative of two similar experiments. In both (A) and (B), fluorescence was excited at 400 nm and emission read at 525 nm.

<15 min there was little difference in the fluorescence of stimulated and unstimulated acini, while at longer times stimulated acini had a significantly lower intensity.

We prefer to think that the results of Fig. 10 mean that CTC does not reach (and therefore does not probe) the stimulation-sensitive compartment at early times. But it is also possible that CTC reaches this compartment quickly but that this compartment is altered only after 15 min of stimulation. To differentiate between these possibilities, we applied bethanechol to acini at different stages of CTC uptake and recorded the acute fluorescence changes. As shown in Fig. 11, application of bethanechol after 10 min of CTC uptake had almost no effect, while application at 22 min or later times produced immediate decreases in fluorescence that increased in magnitude at successively long times up to 62 min. This indicates quite clearly that CTC fluorescence probes a stimulation-induced alteration of an intracellular compartment that occurs quite rapidly, but that

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Figure 11  Effect of bethanechol on the fluorescence of pancreatic acini during CTC uptake. Acini were washed in TR containing no Ca\textsuperscript{2+} or Mg\textsuperscript{2+}, and at time zero resuspended in TR containing 100 μM CTC and 1.28 mM Ca\textsuperscript{2+} (but no Mg\textsuperscript{2+} or BSA). 3-ml aliquots of the acini suspension were incubated in 25-ml flasks, and at 5 min before bethanechol addition they were poured into a fluorometer cuvette, stirred at 37°C, and their fluorescence was monitored continuously. Bethanechol (100 μM) was added to each sample at the arrow after exposure to CTC for the length of time indicated. Fluorescence was excited at 400 nm and emission read at 525 nm; these results are representative of four similar experiments.

CTC reaches this compartment only after 15–20 min.

DISCUSSION

The present study, in which CTC was used as an intracellular probe of divalent cations, provides new and direct evidence for a release of membrane-bound or sequestered calcium during stimulation of pancreatic acinar cells. The utility of CTC as a probe is based on four properties: (a) CTC enters cells easily and incorporates into cell membranes; (b) its fluorescence in a membrane environment is extremely sensitive to divalent cation chelation; (c) its Ca\textsuperscript{2+} and Mg\textsuperscript{2+} complexes can be distinguished spectrally; and (d) it can be localized subcellularly by fluorescence microscopy.

As anticipated, fluorescence of CTC incorporated into membranes of acinar cells is remarkably dependent on divalent cations; addition of calcium to CTC in the presence of sonicated acini rapidly increased the fluorescence intensity by at least 60-fold (Fig. 3 B). In whole acini, the intensity of CTC fluorescence (20-fold greater than equivalent membranes incubated with EDTA) as well as excitation spectra (Fig. 4) confirms that the probe is complexed largely to calcium in a membrane environment. This conclusion is supported by fluorescence micrographs (see Fig. 8 and the following paper) showing that structure such as nuclei and the interior of zymogen granules which contain no membrane are nonfluorescent while other areas containing densely packed membranes are fluorescent.

On the basis of the sensitivity of CTC fluorescence to complexation by the divalent cations present in the membranes of acinar cells, it is reasonable to believe that the loss of CTC fluorescence during stimulation is due to loss of these ions at some intracellular site. This thesis is strengthened by the observation that spectra for CTC fluorescence from unstimulated acini are largely those of CTC complexed to Ca\textsuperscript{2+} while fluorescence from stimulated acini is mainly that of CTC complexed to Mg\textsuperscript{2+} (see Figs. 5 and 6). It implies that loss of fluorescence during stimulation was due to dissociation of Ca\textsuperscript{2+}-CTC complexes, probably followed by CTC partitioning out of the membrane site. Participation of the latter mechanism is suggested by the observation that CTC efflux from these cells increased several fold during stimulation (Fig. 9 A). This event is consistent with dissociation of Ca\textsuperscript{2+}-CTC, since uncomplexed CTC is more soluble in aqueous media than is Ca\textsuperscript{2+}-CTC, and excess CTC formed by dissociation of Ca\textsuperscript{2+}-CTC would be expected to leave the membrane.

In fact, studies with isolated mitochondria show that CTC fluorescence from these organelles is paralleled by the CTC content of their membranes (5). During mitochondrial calcium uptake, both CTC fluorescence and content increase, and spectra indicate the presence of Ca\textsuperscript{2+} complexed to CTC. Upon application of an uncoupler, mitochondrial calcium is released and both CTC fluorescence and content decrease with a similar time-course. At this point, spectra indicate that the CTC remaining in the organelle is complexed to Mg\textsuperscript{2+}. This suggests that the amount of calcium sequestered in mitochondria determines how much CTC partitions into the membranes of the organelle, as well as the amount of the Ca\textsuperscript{2+}-CTC complex that is formed. Thus, the increased exit of CTC from acinar cells during stimulation (Fig. 9), as well as the decreased size of the CTC pool in stimulated cells (Fig. 10), is consistent with the hypothesis that some set of intracellular membranes probed by CTC release calcium during stimulation and that this release results in dissociation of Ca\textsuperscript{2+}-complexed CTC (if present already).
and a lower affinity of this compartment for CTC. Experiments with ANS and OTC ruled out an alternative explanation: that CTC efflux and fluorescence decrease are simply due to increased permeability of acinar cell membranes to CTC during stimulation. Amphipathic molecules like CTC probably cross cell membranes by diffusion rather than specific transport mechanisms (although bacterial membranes may be an exception; see reference 15). It is quite unlikely that changes in membrane permeability to these molecules would affect CTC but not OTC, a nearly identical molecule with four heterocyclic rings but having an extra hydroxyl group and lacking a chloride. Furthermore, the first 20 min of CTC uptake by acini was not affected by stimulation (Fig. 10), suggesting that the plasma membrane permeability is not changed and that at later times CTC uptake was decreased, rather than increased, as would be expected if some intracellular compartment became permeable to CTC.

A number of observations suggest that the calcium release monitored by CTC is related to stimulus-secretion coupling. First, atropine, which is known to block binding of cholinergic agonists to muscarinic receptors, blocks both the CTC fluorescence decrease and the amylase release produced by bethanechol but had no effect on the ability of caerulein to elicit either effect, presumably because it binds to another receptor. Secondly, concentrations of bethanechol which stimulate amylase release also produce fluorescence changes while concentrations too low to stimulate amylase release have no effect on CTC fluorescence. However, it is also clear from the dose-response relationship that the process monitored is not amylase release itself. Maximal amylase release is achieved at a bethanechol concentration which elicited a fluorescence decrease only 30% of maximal (Fig. 2). In addition, stimulation of amylase release is reduced at supramaximal doses of bethanechol (see Fig. 2B and reference 35) while the fluorescence response remained maximal (data not shown). These observations argue against the possibility that CTC associates with zymogen granule contents and is simply "secreted" along with the normal products. In fact, fluorescence micrographs (Fig. 8 and, in the following paper, Fig. 6) show that granule contents are not highly fluorescent.

It is also unlikely that the depolarization accompanying bethanechol or caerulein stimulation (25, 29) in itself initiates the process monitored by CTC fluorescence, since elevation of extracellular KCl to 58 mM, a concentration known to substantially depolarize acinar cells (25), resulted in no changes in CTC fluorescence. This is an important point because plasma membrane depolarization per se is not sufficient to elicit enzyme release from the exocrine pancreas (2, 3, 28). If depolarization had produced CTC fluorescence changes, we would have suspected that the stimulation-induced process monitored by CTC was peripheral rather than necessary for triggering of secretion. That CTC fluorescence might directly probe the plasma membrane potential is highly unlikely because this probe, unlike some fluorescence probes (34), is relatively insensitive to membrane potential (17).

Calcium release as monitored by CTC bears some striking similarities to the increased efflux of isotopic calcium seen at the onset of stimulation (4, 26). Both are initiated with little or no lag (<0.5 min, which is at least as fast as release of amylase can be measured; see reference 26), and both are completed in a relatively short time. (It must be noted, however, that the fluorescence response may be terminated by depletion of CTC at the site of release, rather than by completion of the calcium release process). Dose-response curves show that three- to fivefold greater acetylcholine concentrations are required to elicit maximal 45Ca2+ efflux than are required for maximal amylase release (4, 26). For example, half-maximal amylase release from pancreatic fragments is achieved at about 7 × 10^-8 M acetylcholine, while half-maximal 45Ca2+ efflux occurs at about 3 × 10^-7 M acetylcholine. This situation is comparable to that seen in dose-response relationships for bethanechol-induced fluorescence responses and amylase release from pancreatic acini. Half-maximal amylase release is obtained with 5 μM bethanechol while half-maximal fluorescence responses require 20 μM bethanechol. Thus, increased 45Ca2+ efflux, like the CTC fluorescence response, is seen at doses of cholinergic agonists which elicit enzyme release but are fully developed only at high doses. These similarities suggest the possibility that CTC is probing the calcium release process that is responsible for the sudden increase in 45Ca2+ efflux seen at the onset of secretion. Perhaps only a portion of the calcium potentially available for release is required for maximum amylase release.

In summary, we have provided evidence that CTC is sensitive to divalent cations at intracellular membranes of pancreatic acinar cells and that
changes in CTC fluorescence seen during stimulation result from a release of Ca$^{2+}$ from some intracellular compartment. This release could provide a source of calcium for triggering secretion, since it is initiated at appropriate agonist concentrations and occurs rapidly. The present study, to our knowledge, is the first to use CTC as an "online" probe for calcium redistribution during stimulus-secretion coupling, but it is clear already that this probe may be of use in other secretory systems. Although this technique is most easily carried out with isolated or dissociated cells, it could in the future be extended to intact tissue by use of reflectance fluorometry. The major advantage of CTC fluorescence is that it allows continuous monitoring of membrane-associated Ca$^{2+}$ and Mg$^{2+}$ during secretion in the living cells.

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