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Multiple Fatty Acid Sensing Mechanisms Operate in Enteroendocrine Cells

NOVEL EVIDENCE FOR DIRECT MOBILIZATION OF STORED CALCIUM BY CYTOSOLIC FATTY ACID*

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Fatty acids (FA) with at least 12 carbon atoms increase intracellular Ca^{2+} ($[Ca^{2+}]_i$) to stimulate cholecystokinin release from enteroendocrine cells. Using the murine enteroendocrine cell line STC-1, we investigated whether candidate intracellular pathways transduce the FA signal, or whether FA themselves act within the cell to release Ca^{2+} directly from the intracellular store. STC-1 cells loaded with fura-2 were briefly (3 min) exposed to saturated FA above and below the threshold length (C_8, C_{10}, and C_{12}). C_{12}, but not C_8 or C_{10}, induced a dose-dependent increase in $[Ca^{2+}]_i$, in the presence or absence of extracellular Ca^{2+}. Various signaling inhibitors, including D-*myo*-inositol 1,4,5-triphosphate receptor antagonists, all failed to block FA-induced Ca^{2+} responses. To identify direct effects of cytosolic FA on the intracellular Ca^{2+} store, $[Ca^{2+}]_i$ was measured in STC-1 cells loaded with the lower affinity Ca^{2+} dye magfura-2, permeabilized by streptolysin O. In permeabilized cells, again C_{12} but not C_8 or C_{10}, induced release of stored Ca^{2+}. Although C_{12} released Ca^{2+} in other permeabilized cell lines, only intact STC-1 cells responded to C_{12} in the presence of extracellular Ca^{2+}. In addition, 30 min exposure to C_{12} induced a sustained elevation of $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}, but only a transient response in the absence of extracellular Ca^{2+}. These results suggest that at least two FA sensing mechanisms operate in enteroendocrine cells: intracellularly, FA ($\geq C_{12}$) transiently induce Ca^{2+} release from intracellular Ca^{2+} stores. However, they also induce sustained Ca^{2+} entry from the extracellular medium to maintain an elevated $[Ca^{2+}]_i$.

The ability to sense luminal nutrients after a meal is of fundamental importance in the gut epithelium. This serves to orchestrate digestion and so optimize nutrient assimilation. In addition, epithelial nutrient sensing is central to the short term control of food intake via gut to brain signaling pathways. After a meal, several gastrointestinal peptides are secreted by epithelial enteroendocrine cells (EEC).[1] The pattern of secretion from EEC *in vivo* is complex, being encoded both chemically and anatomically, responding to the presence of specific macronutrient molecules in each luminal region (1–3). This precision implies that a highly specific, nutrient-sensing apparatus must exist at a cellular and molecular level to produce appropriate EEC responses. However, the molecular bases for nutrient sensing by individual EEC are largely uncharacterized.

In the proximal small intestinal epithelium, cholecystokinin (CCK) is a major EEC product and is secreted in response to free fatty acid. Also in this gut region, glucose evokes glucagon-like peptide 1 and 5-hydroxytryptamine secretion, amino acids induce gastrin release, and luminal acid causes secretin release. This categorization is a little oversimplified; for instance, dietary proteins can also stimulate CCK release (4, 5). Nonetheless, specific information about the nutrient environment in the lumen is transduced across the epithelium by EEC to activate local and distant reflexes. Of the various EEC cellular mechanisms, those involved in glucose-induced glucagon-like peptide 1 secretion by L-cells (6, 7) are best understood.

Lipid sensing is less well explained. Our earlier studies demonstrate that 12 or more carbon atoms (C_{12}) are required in the acyl chain for saturated fatty acids to stimulate CCK release in humans (8) and in the murine enteroendocrine cell line STC-1, where fatty acid exposure causes a reversible increase in intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) (9).

Fatty acids are the most difficult nutrients to study, because they display complex physicochemical behavior in an aqueous environment. On account of their hydrophobic nature, once they exceed their limit of solubility they preferentially form insoluble aggregates unless a detergent such as bile is present. Bile salt secretion and, hence, this dissolution step, only occurs as a secondary event in response to the detection of fatty acids. The delivery of bile to the duodenum by gall bladder contraction is mediated by CCK. Therefore the system must initially be able to identify these unsolubilized fatty acids to secrete CCK in the first place.

Our recent data have demonstrated that STC-1 cells are indeed able to respond to such unsolubilized particulate material, either lipid or nonlipid in origin, and this may underpin a component of the fatty acid response (10, 11). However, this cannot be the sole mechanism because, under different physicochemical conditions (*e.g.* a Ca^{2+}-free milieu), the same saturated fatty acids are far more soluble and effectively nonparticulate, yet still evoke a response from STC-1 cells. Hydrophobic lipids will rapidly leave aqueous solution to enter

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[1] The abbreviations used are: EEC, enteroendocrine cells; CCK, cholecystokinin; 2-APB, 2-aminoethyldiphenyl borate; SLO, streptolysin-O; ER, endoplasmic reticulum; TG, thapsigargin.

the lipid plasma membrane and, as our previous data show, are rapidly accumulated in the cytoplasm of STC-1 cells (12). This is germane to the critical but unresolved issue as to whether fatty acids act on EEC at an extracellular or intracellular site. The relevant but uncharacterized signal transduction pathways activated by fatty acids clearly require identification.

Intracellular fatty acid effects are the focus of the current study. We have analyzed in STC-1 cells the role of extracellular Ca^{2+} and intracellular Ca^{2+} pools in the C_{12}-induced increase in $[Ca^{2+}]_i$, and the possible involvement of candidate signal transduction pathways. In light of the results from the initial studies, we then formulated and tested a novel hypothesis, that intracellular fatty acids can act directly and independently to induce Ca^{2+} release from intracellular Ca^{2+} stores.

EXPERIMENTAL PROCEDURES

Materials—Cell culture consumables (Dulbecco's modified Eagle's medium, horse serum, fetal bovine serum, penicillin/streptomycin, trypsin, and EDTA solution) were purchased from Invitrogen. Saturated fatty acids (C_8, C_{10}, and C_{12}), bombesin, poly-L-lysine solution (0.1% solution), U-73122, 2-aminoethyldiphenyl borate (2-APB), ryanodine, dantrolene, ruthenium red, thapsigargin, D-*myo*-inositol 1,4,5-triphosphate sodium salt (IP_3), antimycin, and oligomycin were purchased from Sigma. Fura-2-AM, magfura-2-AM, and pluronic F-127 were obtained from Molecular Probes (Leiden, Netherlands). Genistein, adenosine 3′,5′-cyclic monophosphorothioate, 8-bromo-, R_p-isomer ((R_p)-8-Br-cAMPs), and xestospongin C were from Calbiochem (San Diego, CA). ONO-RS-082 was obtained from Biomol (Plymouth Meeting, PA). Streptolysin O was provided from Murex Diagnostics, NorCross, GA.

Cell Culture—STC-1 cells (a gift from D. Hanahan, University of California, San Francisco, CA) were grown in Dulbecco's modified Eagle's medium (Invitrogen, number 41965-039) supplemented with 15% horse serum, 2.5% fetal bovine serum, 50 IU/ml penicillin, and 500 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. Cells were routinely subcultured by trypsinization upon reaching 80–90% confluency. For fluorescence imaging of intracellular Ca^{2+}, cells were grown on 0.025% poly-L-lysine-coated coverslips (1.3 cm^2) at a density of 1–2 $\times$ 10^5 cells/cm^2 in 24-well plates and used 24–48 h after seeding. Cells between passages 50 and 60 were used. Other cell lines, PC12 (rat pheochromocytoma, ATCC), BON (human enterochromaffin, Dr. C. M. Townsend Jr., University of Texas Medical Branch, Galveston, TX), Caco-2 (human colon epithelial, ATCC), and IIC9 (Chinese hamster embryo fibroblast, ATCC) cells, were handled similarly to STC-1 cells. Cell viability was estimated by trypan blue exclusion and was always greater than 95%.

Preparation of Fatty Acids—Fatty acids were dissolved in 99.6% ethanol and then diluted into extracellular or intracellular buffer (compositions described below), so as to produce working solutions with fatty acid concentrations between 100 and 500 μM. Each fatty acid solution was sonicated prior to use (SONICATOR XL, Misonix Inc., Farmingdale, NY) at level 9 for 3 min.

Measurement of Intracellular Ca^{2+} Concentration in Intact STC-1 Cells—The cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined using dual-excitation fluorescence microscopy with the calcium-sensitive ratiometric dye fura-2-AM, as previously described (10–12). Briefly, cells were loaded with 2 μM fura-2-AM dissolved in extracellular buffer, containing 0.015% Pluronic F-127 at 37 °C for 20 min. After loading, coverslips were mounted into a perfusion chamber and washed with extracellular buffer or Ca^{2+}-free extracellular buffer. The chamber was placed on the stage of an inverted epifluorescence microscope (Nikon Diaphot, Tokyo, Japan). Fluorescence images were observed via a $\times$40 oil immersion lens, at an emission wavelength of 510 nm and were captured by a cooled slow scan CCD camera (Digital Pixel Ltd., Brighton, UK) at excitation wavelengths of 340 and 380 nm. Images were acquired every 15 or 20 s, and the 340/380 nm ratio images were constructed and analyzed using Lucida 3.5 software (Kinetic Imaging Ltd., Bromborough, Wirral, UK). Normal extracellular buffer (pH 7.4) had the following composition (in mM): 140 NaCl, 4.5 KCl, 10 Hepes, 10 Hepes salt, 1.2 $CaCl_2$, 1.2 $MgCl_2$, and 10 glucose. In Ca^{2+}-free extracellular buffer, $CaCl_2$ was omitted and 0.2 mM EGTA was included. The pH of both buffers was adjusted to 7.4.

Measurement of Ca^{2+} Release from Intracellular Ca^{2+} Stores in Streptolysin O-permeabilized STC-1 Cells—To measure the release of Ca^{2+} from intracellular Ca^{2+} stores, cells were loaded with the low affinity Ca^{2+} indicator magfura-2 and then permeabilized with the bacterial protein streptolysin-O (SLO) as previously described (13). STC-1 cells on coverslips were exposed to extracellular buffer containing 2 μM magfura-2-AM and 0.015% Pluronic F-127 at 37 °C for 20 min. The coverslip was mounted into the perfusion chamber and washed with permeabilization buffer on the stage of the microscope as described above. Permeabilization buffer contained 135 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM EGTA, and 20 mM Hepes/KOH (pH 7.1). The free Mg^{2+} concentration was calculated as 0.9 mM, being adjusted with $MgCl_2$ according to a previously described method (14). To observe the real time process of cytosolic dye leakage, cells loaded with magfura-2 were excited at 360 nm, the isosbestic wavelength for magfura-2, and the permeabilization procedure was performed on the microscope stage in permeabilization buffer containing 0.5 units/ml SLO. After exposure to SLO solution for 5–10 min, cytosolic magfura-2 was visibly lost and the fluorescence intensity at 360 nm significantly dropped. Permeabilized cells were then washed with Ca^{2+}-free intracellular buffer containing: 1 mM ATP, 135 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM EGTA, 20 mM Hepes/KOH (pH 7.1), and 0.9 mM free Mg^{2+}, for 5 min to remove residual SLO. To load Ca^{2+} into the intracellular Ca^{2+} stores, permeabilized cells were exposed to intracellular buffer containing 0.2 μM free Ca^{2+}, 0.9 mM free Mg^{2+}, 1 mM ATP, 135 mM KCl, 1.2 mM KH_2PO_4, 0.5 mM EGTA, 20 mM Hepes/KOH (pH 7.1). For Ca^{2+} uptake experiments, cells were washed with the permeabilization buffer (free of ATP and Ca^{2+}), then exposed to the buffer containing Ca^{2+} but devoid of ATP for 2 min. Uptake of Ca^{2+} was then initiated by exposure to the intracellular buffer containing ATP and Ca^{2+}. After Ca^{2+} loading for 5 min, permeabilized cells were exposed to test agents to examine stimulatory effects on Ca^{2+} release from intracellular Ca^{2+} stores. Because magfura-2 has spectral properties similar to fura-2, images were acquired and analyzed as described above for fura-2.

Data Analysis—Data were calculated by determining ratio values for each of the individual cells (10–40 cells) in a microscope field. All data are representative of at least three individual experiments. Significant differences were determined by Student's unpaired *t* test.

RESULTS

Fatty Acids Elevate $[Ca^{2+}]_i$ in the Absence of Extracellular Ca^{2+}—To investigate the contribution of intracellular Ca^{2+} stores to fatty acid-induced $[Ca^{2+}]_i$ responses, STC-1 cells were exposed to fatty acids in the presence or absence of extracellular Ca^{2+}. In both cases, C_{12} at 500 μM induced a rise in $[Ca^{2+}]_i$. However, in the absence of extracellular Ca^{2+}, the rise was more rapid (Fig. 1*A*). This response was dose dependent (Fig. 1*B*). STC-1 cells were also exposed to different chain length fatty acids. As shown in Fig. 1*C*, in the absence of extracellular Ca^{2+}, C_{12} but not C_8 or C_{10}, induced an increase in $[Ca^{2+}]_i$. This chain length specificity corresponds to that reported by us both in humans (8) and in STC-1 cells under Ca^{2+} containing conditions (9).

In experiments measuring $[Ca^{2+}]_i$, the uncalibrated 340/380 nm ratio signal is generally presented as a surrogate for $[Ca^{2+}]_i$, because absolute estimates of $[Ca^{2+}]_i$ are not routinely derived from ratio values. However, a two-point calibration of the fura-2 signal was carried out on a limited number of STC-1 cells as described elsewhere (12). Individual cells were initially treated with 1 μM thapsigargin and 1 μM ionomycin in Ca^{2+}-free medium (containing 2 mM EGTA) to obtain fluorescence parameters for fura-2 under Ca^{2+}-free conditions (R_{min}). The cells were subsequently superfused with thapsigargin and ionomycin in Ca^{2+}-supplemented medium to obtain fluorescence parameters for Ca^{2+}-saturated fura-2 (R_{max}). The 340/380 nm ratio signal was 0.99 $\pm$ 0.10 in resting cells (n = four experiments), corresponding to an estimated $[Ca^{2+}]_i$ of 134 $\pm$ 22 nM (the K_d of fura-2 at 22 °C was taken as 135 nM (15)). In Ca^{2+}-containing conditions C_{12} (500 μM) typically raised the 340/380 fluorescence ratio by 0.25 $\pm$ 0.03, which corresponds to an estimated increase in $[Ca^{2+}]_i$ of 68 $\pm$ 7 nM, which is around 200 nM, a value similar to those in our previous STC-1 studies (9, 12) and in other endocrine cell types (16–18). In Ca^{2+}-free conditions C_{12} (500 μM) typically raised the 340/380 fluorescence ratio by 0.35 $\pm$ 0.02, which corresponds to an estimated increase in $[Ca^{2+}]_i$ of 83 $\pm$ 2 nM, which is around 220 nM.

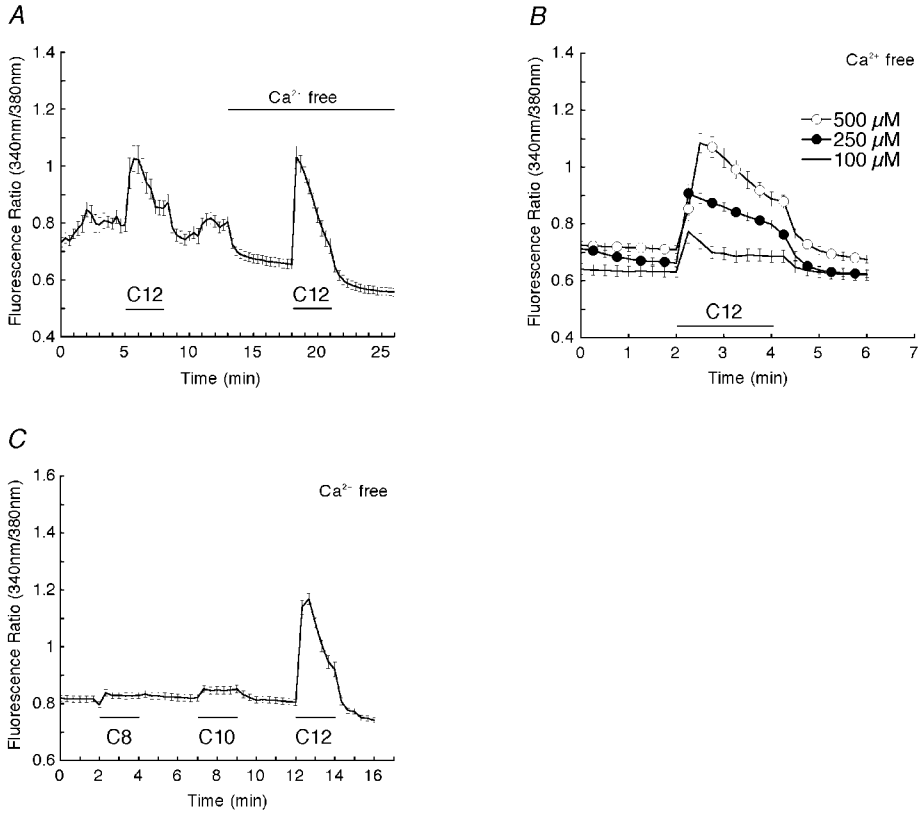

FIG. 1. **Fatty acid (C_{12}) induces intracellular Ca^{2+} mobilization in the absence of extracellular Ca^{2+} in STC-1 cells.** *A*, STC-1 cells were exposed to 500 μM C_{12} for 3 min (*horizontal bars*) in the presence of extracellular Ca^{2+} (1.2 mM), then cells were exposed to Ca^{2+}-free extracellular buffer for 5 min followed by exposure to C_{12} solution for 3 min. *B*, cells were exposed to various concentrations (100–500 μM) of C_{12} for 2 min in the absence of extracellular Ca^{2+}. *C*, STC-1 cells were sequentially exposed to 500 μM C_8, C_{10}, or C_{12} for 2 min (*horizontal bars*) in the absence of extracellular Ca^{2+}. Values are mean fluorescence ratio ± S.E. of 10–30 cells obtained from a single experiment, and data are representative of at least three separate experiments.

Finally, KCl (70 mmol) typically raised the 340/380 fluorescence ratio by 0.45 ± 0.05, which corresponds to an estimated increase in $[Ca^{2+}]_i$ of 116 ± 11 nM, which is around 250 nM (Fig. 7*A*).

C_{12} Mobilizes Ca^{2+} from Thapsigargin- and IP_3-sensitive Intracellular Ca^{2+} Stores—When intracellular Ca^{2+} stores were depleted by the Ca^{2+}-ATPase inhibitor thapsigargin (TG) (Fig. 2*A*), C_{12} failed to induce $[Ca^{2+}]_i$ responses in the absence of extracellular Ca^{2+}. A similar result was obtained using the neuroendocrine peptide bombesin, which is known to activate the IP_3 pathway in STC-1 cells (19). As expected, bombesin induced a rapid increase in $[Ca^{2+}]_i$, in the absence of extracellular Ca^{2+}, indicating release of Ca^{2+} from intracellular Ca^{2+} stores. After Ca^{2+} release by bombesin, C_{12} failed to increase $[Ca^{2+}]_i$ (Fig. 2*B*). These data suggest that C_{12} releases Ca^{2+} from the same intracellular store mobilized by IP_3.

Potential Pathways Involved in Fatty Acid-induced Ca^{2+} Release from Intracellular Stores—To explore the signaling pathways involved in fatty acid-induced release of Ca^{2+} from intracellular stores, we examined the effect of pretreatment with several agents known to block intracellular signal transduction pathways linked to Ca^{2+} store mobilization. In the first instance, since C_{12} mobilizes Ca^{2+} from IP_3-sensitive stores (Fig. 2*A*), pretreatment was undertaken with a PLC inhibitor, U73122 (10 μM, Fig. 3*B*), or an IP_3 receptor antagonist, 2-APB (100 μM, Fig. 3*C*). However, both agents failed to block the C_{12}-induced $[Ca^{2+}]_i$ response although, as expected, both fully blocked the effects of 10 nM bombesin, a positive control of PLC/IP_3-dependent Ca^{2+} release.

Several other agents tested (data not shown), namely the IP_3 antagonist xestospongin C, and a panel of ryanodine receptor antagonists (dantrolene, ruthenium red, and ryanodine used at >10 μM) also failed to block C_{12}-induced $[Ca^{2+}]_i$ responses, as did pertussis toxin, which inhibits G_i- and G_o-coupled pathways, and genistein, which inhibits tyrosine kinase-linked receptor pathways. Although previous papers have demonstrated cAMP-dependent CCK release in STC-1 cells (20, 21), the cAMP antagonist (R_p)-8-Br-cAMPs also failed to block C_{12}-induced $[Ca^{2+}]_i$ responses. Finally, thromboxane A_2, an arachidonic acid cascade product generated by phospholipase A_2 has been reported to induce Ca^{2+} release from intracellular stores (22). Therefore the phospholipase A_2 inhibitor ONO-RS-082 was tested, but it too failed to block the C_{12}-evoked Ca^{2+} responses.

This evolving mass of negative data raised the alternative hypothesis: that C_{12} itself, which gains rapid access to the intracellular compartment (12), was transducing its own signal. To assess this possibility, we developed a permeabilized STC-1 cell system in which the effect of C_{12} on the intracellular Ca^{2+} store can be assessed directly.

Ca^{2+} Stores Remain Functional in Permeabilized STC-1 Cells—The fluorescence image of magfura-2-loaded STC-1 cells was monitored while excited at 360 nm, the dye isosbestic wavelength, before and after treatment of cells with SLO. In intact cells, all cell compartments including the cytosol and nucleus were stained with magfura-2 after 20 min of loading. Three minutes after exposure to 0.5 units/ml SLO, fluorescence intensity began to decrease, and full permeabilization was achieved within 10 min. As a consequence of the loss of cytosolic magfura-2, together with the soluble cytosolic contents (including soluble signaling molecules), magfura-2 fluorescence became punctate, indicating that residual dye was compartmentalized into cell organelles including the endoplasmic reticulum (ER) Ca^{2+} stores.

To demonstrate that stores retained physiological functions in permeabilized cells, Ca^{2+} uptake and Ca^{2+} release were evaluated under several conditions. The experimental protocol was adapted by initially exposing cells to Ca^{2+}-free intracellular buffer containing 0.9 mM free Mg^{2+} and 1 mM ATP to rule out the possibility that the changes in the magfura-2 ratio were because of changes in intraorganelle $[Mg^{2+}]$, rather than $[Ca^{2+}]$. Cells were then exposed to the same buffer, but con-

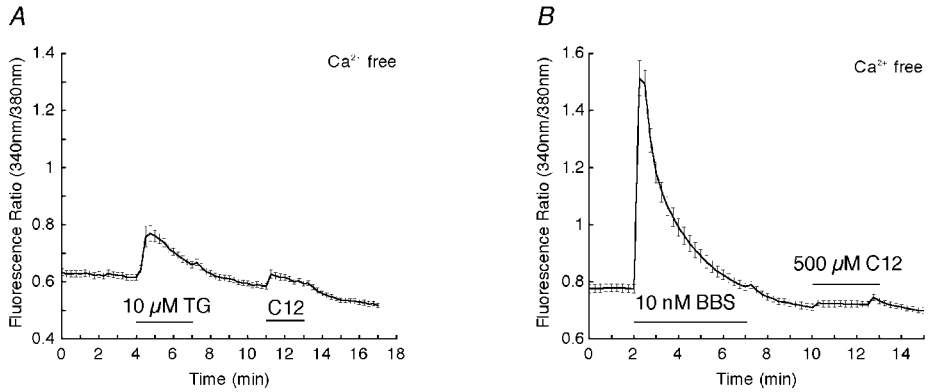

FIG. 2. **C_{12} induces Ca^{2+} release from intracellular Ca^{2+} stores.** In the absence of extracellular Ca^{2+}, STC-1 cells were exposed to (*A*) 10 μM TG for 3 min, or (*B*) 10 nM bombesin (*BBS*) for 5 min before exposure to 500 μM C_{12}. Values are mean fluorescence ratio ± S.E. of 10–30 cells obtained from a single experiment, and the data are representative of at least four separate experiments.

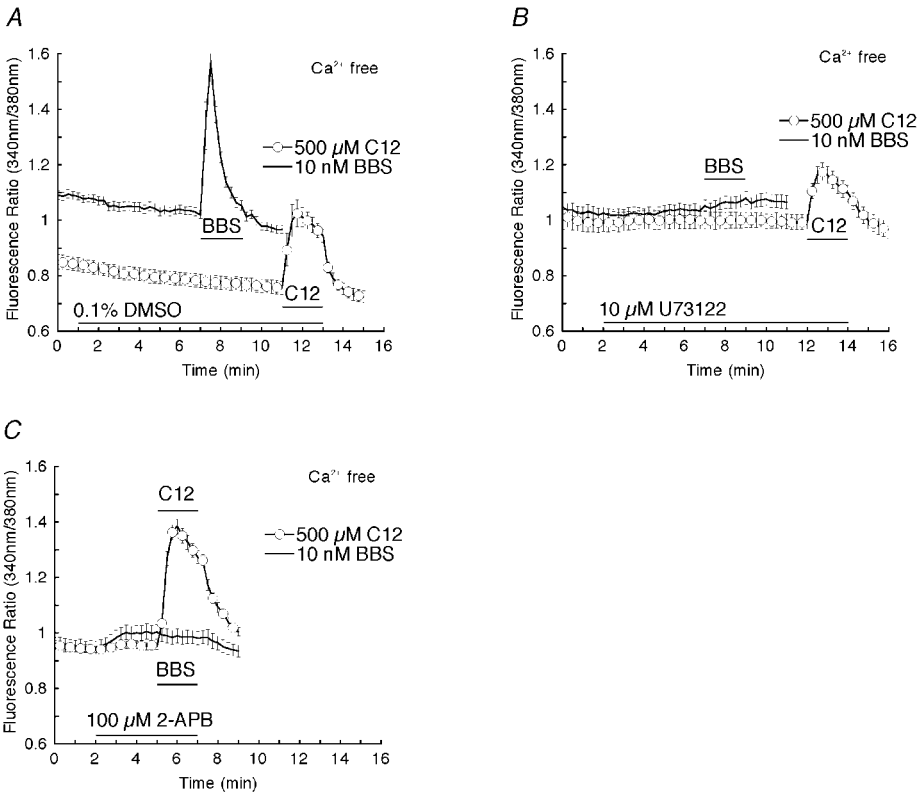

FIG. 3. **Phospholipase C-IP_3 pathway is not involved in C_{12}-induced $[Ca^{2+}]_i$ responses.** STC-1 cells were exposed to 500 μM C_{12} (*open circles*) or 10 nM bombesin (*BBS*; *no symbols*)) for 2 min in the presence of 0.1% (v/v) dimethyl sulfoxide (*DMSO*) (*A*), 10 μM U73122 (*B*), or 100 μM 2-APB (*C*). Cells were exposed to each drug for 3–10 min prior to exposure to C_{12} or bombesin in the presence of Me_2SO, U73122, or 2-APB. Compounds were dissolved in Me_2SO to make a stock solution (10 mM for U73122, 100 mM for 2-APB), respectively, and were diluted into extracellular buffer at 1:1000. Values are mean fluorescence ratio ± S.E. of 10–30 cells obtained from a single experiment, and data are representative of at least three separate experiments.

taining in addition 0.2 μM free Ca^{2+}, which resulted in an appropriate increase in the magfura-2 ratio as Ca^{2+} was sequestered into the organelles (data not shown). Because free Mg^{2+} concentration was maintained constant at 0.9 mM, any increase in magfura-2 ratio indicates an increase in $[Ca^{2+}]$ in cell organelles. The magfura-2 ratio was not changed by adding Ca^{2+} in the absence of ATP, but was increased in the presence of ATP, an effect that was appropriately prevented by 1 μM thapsigargin pretreatment (data not shown). These results confirmed that Ca^{2+} was sequestered into the organelles by a sarco/endoplasmic reticulum calcium ATPase-type Ca^{2+}-ATPase.

Exposing permeabilized cells to IP_3 resulted in a rapid, dose-dependent release of stored Ca^{2+} (Fig. 4, *A* and *D*). By contrast, thapsigargin induced a slow and continuous decrease in the magfura-2 ratio, indicating the existence of a slow efflux of Ca^{2+} from intracellular stores that is normally masked by sarco/endoplasmic reticulum calcium ATPase-mediated Ca^{2+} re-uptake (Fig. 4*B*). When applied in combination with thapsigargin, IP_3 induced a larger Ca^{2+} release than did IP_3 or thapsigargin alone, almost completely depleting the stores (Fig. 4, *C* and *D*).

These validation studies confirm that in permeabilized cells Ca^{2+} is still functionally sequestered into intracellular Ca^{2+} stores via Ca^{2+}-ATPase, and that these stores are the source of the fluorescence we measured. Intracellular Ca^{2+} stores are not damaged by SLO permeabilization, as they are still appropriately responsive to physiological and pharmacological stimuli.

C_{12} Induces Ca^{2+} Release from Intracellular Ca^{2+} Stores in Permeabilized STC-1 Cells—Having validated the model of permeabilized STC-1 cells, we next examined whether intracellular fatty acids can induce Ca^{2+} release. Permeabilized cells were exposed to C_{12} after loading Ca^{2+} into the stores. Exposure to C_{12} at ≥250 μM induced a rapid decrease in magfura-2 ratio (corresponding to release of 50% of loaded Ca^{2+}), and the ratio dropped irreversibly to the basal value on exposure to 500 μM C_{12} (Fig. 5*A*). The magfura-2 ratio did not recover after removing C_{12}. This might suggest that C_{12} has a nonspecific permeabilization effect on the ER membrane. To exclude this possibility, we isolated and analyzed the time course data for cells excited at 380 nm. As the denominator in the 340/380 ratio, this signal appropriately falls as calcium is loaded into the stores. Simple leakage of store contents would have caused this signal to fall upon C_{12} exposure because of dye

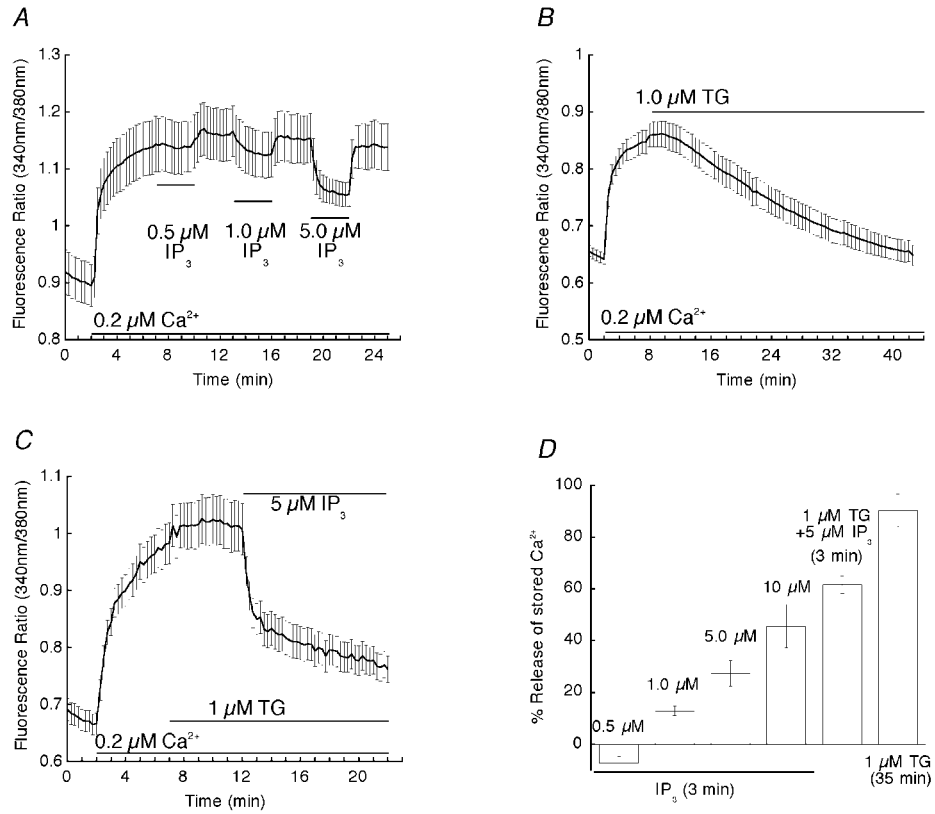

FIG. 4. **IP_3 and thapsigargin induce Ca^{2+} release from intracellular stores in permeabilized STC-1 cells.** Permeabilized cells were washed with the intracellular buffer containing 1 mM ATP and 0.9 mM free Mg^{2+} in the absence of Ca^{2+}, then cells were loaded with 0.2 μM free Ca^{2+} for 5 min. *A*, Ca^{2+}-loaded cells were sequentially exposed to 0.5, 1.0, and 5.0 μM IP_3 for 3 min. *B*, Ca^{2+}-loaded cells were exposed to 1.0 μM TG for 35 min. *C*, Ca^{2+}-loaded cells were exposed to 1.0 μM TG for 5 min, and exposed to 5.0 μM IP_3 in the presence of 1.0 μM TG. Values are mean fluorescence ratio ± S.E. of 10–30 cells obtained from a single experiment, and data are representative of at least three different experiments. *D*, mean percent value (%) of Ca^{2+} released by various concentrations of IP_3 (in 3 min), by a combination of IP_3 and TG (in 3 min), and by 1.0 μM TG alone (in 35 min). Values are mean ± S.E. obtained from different experiments (n = 6 for 1–5 μM IP_3, n = 5 for 10 μM IP_3, n = 9 for IP_3 + TG, n = 5 for TG, respectively).

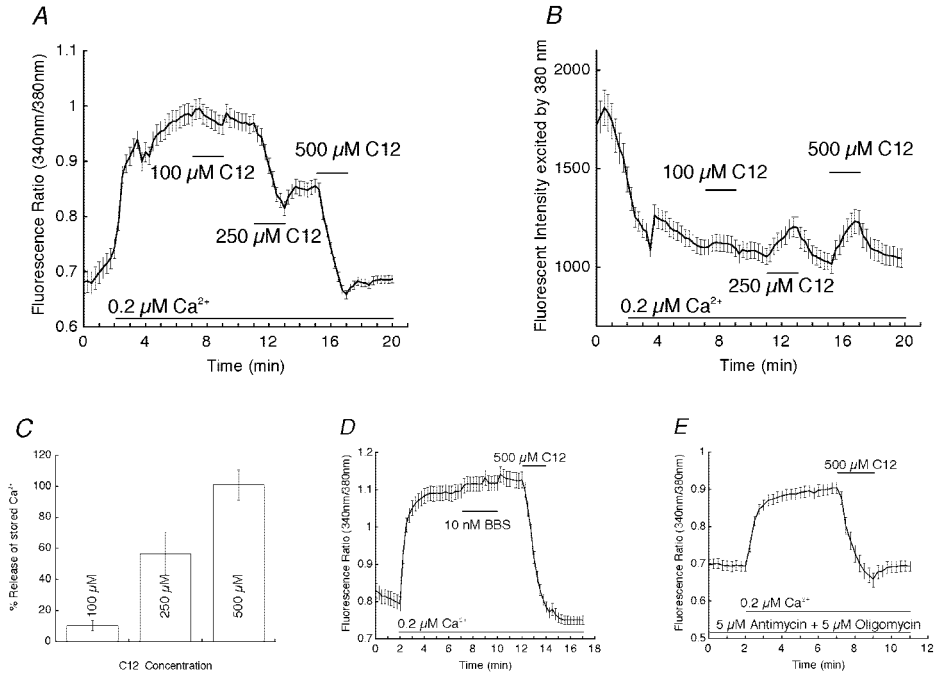

FIG. 5. **C_{12} induces Ca^{2+} release from intracellular stores in permeabilized STC-1 cells.** Permeabilized cells were washed with the intracellular buffer containing 1 mM ATP and 0.9 mM free Mg^{2+} in the absence of Ca^{2+}, then cells were loaded with 0.2 μM free Ca^{2+} for 5 min. Changes in fluorescence ratio (*A*, 340:380) and fluorescence intensity excited by 380 nm (*B*) in Ca^{2+}-loaded cells exposed to 100, 250, and 500 μM C_{12} for 3 min sequentially. *C*, mean percent value (%) of Ca^{2+} released from intracellular stores by exposure to various concentrations of C_{12} for 3 min. Values are mean ± S.E. obtained from different experiments (n = 5 for 100 μM, n = 4 for 250 μM, n = 7 for 500 μM C_{12}, respectively). *D*, Ca^{2+}-loaded cells were sequentially exposed to 10 nM bombesin (*BBS*) and 500 μM C_{12}. *E*, permeabilized cells were washed and loaded with 0.2 μM free Ca^{2+} in the buffer containing mitochondrial Ca^{2+} uptake inhibitors (5 μM antimycin and 5 μM oligomycin). Ca^{2+}-loaded cells were exposed to 500 μM C_{12} in the presence of mitochondria Ca^{2+} uptake inhibitors. Values in *A*, *B*, and *D* are mean fluorescence ratio or fluorescence intensity ± S.E. of 10–30 cells obtained from a single experiment, and data are representative of at least three different experiments. *E* presents mean data pooled from three experiments.

loss. However, the 380 nm fluorescence rose upon C_{12} exposure, so that the overall 340/380 ratio fell. This is in keeping with a selective transmembrane flux of calcium (Fig. 5*B*). The effect of C_{12} on magfura-2 ratio was dose dependent (Fig. 5*C*). In contrast to the result in intact cells (Fig. 3*C*), exposure to 10 nM bombesin did not change the magfura-2 ratio in permeabilized

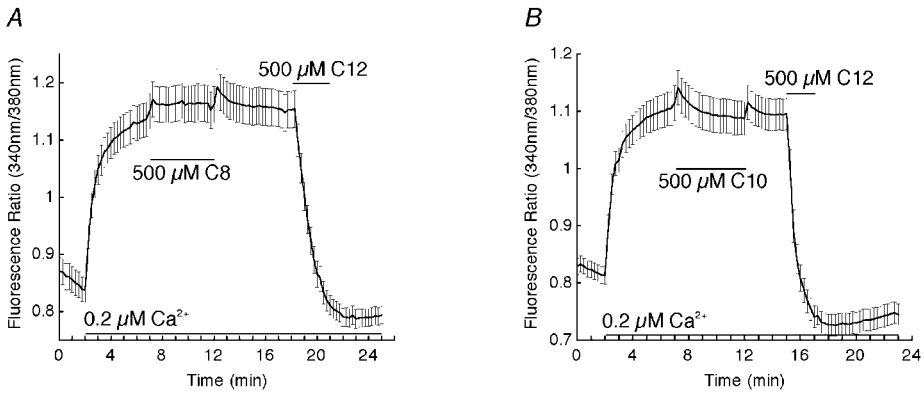

FIG. 6. **C_8 and C_{10} do not induce Ca^{2+} release from intracellular stores in permeabilized STC-1 cells.** Permeabilized cells were washed with the intracellular buffer containing 1 mM ATP and 0.9 mM free Mg^{2+} in the absence of Ca^{2+}, then cells were loaded with 0.2 μM free Ca^{2+} for 5 min. Ca^{2+}-loaded cells were sequentially exposed to: *A,* 500 μM C_8 for 5 min and 500 μM C_{12} for 2 min; *B*, 500 μM C_{10} for 5 min and 500 μM C_{12} for 2 min. Values are mean fluorescence ratio ± S.E. of 10–30 cells obtained from a single experiment, and data are representative of at least three different experiments.

cells (Fig. 5*D*) even though C_{12} induced a decrease in magfura-2 ratio in the same cell preparations. Mitochondrial Ca^{2+} uptake inhibitors (23) antimycin (5 μM) and oligomycin (5 μM) did not impair the decrease in magfura-2 ratio induced by C_{12} (Fig. 5*E*). As in intact cells (Fig. 1*B*), C_8 and C_{10} (500 μM) did not affect magfura-2 ratio, but subsequent C_{12} was still effective (Fig. 6, *A* and *B*). In summary, C_{12} evokes Ca^{2+} release from non-mitochondrial stores in permeabilized STC-1 cells.

Extracellular Ca^{2+} Changes the Response Pattern to C_{12} and Is Necessary for Continuous $[Ca^{2+}]_i$ Elevation—The above data indicate a direct effect of C_{12} on intracellular calcium stores. However, previous data have also shown that responses to C_{12} in Ca^{2+}-containing medium depend largely on Ca^{2+} entry (9, 12), suggesting that fatty acids may influence intracellular Ca^{2+} homeostasis in more than one manner, probably depending on their mode of presentation. During short term exposure of calcium-free C_{12} to STC-1 cells, the $[Ca^{2+}]_i$ response showed a very brisk rate of onset and decline. We went on to examine the Ca^{2+} dependence of C_{12} responses during longer exposures. Accordingly, STC-1 cells were exposed to 500 μM C_{12} for 30 min in the presence or absence of extracellular Ca^{2+}. In the presence of extracellular Ca^{2+}, the C_{12}-induced elevation of $[Ca^{2+}]_i$ was maintained throughout the exposure (Fig. 7*A*). This response was also reversible, $[Ca^{2+}]_i$ rapidly returning to basal values when C_{12} was washed out. In addition, STC-1 cells tolerate prolonged C_{12} exposure, responding promptly to depolarization (70 mM KCl) even after 30 min exposure to C_{12}. In contrast, in the absence of extracellular Ca^{2+}, C_{12} induced only a transient Ca^{2+} spike, and the elevated $[Ca^{2+}]_i$ returned to basal value within 10 min of starting continuous exposure to C_{12} (Fig. 7*B*).

Depletion of Intracellular Ca^{2+} Stores Does Not Prevent C_{12}-induced Ca^{2+} Response in the Presence of Extracellular Ca^{2+}—In calcium-free conditions, intracellular store depletion prevents a rise in $[Ca^{2+}]_i$ in response to C_{12} (Fig. 2). However, we have previously suggested that external Ca^{2+} entry through a L-type Ca^{2+} channel is involved in fatty acid-induced responses (9, 12). The dual kinetics presented in Fig. 7 suggested that both mechanisms may in fact operate. To further investigate the involvement of extracellular Ca^{2+} in the fatty acid sensing mechanism, the intracellular Ca^{2+} store was depleted by thapsigargin (TG) as before (Fig. 2), but this time in the presence of extracellular Ca^{2+} and before fatty acid exposure. TG at 10 μM induced Ca^{2+} mobilization because of Ca^{2+} release from intracellular stores (Fig. 8*A*). Vehicle alone (Me_2SO at 0.1%) did not affect $[Ca^{2+}]_i$ (data not shown). After treatment with vehicle, both C_{12} and bombesin induced Ca^{2+} mobilization. Intracellular $[Ca^{2+}]_i$ returned nearly to basal values 15 min after TG treatment, at which time cells were exposed to 500 μM C_{12} or 10 nM bombesin in the continued presence of TG. Bombesin at 10 nM, which had been shown to induce Ca^{2+} release from the store only (Figs. 2 and 3), failed to induce any further increase in $[Ca^{2+}]_i$ after TG treatment (Fig. 8*A*), confirming that Ca^{2+} stores were depleted completely by TG treatment. However, C_{12} was still effective in inducing a further rise in $[Ca^{2+}]_i$ after TG treatment. This contrasted with the inability of C_{12} to cause any additional increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} (Fig. 2). Fig. 8*B* shows the area under the curve for the fluorescence ratio changes during 2 min exposure to C_{12} or bombesin after TG or vehicle treatment. The bombesin-induced Ca^{2+} response was abolished by TG treatment ($p = 0.0027$), but the C_{12}-induced Ca^{2+} response was unchanged ($p = 0.9152$).

C_{12} Release Ca^{2+} from the Store After Permeabilization, and in the Absence of Extracellular Ca^{2+} in Several Cell Types—To investigate whether the effect of C_{12} on $[Ca^{2+}]_i$ is specific to this particular CCK-producing enteroendocrine cell, several additional cell lines were exposed to C_{12} in the presence or absence of extracellular Ca^{2+}, and also exposed to C_{12} after magfura loading and SLO permeabilization (Table I). In the presence of extracellular Ca^{2+}, only STC-1 cells responded to C_{12}. On the other hand, in the absence of extracellular Ca^{2+} in intact cells, and in SLO-permeabilized cells, all cell lines showed some release of stored Ca^{2+} by 500 μM C_{12}. However, responses in STC-1 cells remained greater than in all the other cell lines studied.

DISCUSSION

The cellular mechanisms by which fatty acids induce chain length-specific responses in enteroendocrine cells are still uncharacterized. The present study has demonstrated that fatty acids can act directly on endoplasmic reticulum Ca^{2+} stores in a manner that retains the key chain length specificity. Nonetheless, fatty acids clearly also induce Ca^{2+} entry from extracellular sources. Importantly, the $[Ca^{2+}]_i$ kinetics observed for each site of action of fatty acid are distinctive, in keeping with the coexistence of at least two discrete sensing mechanisms. The evidence supports both of the outlined possibilities.

In the presence of extracellular Ca^{2+}, C_{12} forms insoluble aggregates that must be dispersed by sonication. The time elapsed after sonication affects solubility, and re-aggregation of fatty acid occurs, which in turn affects the cellular responses to C_{12} (10). This has led to the suggestion that fatty acid effects may in part be exerted by the extracellular aggregates themselves (10, 11). Removing Ca^{2+} from the buffer renders C_{12} far more soluble and stable in its physicochemical state, yet results in $[Ca^{2+}]_i$ responses that are rapid, reversible, and more reproducible than in the presence of extracellular Ca^{2+}. Ca^{2+}-free conditions permit detailed and separate study of the intracellular Ca^{2+} releasing mechanism, under more constant and controlled physicochemical conditions. The responses showed the same chain length dependence as previously described *in*

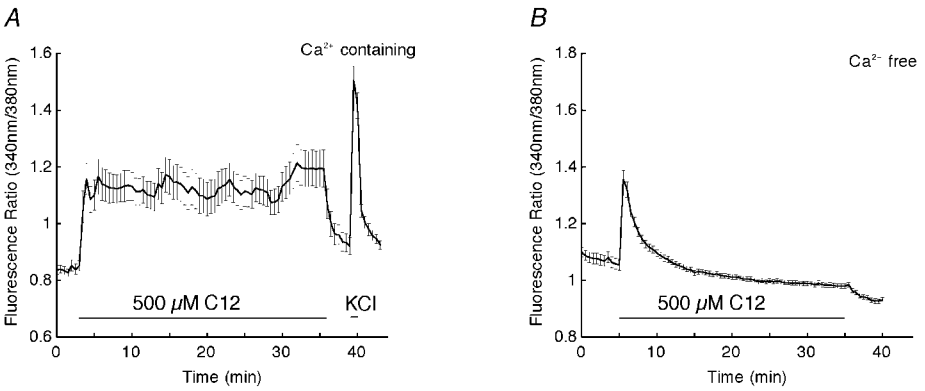

FIG. 7. **Changes in $[Ca^{2+}]_i$ in response to C_{12} exposure for 30 min in the presence or absence of extracellular Ca^{2+} in STC-1 cells.** STC-1 cells were exposed to 500 μM C_{12} for 30 min in the presence (*A*) or absence (*B*) of extracellular Ca^{2+}. In the presence of extracellular Ca^{2+}, cells were depolarized with 70 mM KCl after C_{12} exposure. Values are mean fluorescent ratio value ± S.E., and data are representative of three individual experiments.

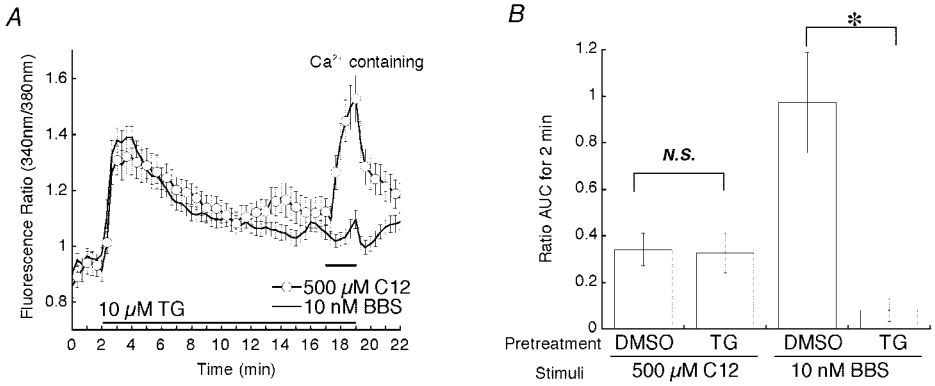

FIG. 8. **Changes in $[Ca^{2+}]_i$ in response to C_{12} or bombesin after TG treatment in the presence of extracellular Ca^{2+} in STC-1 cells.** In the presence of extracellular Ca^{2+}, STC-1 cells were exposed to 10 μM TG or vehicle (0.1% dimethyl sulfoxide (*DMSO*)) for 15 min followed by exposure to 500 μM C_{12} (*open circles*) or 10 nM bombesin (*BBS*; *no symbols*) for 2 min. *A*, values are mean fluorescence ratio ± S.E. of 10–30 cells obtained from a single experiment. Data are representative of at least four separate experiments. *B*, values are mean area under curve (*AUC*) of changes in fluorescence ratio induced by 500 μM C_{12} or 10 nM bombesin exposure for 2 min after TG or vehicle treatment (n = 4, 7, 4, and 5 for Me_2SO + C12, TG + C_{12}, Me_2SO + bombesin, and TG + bombesin, respectively). *N.S.*, not significantly different between Me_2SO and TG treatment. *, significantly different between Me_2SO and TG treatment by Student's unpaired t test, $p < 0.05$.

TABLE I

Changes in fluorescent ratio (340/380 nm) by 500 μM C_{12} exposure in intact cells in the presence or absence of extracellular Ca^{2+}, or in permeabilized cells

Values are the mean fluorescent ratio changes (340/380 nm) in 30–133 cells from two to seven separate experiments. The intracellular Ca^{2+} level was measured in fura-2-loaded intact cells in the presence or absence of extracellular Ca^{2+} (Ca^{2+}_o), and in magfura-2-loaded cells permeabilized by SLO. In permeabilized cells, values decreased the fluorescent ratio of C_{12} in 3 min.

	Intact cells		Permeabilized cells
	With Ca^{2+}_o	Without Ca^{2+}_o	
STC-1	0.349	0.428	−0.326
PC12	0.025	0.086	−0.089
BON	0.037	0.276	−0.215
Caco-2	−0.052	0.107	−0.175
IIC9	0.019	0.143	−0.173

vivo (8), indicating that the same basic sensing mechanisms were probably involved.

The entry of extracellular Ca^{2+} induced by C_{12} has previously been shown to be via L-type Ca^{2+} channels (9, 12). In the absence of extracellular Ca^{2+}, C_{12} mobilized Ca^{2+} from intracellular stores, as confirmed by prior depletion of intracellular Ca^{2+} stores using thapsigargin (10 μM). This observation contrasts with data we have reported in a previous study, but the discrepancy can be explained by use of a lower concentration of thapsigargin (1 μM) or by the shorter incubation time (7 min) employed in the earlier study (12). Indeed, Fig. 6*B* shows that emptying of Ca^{2+} stores by thapsigargin treatment in STC-1 cells is a slow process. In the present study, the inclusion of bombesin as a positive control confirmed store emptying by thapsigargin, and therefore supports the current interpretation.

To probe the transduction mechanisms by which C_{12} acts to mobilize Ca^{2+} from intracellular stores, we initially employed specific blockers for several known cellular signaling pathways linked to Ca^{2+} mobilization. Where appropriate, the bombesin pathway was used as a positive control. These data were particularly important in excluding a role for IP_3. All the blockers tested were ineffective. Although clearly many other transcytosolic signaling pathways could be involved, these data tend to rule out several obvious candidates.

Our subsequent data using permeabilized cells largely overcome the theoretical limitations associated with purely negative results, and with the need for suitable controls for every putative pathway, because in this model system the cytoplasm is replaced with an artificial intracellular buffer. This technique has been previously applied to study intracellular Ca^{2+} stores or exocytosis in several cell types including gastric epithelial cells (24), pancreatic acinar cells (13, 25), and platelets (26). We optimized and validated this method for use in STC-1 cells. These manipulations indicate that permeabilized STC-1 cells remain physiologically intact in being capable of accumulating Ca^{2+} into intracellular stores by an energy-dependent process, then releasing it in response to C_{12}. Crucially, the specificity of response to fatty acid chain length remained identical to that seen in intact cells. It was important to exclude a nonspecific detergent effect of C_{12} causing leakage of all ER contents. This was confirmed by monitoring the 380 nm excitation data alone, in addition to the 340/380 ratio. A reduction in 380 nm intensity on C_{12} exposure would have been expected if leakage of dye were responsible for the fall in 340/380 fluorescence ratio. However, the 380 nm intensity actually rose

after exposure to C_{12} (Fig. 5*B*). Indeed, magfura-2 is a small molecule (M_r 722), so its retention in the ER demonstrates retained membrane selectivity, and the observations following C_{12} can be ascribed to Ca^{2+} flux, rather than general membrane permeabilization with redistribution of Ca^{2+} dye.

Permeabilization was monitored in real time on the fluorescence microscope to ensure that cytoplasmic magfura-2 was lost while washing repeatedly with the intracellular buffer. Hence cytosolic signaling molecules such as phospholipases, protein kinases, IP_3, and cAMP must be lost together with cytosolic magfura-2. Generation of new signaling molecules at the plasma membrane that could diffuse into the intracellular buffer cannot be totally excluded, but it seems unlikely that such cascades could be reconstituted quickly enough to explain the rapid time course demonstrated in response to C_{12}. Moreover, if restoration of washed out signaling molecules occurred by regeneration *in situ*, bombesin would be expected to evoke a $[Ca^{2+}]_i$ response in permeabilized cells, but this was not the case. Recently, there has been a resurgence of interest in the possibility that mitochondrial Ca^{2+} is involved in intracellular signaling (27, 28). However, Ca^{2+} release was unaffected in STC-1 cells in the presence of mitochondrial Ca^{2+} uptake inhibitors (Fig. 5*E*), suggesting that C_{12} releases Ca^{2+} from the ER Ca^{2+} store and not from mitochondria.

How do fatty acids induce Ca^{2+} release from the endoplasmic reticular stores? Perhaps there is a specific fatty acid receptor awaiting characterization, expressed in the ER membrane and working in parallel to the IP_3 or ryanodine receptors. Another possibility is that fatty acids may directly act on Ca^{2+} channels or pumps on the store membrane. Alternatively, fatty acids incorporated into the ER membrane may modify a biophysical membrane property to rapidly enhance efflux of Ca^{2+}. Answering these fundamental questions is an important aim for future studies.

In contrast to Ca^{2+} release evoked by IP_3, the Ca^{2+} response to C_{12} in permeabilized cells was irreversible. This is most likely because of loss of cytoplasmic fatty acid shuttling (or buffering) proteins that are responsible for the rapid permeation of C_{12} throughout the intact cell, or perhaps reflects an inability to wash out fatty acid that enters the ER membrane in these modified conditions. The loss of counter-regulatory systems is an inevitable drawback of cellular permeabilization.

Comparison with several other cell types showed that intact STC-1 cells are clearly specialized and sensitive as fatty acid sensors. However, other cells become responsive to C_{12} when the fatty acid is rendered more available (*i.e.* presented in Ca^{2+}-free medium or following permeabilization). This suggests that if adequate C_{12} enters any cell type, it can act on the Ca^{2+} store. It is likely that, in the absence of extracellular Ca^{2+}, soluble C_{12} fatty acids are readily able to cross the plasma membrane, because of their hydrophobic properties and relatively small size. The fatty acid can then induce Ca^{2+} release via an intracellular site of action. However, only STC-1 cells responded to the less soluble fatty acids presented in the presence of extracellular Ca^{2+}. Fatty acids have been shown to rapidly permeate STC-1 cells (12), so a theoretical component of the intact STC-1 cell fatty acid sensing mechanism may be a high affinity uptake system.

Taken together with our previous data, the current results strongly suggest the involvement of two pathways, one initiated at the plasma membrane to trigger influx of extracellular Ca^{2+} and another operated by fatty acid arriving at the endoplasmic reticulum store to trigger Ca^{2+} release. This duality may explain the small discrepancies in chain length dependence of fatty acid stimulation: C_8 and C_{10} had a small effect on the Ca^{2+} response in the presence of extracellular Ca^{2+} (9, 12), but they had no direct effect on Ca^{2+} release from the store as shown in the present study (Figs. 1 and 6). The specificity of the STC-1 cell as a lipid sensor is likely to be explained by a combination of one or more specialized cell surface detection systems, and high avidity fatty acid uptake that allows rapid access to deeper compartments.

In vivo, it is likely that fatty acids in the gut lumen after a meal co-exist as a mixture of aggregate and soluble states, and that EEC are able to respond to both fatty acid states. Therefore, both pathways may be biologically important, because the small intestinal epithelium is the only organ that will ordinarily be exposed to such high concentrations of free fatty acids. In all other biological compartments, free fatty acids are transported mainly bound to protein, or repackaged in esterified form to circulate with lipoproteins.

In conclusion, medium chain fatty acid that releases CCK (C_{12}, but not C_8 or C_{10}) induces intracellular Ca^{2+} release from ER stores in EEC. It also induces Ca^{2+} entry from the extracellular medium to maintain a high intracellular $[Ca^{2+}]_i$, via a different sensing mechanism. Cell surface receptors or EEC-specific transport systems may be involved in the two mechanisms.

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