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\(_{12}\), C\(_{16}\), and C\(_{18}\)), but not C\(_{10}\) or C\(_{14}\) induced a dose-dependent increase in [Ca\(^{2+}\)]\(_i\) in the presence or absence of extracellular Ca\(^{2+}\). Various signaling inhibitors, including L-1-myoinositol 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\(_{10}\) or C\(_{14}\) 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). The pattern of secretion from EEC in vivo is complex, being encoded both chemically and anatomically, responding to the presence of specific macro-nutrient 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 demonstrated 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 the lumen to be sensed by CCK in the enteroendocrine cells.

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

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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\(_t\)-induced increase in [Ca\(^{2+}\)], and the possible involvement of candidate signal transduction pathways. In light of the results from the above 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\(_6\), C\(_10\), and C\(_12\)), benzoin, poly-l-lysine solution (0.1% solution), U-73122, 2-aminoethyldiphenylborate (2-APB), ryanodine, dantrolene, ruthenium red, thapsigargin, D-myoinositol 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-, and 5-bromo-2′-deoxyadenosine 3′,5′-cyclic monophosphorothioate, 8-bromo-, R,3′-isomer (R,3′)-8-BrcAMPs, and xestospongin C were from Calbiochem (San Diego, CA). ORO-BS-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. Coverslips were mounted in a perfusion chamber and washed with extracellular buffer containing 0.5 units/ml SLO. After exposure to SLO solution for 5–10 min, the coverslip was washed with extracellular buffer and then washed with Ca\(^{2+}\)-free intracellular buffer containing 1 mM ATP, 135 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 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\(^{2+}\), and then washed with Ca\(^{2+}\)-free intracellular buffer containing: 1 mM ATP, 135 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 0.5 mM EGTA, 20 mM Hepes/KOH (pH 7.1) for Ca\(_t\)-uptake experiments, cells were washed with Mg\(^{2+}\)-free intracellular buffer (free of ATP and Ca\(_t\)), then exposed to the buffer containing Ca\(_t\) but devoid of ATP for 2 min. Uptake of Ca\(_t\) was then initiated by exposure to the intracellular buffer containing ATP and Ca\(_t\). After Ca\(_t\)-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\(^{2+}\)] in the Absence of Extracellular Ca\(^{2+}\)**—To investigate the contribution of intracellular Ca\(^{2+}\) stores to fatty acid-induced [Ca\(_t\)]\(^{2+}\) responses, STC-1 cells were exposed to fatty acids in the presence or absence of extracellular Ca\(^{2+}\). In both cases, C\(_6\) at 500 μM induced a rise in [Ca\(_t\)]\(^{2+}\). However, in the absence of extracellular Ca\(^{2+}\), the rise was more rapid (Fig. 1A). This response was dose dependent (Fig. 1B). STC-1 cells were also exposed to different chain length fatty acids. As shown in Fig. 1C, in the absence of extracellular Ca\(_t\), C\(_12\) but not C\(_8\) or C\(_10\), induced an increase in [Ca\(_t\)]\(^{2+}\). This chain length specificity corresponds to that reported by us both in humans (8) and in STC-1 cells under Ca\(_t\)-containing conditions (9).

In experiments measuring [Ca\(_t\)]\(^{2+}\), the uncalibrated 340/380 nm ratio signal is generally presented as a surrogate for [Ca\(_t\)]\(^{2+}\], because absolute estimates of [Ca\(_t\)]\(^{2+}\) 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\(_{340\text{nm}}\)). The cells were subsequently superfused with thapsigargin and ionomycin in Ca\(^{2+}\)-supplemented medium to obtain fluorescence parameters for Ca\(_t\)-saturated fura-2 (R\(_{340\text{nm}\text{max}}\)). The 340/380 nm ratio signal was 0.99 ± 0.10 in resting cells (n = four experiments), corresponding to an estimated [Ca\(_t\)]\(^{2+}\) of 134 ± 22 nM (the K\(_d\) of fura-2 at 22 °C was taken as 135 nM (15)). In Ca\(_t\)-containing conditions C\(_12\) (500 μM) typically raised the 340/380 fluorescence ratio by 0.25 ± 0.03, which corresponds to an estimated increase in [Ca\(_t\)]\(^{2+}\), of 68 ± 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\(_t\)-free conditions C\(_12\) (500 μM) typically raised the 340/380 fluorescence ratio by 0.35 ± 0.02, which corresponds to an estimated increase in [Ca\(_t\)]\(^{2+}\), of 83 ± 2 nM, which is around 220 nM.
Finally, KCl (70 mmol) typically raised the 340/380 fluorescence ratio by 0.45 ± 0.05, which corresponds to an estimated increase in [Ca\(^{2+}\)], of 116 ± 11 mM, which is around 250 mM (Fig. 7A).

**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. 2A), C\(_{12}\) failed to induce [Ca\(^{2+}\)], 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\(^{2+}\)], 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\(^{2+}\)], (Fig. 2B). 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. 2A), pretreatment was undertaken with a PLC inhibitor, U73122 (10 \(\mu\)M, Fig. 3B), or an IP\(_{3}\) receptor antagonist, 2-APB (100 \(\mu\)M, Fig. 3C). However, both agents failed to block the C\(_{12}\)-induced [Ca\(^{2+}\)], response although, as expected, both fully blocked the effects of 10 \(\mu\)M 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 \(\mu\)M) also failed to block C\(_{12}\)-induced [Ca\(^{2+}\)], 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\(^{2+}\)], 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 isoabsorbent 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 intraganelle [Mg\(^{2+}\)], rather than [Ca\(^{2+}\)]. Cells were then exposed to the same buffer, but con-
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\(^{2+}\)]\(_{\text{i}}\) 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 sarcoplasmic reticulum calcium ATPase-mediated Ca\(^{2+}\) re-uptake (Fig. 4B). 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 stored Ca\(^{2+}\)), and the ratio dropped irreversibly to the basal value on exposure to 500 μM C\(_12\) (Fig. 5A). 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
loss. However, the 380 nm fluorescence rose upon C12 exposure, so that the overall 340/380 ratio fell. This is in keeping with a selective transmembrane flux of calcium (Fig. 5B). The effect of C12 on magfura-2 ratio was dose dependent (Fig. 5C). In contrast to the result in intact cells (Fig. 3C), exposure to 10 nM bombesin did not change the magfura-2 ratio in permeabilized...
cells (Fig. 5D) even though C12 induced a decrease in magfura-2 ratio in the same cell preparations. Mitochondrial Ca2+ uptake inhibitors (23) antimycin (5 μM) and oligomycin (5 μM) did not impair the decrease in magfura-2 ratio induced by C12 (Fig. 5E). As in intact cells (Fig. 1B), C8 and C10 (500 μM) did not affect magfura-2 ratio, but subsequent C12 was still effective (Fig. 6, A and B). In summary, C12 evokes Ca2+ release from non-mitochondrial stores in permeabilized STC-1 cells.

**Extracellular Ca2+ Changes the Response Pattern to C12 and Is Necessary for Continuous [Ca2+]i Elevation.**—The above data indicate a direct effect of C12 on intracellular calcium stores. However, previous data have also shown that responses to C12 in Ca2+-containing medium depend largely on Ca2+ entry (9, 12), suggesting that fatty acids may influence intracellular Ca2+ homeostasis in more than one manner, probably depending on their mode of presentation. During short term exposure of calcium-free C12 to STC-1 cells, the [Ca2+]i response showed a very brisk rate of onset and decline. We went on to examine the Ca2+ dependence of C12 responses during longer exposures. Accordingly, STC-1 cells were exposed to 500 μM C12 for 30 min in the presence or absence of extracellular Ca2+. In the presence of extracellular Ca2+, the C12-induced elevation of [Ca2+] was maintained throughout the exposure (Fig. 7A). This response was also reversible, [Ca2+]i, rapidly returning to basal values when C12 was washed out. In addition, STC-1 cells tolerate prolonged C12 exposure, responding promptly to depolarization (70 mM KCl) even after 30 min exposure to C12. In contrast, in the absence of extracellular Ca2+, C12 induced only a transient Ca2+ spike, and the elevated [Ca2+]i, returned to basal value within 10 min of starting continuous exposure to C12 (Fig. 7B).

**Depletion of Intracellular Ca2+ Stores Does Not Prevent C12-Induced Ca2+ Response in the Presence of Extracellular Ca2+.**—In calcium-free conditions, intracellular store depletion prevents a rise in [Ca2+]i, in response to C12 (Fig. 1). However, we have previously suggested that external Ca2+ entry through a L-type Ca2+ 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 Ca2+ in the fatty acid sensing mechanism, the intracellular Ca2+ store was depleted by thapsigargin (TG) as before (Fig. 2), but this time in the presence of extracellular Ca2+ and before fatty acid exposure. TG at 10 μM induced Ca2+ mobilization because of Ca2+ release from intracellular stores (Fig. 8A). Vehicle alone (Me2SO at 0.1%) did not affect [Ca2+]i (data not shown). After treatment with vehicle, both C12 and bombesin induced Ca2+ mobilization. Intracellular [Ca2+]i returned nearly to basal values 15 min after TG treatment, at which time cells were exposed to 500 μM C12 or 10 nM bombesin in the continued presence of TG. Bombesin at 10 nM, which had been shown to induce Ca2+ release from the store only (Figs. 2 and 3), failed to induce any further increase in [Ca2+]i after TG treatment (Fig. 8A), confirming that Ca2+ stores were depleted completely by TG treatment. However, C12 was still effective in inducing a further rise in [Ca2+]i after TG treatment. This contrasted with the inability of C12 to cause any additional increase in [Ca2+]i, in the absence of extracellular Ca2+ (Fig. 2). Fig. 8B shows the area under the curve for the fluorescence ratio changes during 2 min exposure to C12 or bombesin after TG or vehicle treatment. The bombesin-induced Ca2+ response was abolished by TG treatment (p = 0.0027), but the C12-induced Ca2+ response was unchanged (p = 0.9152).

C12 Release Ca2+ from the Store After Permeabilization, and in the Absence of Extracellular Ca2+ in Several Cell Types.—To investigate whether the effect of C12 on [Ca2+]i is specific to this particular CCK-producing enteroendocrine cell, several additional cell lines were exposed to C12 in the presence or absence of extracellular Ca2+, and also exposed to C12 after magfura loading and SLO permeabilization (Table I). In the presence of extracellular Ca2+, only STC-1 cells responded to C12. On the other hand, in the absence of extracellular Ca2+ in intact cells, and in SLO-permeabilized cells, all cell lines showed some release of stored Ca2+ by 500 μM C12. 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 Ca2+ stores in a manner that retains the key chain length specificity. Nonetheless, fatty acids clearly also induce Ca2+ entry from extracellular sources. Importantly, the [Ca2+]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 Ca2+, C12 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 C12 (10). This has led to the suggestion that fatty acid effects may in part be exerted by the extracellular aggregates themselves (10, 11). Removing Ca2+ from the buffer renders C12 far more soluble and stable in its physicochemical state, yet results in [Ca2+]i responses that are rapid, reversible, and more reproducible than in the presence of extracellular Ca2+. Ca2+-free conditions permit detailed and separate study of the intracellular Ca2+ 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\(^{2+}\)] 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 \(\mu\)M 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.

![Image](image1.png)

**FIG. 8.** Changes in [Ca\(^{2+}\)], 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 \(\mu\)M TG or vehicle (0.1% dimethyl sulfoxide (DMSO)) for 15 min followed by exposure to 500 \(\mu\)M C\(_{12}\) (open circles) or 10 nM bombesin (BBS; no symbols) for 2 min. A, values are mean fluorescent 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 fluorescent ratio induced by 500 \(\mu\)M C\(_{12}\) or 10 nM bombesin exposure for 2 min after TG or vehicle treatment (n = 4, 7, 4, and 5 for Me\(_{2}\)SO + C\(_{12}\), TG + C\(_{12}\), Me\(_{2}\)SO + bombesin, and TG + bombesin, respectively). N.S., not significantly different between Me\(_{2}\)SO and TG treatment. *, significantly different between Me\(_{2}\)SO and TG treatment by Student’s unpaired \(t\) test, \(p < 0.05\).

![Image](image2.png)

**TABLE I**

|                | In intact cells | Permeabilized cells |
|----------------|----------------|---------------------|
|                | With Ca\(^{2+}\)_e | Without Ca\(^{2+}\)_e |                     |
| 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              |

*Changes in fluorescent ratio (340/380 nm) by 500 \(\mu\)M 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\(^{2+}\)_e 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.

In 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 \(\mu\)M). 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 \(\mu\)M) or by the shorter incubation time (7 min) employed in the earlier study (12). Indeed, Fig. 6B 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. 5B). Indeed, magfura-2 is a small molecule (M, 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. 5E), 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 shutting (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+}], via a different sensing mechanism. Cell surface receptors or EEC-specific transport systems may be involved in the two mechanisms.

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REFERENCES
1. Buchan, A. (1999) Am. J. Physiol. 277, G1109–G1107
2. Furness, J. B., Kunze, W., and Clerc, N. (1999) Am. J. Physiol. 277, G922–G928
3. Baylouird, H. E. (1999) Am. J. Physiol. 277, G751–G755
4. Hira, T., Hara, H., and Aoyama, Y. (1999) Biosci. Biotechnol. Biochem. 63, 1192–1196
5. Nishi, T., Hara, H., Hira, T., and Tomita, F. (2001) Exp. Biol. Med. 226, 1031–1036
6. Reimann, F., and Gribble, F. M. (2002) Diabetes 51, 2757–2763
7. Gribble, F. M., Williams, L., Simpson, A. K., and Reimann, F. (2003) Diabetes 52, 1147–1154
8. McLaughlin, J. T., Grazia Luca, M., Jones, M. N., D’Amato, M., Dockray, G. J., and Thompson, D. G. (1999) Gastroenterology 116, 46–53
9. McLaughlin, J. T., Lenox, B. H., Hall, L., Dockray, G. J., Thompson, D. G., and Warhurst, G. (1998) J. Physiol. 513, 11–18
10. Benson, R. S., Sidhu, S., Jones, M. N., Case, R. M., and Thompson, D. G. (2002) J. Physiol. 538, 121–133
11. Kazmi, S., Sidhu, S. S., Donoho, T. J., Wickham, M., Jones, M. N., Thompson, D. G., Case, R. M., and Benson, R. S. (2003) J. Physiol. 553, 759–773
12. Sidhu, S. S., Thompson, D. G., Warhurst, G., Case, R. M., and Benson, R. S. (2000) J. Physiol. 528, 165–176
13. van de Put, F. H., and Elliott, A. C. (1996) J. Biol. Chem. 271, 4999–5006
14. Schoonmaker, T. J., Visser, G. J., Flik, G., and Theuvenet, A. P. (1992) BioTechniques 12, 870–874, 876–879
15. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 3440–3450
16. Menezes, A., Zeman, R., and Sabban, E. (1996) J. Neurochem. 67, 2316–2324
17. Karlsson, S., Sundler, F., and Ahren, B. (2001) Biochem. Biophys. Res. Commun. 280, 610–614
18. Hoenig, M., and Sharp, G. W. (1986) Endocrinology 119, 2502–2507
19. Chang, C. H., Chey, W. Y., Erway, H., Coy, D. H., and Chang, T. M. (1998) Am. J. Physiol. 275, G192–G202
20. Chang, C. H., Chey, W. Y., Sun, Q., Leiter, A., and Chang, T. M. (1994) Biochim. Biophys. Acts 1221, 339–347
21. Priep, V., Basavappa, S., Liddle, R. A., and Mangel, A. W. (1994) Biochem. Biophys. Res. Commun. 201, 1483–1489
22. Hertelendy, F., Molnar, M., and Jamaluddin, M. (1992) Mol. Cell. Endocrinol. 83, 173–181
23. van de Put, F. H., and Elliott, A. C. (1997) J. Biol. Chem. 272, 27764–27770
24. Hofer, A. M., and Machen, T. E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2598–2602
25. Padfield, P. J., and Panesar, N. (1995) Am. J. Physiol. 269, G647–G652
26. Padfield, P. J., Panesar, N., Henderson, P., and Baldassare, J. J. (1996) Biochem. J. 314, 123–128
27. Pozzan, T., Magalhaes, P., and Rizzuto, R. (2000) Cell Calcium 28, 279–283
28. Sanders, K. M. (2001) J. Appl. Physiol. 91, 1438–1449