



Title	Multiple Fatty Acid Sensing Mechanisms Operate in Enteroendocrine Cells
Author(s)	Hira, Tohru; Elliott, Austin C.; Thompson, David G. et al.
Citation	Journal of Biological Chemistry, 279(25), 26082-26089 <a href="https://doi.org/10.1074/jbc.M400098200">https://doi.org/10.1074/jbc.M400098200</a>
Issue Date	2004
Doc URL	<a href="https://hdl.handle.net/2115/84549">https://hdl.handle.net/2115/84549</a>
Rights	This research was originally published in the Journal of Biological Chemistry. Author(s). Multiple Fatty Acid Sensing Mechanisms Operate in Enteroendocrine Cells. J. Biol. Chem. 2004; Vol.279: 26082-26089. © the American Society for Biochemistry and Molecular Biology or © the Author(s).
Rights(URL)	<a href="https://creativecommons.org/licenses/by/4.0/">https://creativecommons.org/licenses/by/4.0/</a>
Type	journal article
File Information	PIIS0021925820855859.pdf



# Multiple Fatty Acid Sensing Mechanisms Operate in Enteroendocrine Cells

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

Received for publication, January 6, 2004, and in revised form, March 19, 2004  
Published, JBC Papers in Press, April 5, 2004, DOI 10.1074/jbc.M400098200

Tohru Hira<sup>‡§</sup>, Austin C. Elliott<sup>‡</sup>, David G. Thompson<sup>¶</sup>, R. Maynard Case<sup>‡</sup>,  
and John T. McLaughlin<sup>¶</sup>

From the <sup>‡</sup>School of Biological Sciences, G.38 Stopford Building, University of Manchester, Oxford Road, Manchester M13 9PT, United Kingdom, the <sup>§</sup>Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kita-ku, Sapporo, 060-8589, Japan, and <sup>¶</sup>Gastrointestinal Sciences, Clinical Sciences Building, Hope Hospital, Stott Lane, Salford M6 8HD, United Kingdom

**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).<sup>1</sup> 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

\* This work was supported by the Digestive Disorders Foundation, UK, and the Japan Society for the Promotion of Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 44-161-206-4362; Fax: 44-161-206-1495; E-mail: john.mclaughlin@man.ac.uk.

<sup>1</sup> The abbreviations used are: EEC, enteroendocrine cells; CCK, cho-

lecystokinin; 2-APB, 2-aminoethyl diphenyl 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  $\text{Ca}^{2+}$  and intracellular  $\text{Ca}^{2+}$  pools in the  $\text{C}_{12}$ -induced increase in  $[\text{Ca}^{2+}]_i$ , 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  $\text{Ca}^{2+}$  release from intracellular  $\text{Ca}^{2+}$  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 ( $\text{C}_8$ ,  $\text{C}_{10}$ , and  $\text{C}_{12}$ ), bombesin, poly-L-lysine solution (0.1% solution), U-73122, 2-aminoethylidiphenyl borate (2-APB), ryanodine, dantrolene, ruthenium red, thapsigargin, D-myo-inositol 1,4,5-triphosphate sodium salt ( $\text{IP}_3$ ), 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  $\mu\text{g}/\text{ml}$  streptomycin in a humidified 5%  $\text{CO}_2$  atmosphere at 37 °C. Cells were routinely subcultured by trypsinization upon reaching 80–90% confluency. For fluorescence imaging of intracellular  $\text{Ca}^{2+}$ , cells were grown on 0.025% poly-L-lysine-coated coverslips (1.3  $\text{cm}^2$ ) at a density of  $1-2 \times 10^5$  cells/ $\text{cm}^2$  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  $\mu\text{M}$ . Each fatty acid solution was sonicated prior to use (SONICATOR XL, Misonix Inc., Farmingdale, NY) at level 9 for 3 min.

**Measurement of Intracellular  $\text{Ca}^{2+}$  Concentration in Intact STC-1 Cells**—The cytoplasmic free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_i$ ) 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  $\mu\text{M}$  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  $\text{Ca}^{2+}$ -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  $\text{CaCl}_2$ , 1.2  $\text{MgCl}_2$ , and 10 glucose. In  $\text{Ca}^{2+}$ -free extracellular buffer,  $\text{CaCl}_2$  was omitted and 0.2 mM EGTA was included. The pH of both buffers was adjusted to 7.4.

**Measurement of  $\text{Ca}^{2+}$  Release from Intracellular  $\text{Ca}^{2+}$  Stores in Streptolysin O-permeabilized STC-1 Cells**—To measure the release of  $\text{Ca}^{2+}$  from intracellular  $\text{Ca}^{2+}$  stores, cells were loaded with the low affinity  $\text{Ca}^{2+}$  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  $\mu\text{M}$  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  $\text{KH}_2\text{PO}_4$ , 0.5 mM EGTA, and 20 mM Hepes/KOH (pH 7.1). The free  $\text{Mg}^{2+}$  concentration was calculated as 0.9 mM, being adjusted with  $\text{MgCl}_2$  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  $\text{Ca}^{2+}$ -free intracellular buffer containing: 1 mM ATP, 135 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 0.5 mM EGTA, 20 mM Hepes/KOH (pH 7.1), and 0.9 mM free  $\text{Mg}^{2+}$ , for 5 min to remove residual SLO. To load  $\text{Ca}^{2+}$  into the intracellular  $\text{Ca}^{2+}$  stores, permeabilized cells were exposed to intracellular buffer containing 0.2  $\mu\text{M}$  free  $\text{Ca}^{2+}$ , 0.9 mM free  $\text{Mg}^{2+}$ , 1 mM ATP, 135 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 0.5 mM EGTA, 20 mM Hepes/KOH (pH 7.1). For  $\text{Ca}^{2+}$  uptake experiments, cells were washed with the permeabilization buffer (free of ATP and  $\text{Ca}^{2+}$ ), then exposed to the buffer containing  $\text{Ca}^{2+}$  but devoid of ATP for 2 min. Uptake of  $\text{Ca}^{2+}$  was then initiated by exposure to the intracellular buffer containing ATP and  $\text{Ca}^{2+}$ . After  $\text{Ca}^{2+}$  loading for 5 min, permeabilized cells were exposed to test agents to examine stimulatory effects on  $\text{Ca}^{2+}$  release from intracellular  $\text{Ca}^{2+}$  stores. Because magfura-2 has spectral properties similar to fura-2, images were acquired and analyzed as described above for fura-2.

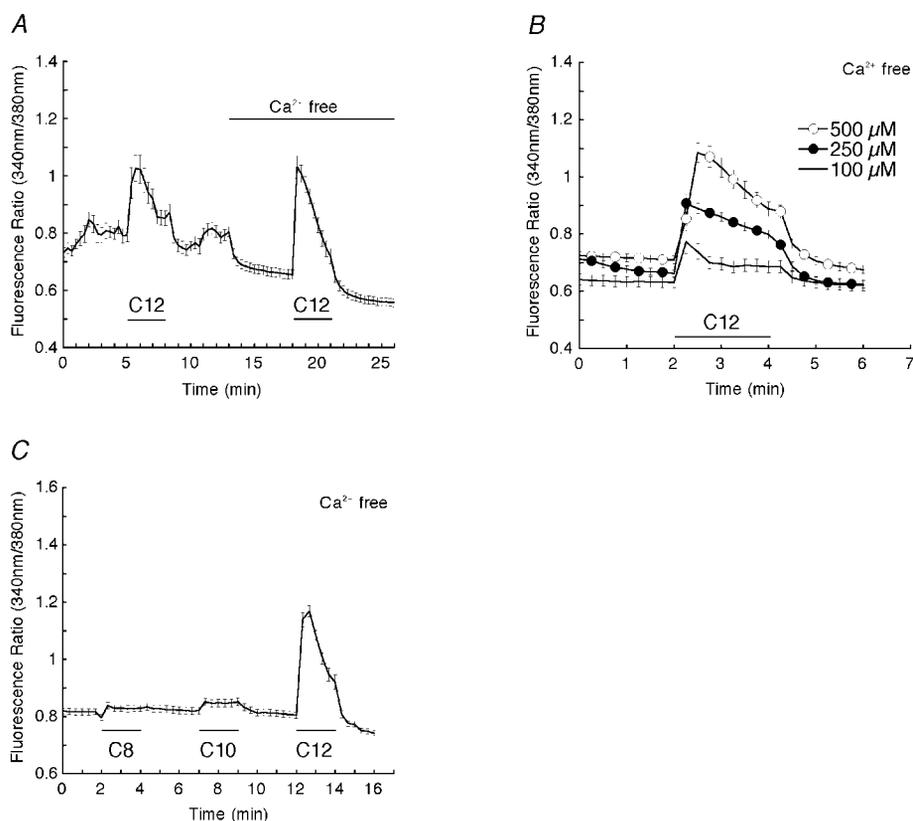
**Data Analysis**—Data were calculated by determining ratio values for each of the individual cells (10–40 cells) in a microscope field. All data are representative of at least three individual experiments. Significant differences were determined by Student's unpaired *t* test.

#### RESULTS

**Fatty Acids Elevate  $[\text{Ca}^{2+}]_i$  in the Absence of Extracellular  $\text{Ca}^{2+}$** —To investigate the contribution of intracellular  $\text{Ca}^{2+}$  stores to fatty acid-induced  $[\text{Ca}^{2+}]_i$  responses, STC-1 cells were exposed to fatty acids in the presence or absence of extracellular  $\text{Ca}^{2+}$ . In both cases,  $\text{C}_{12}$  at 500  $\mu\text{M}$  induced a rise in  $[\text{Ca}^{2+}]_i$ . However, in the absence of extracellular  $\text{Ca}^{2+}$ , 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  $\text{Ca}^{2+}$ ,  $\text{C}_{12}$  but not  $\text{C}_8$  or  $\text{C}_{10}$ , induced an increase in  $[\text{Ca}^{2+}]_i$ . This chain length specificity corresponds to that reported by us both in humans (8) and in STC-1 cells under  $\text{Ca}^{2+}$  containing conditions (9).

In experiments measuring  $[\text{Ca}^{2+}]_i$ , the uncalibrated 340/380 nm ratio signal is generally presented as a surrogate for  $[\text{Ca}^{2+}]_i$ , because absolute estimates of  $[\text{Ca}^{2+}]_i$  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  $\mu\text{M}$  thapsigargin and 1  $\mu\text{M}$  ionomycin in  $\text{Ca}^{2+}$ -free medium (containing 2 mM EGTA) to obtain fluorescence parameters for fura-2 under  $\text{Ca}^{2+}$ -free conditions ( $R_{\text{min}}$ ). The cells were subsequently superfused with thapsigargin and ionomycin in  $\text{Ca}^{2+}$ -supplemented medium to obtain fluorescence parameters for  $\text{Ca}^{2+}$ -saturated fura-2 ( $R_{\text{max}}$ ). The 340/380 nm ratio signal was  $0.99 \pm 0.10$  in resting cells ( $n = \text{four}$  experiments), corresponding to an estimated  $[\text{Ca}^{2+}]_i$  of  $134 \pm 22$  nM (the  $K_d$  of fura-2 at 22 °C was taken as 135 nM (15)). In  $\text{Ca}^{2+}$ -containing conditions  $\text{C}_{12}$  (500  $\mu\text{M}$ ) typically raised the 340/380 fluorescence ratio by  $0.25 \pm 0.03$ , which corresponds to an estimated increase in  $[\text{Ca}^{2+}]_i$  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  $\text{Ca}^{2+}$ -free conditions  $\text{C}_{12}$  (500  $\mu\text{M}$ ) typically raised the 340/380 fluorescence ratio by  $0.35 \pm 0.02$ , which corresponds to an estimated increase in  $[\text{Ca}^{2+}]_i$  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  $500 \mu M$   $C_{12}$  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  $\mu M$ ) of  $C_{12}$  for 2 min in the absence of extracellular  $Ca^{2+}$ . *C*, STC-1 cells were sequentially exposed to 500  $\mu M$   $C_8$ ,  $C_{10}$ , or  $C_{12}$  for 2 min (horizontal bars) in the absence of extracellular  $Ca^{2+}$ . Values are mean fluorescence ratio  $\pm$  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  $0.45 \pm 0.05$ , which corresponds to an estimated increase in  $[Ca^{2+}]_i$  of  $116 \pm 11$  nM, which is around 250 nM (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  $[Ca^{2+}]_i$  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. 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  $[Ca^{2+}]_i$  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  $>10 \mu M$ ) 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

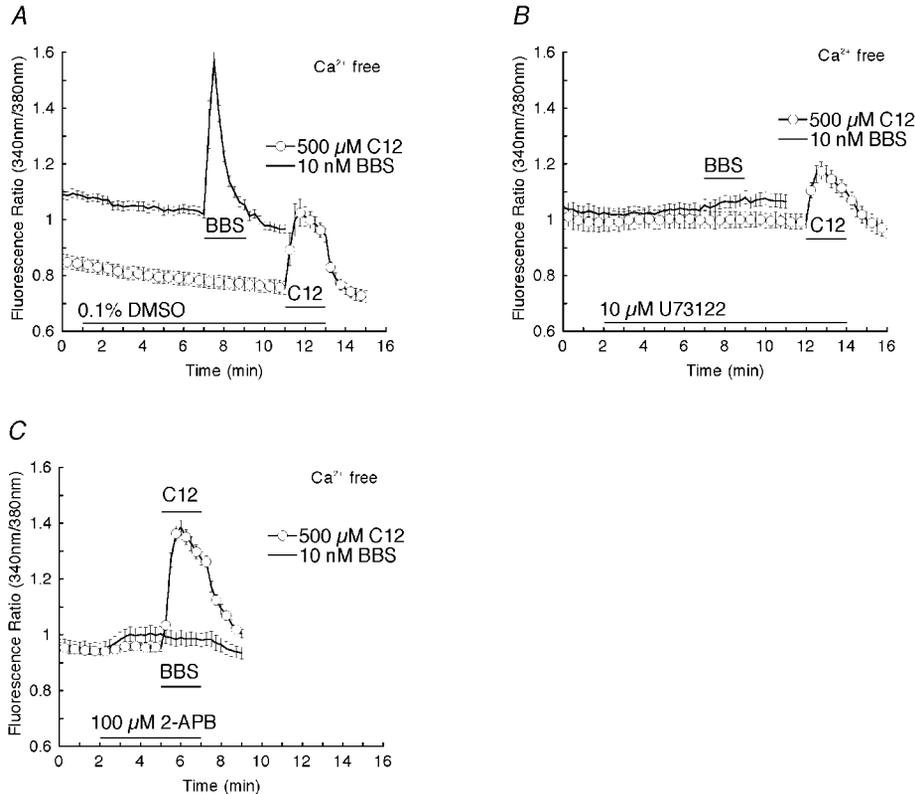
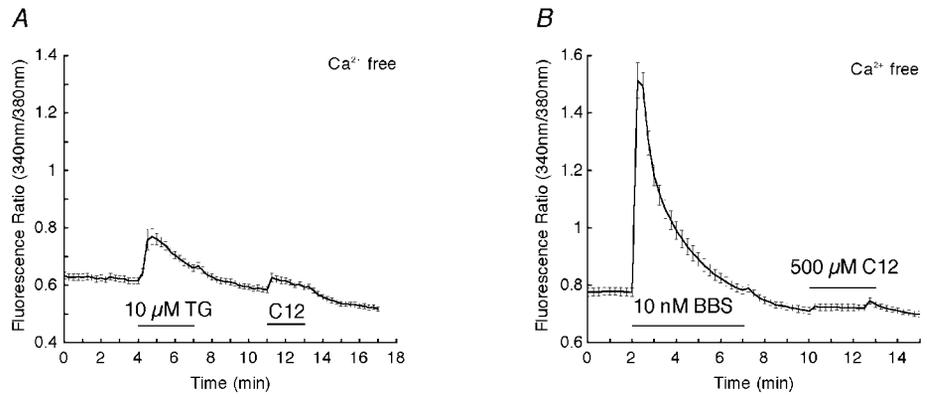
cAMP-dependent CCK release in STC-1 cells (20, 21), the cAMP antagonist ( $R_p$ )-8-Br-cAMPs also failed to block  $C_{12}$ -induced  $[Ca^{2+}]_i$  responses. Finally, thromboxane  $A_2$ , an arachidonic acid cascade product generated by phospholipase  $A_2$  has been reported to induce  $Ca^{2+}$  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)  $10 \mu M$  TG for 3 min, or (B)  $10 nM$  bombesin (BBS) for 5 min before exposure to  $500 \mu M$   $C_{12}$ . Values are mean fluorescence ratio  $\pm$  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  $500 \mu M$   $C_{12}$  (open circles) or  $10 nM$  bombesin (BBS; no symbols) for 2 min in the presence of 0.1% (v/v) dimethyl sulfoxide (DMSO) (A),  $10 \mu M$  U73122 (B), or  $100 \mu M$  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  $\pm$  S.E. of 10–30 cells obtained from a single experiment, and data are representative of at least three separate experiments.

taining in addition  $0.2 \mu M$  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  $[Ca^{2+}]_i$  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  $1 \mu M$  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.

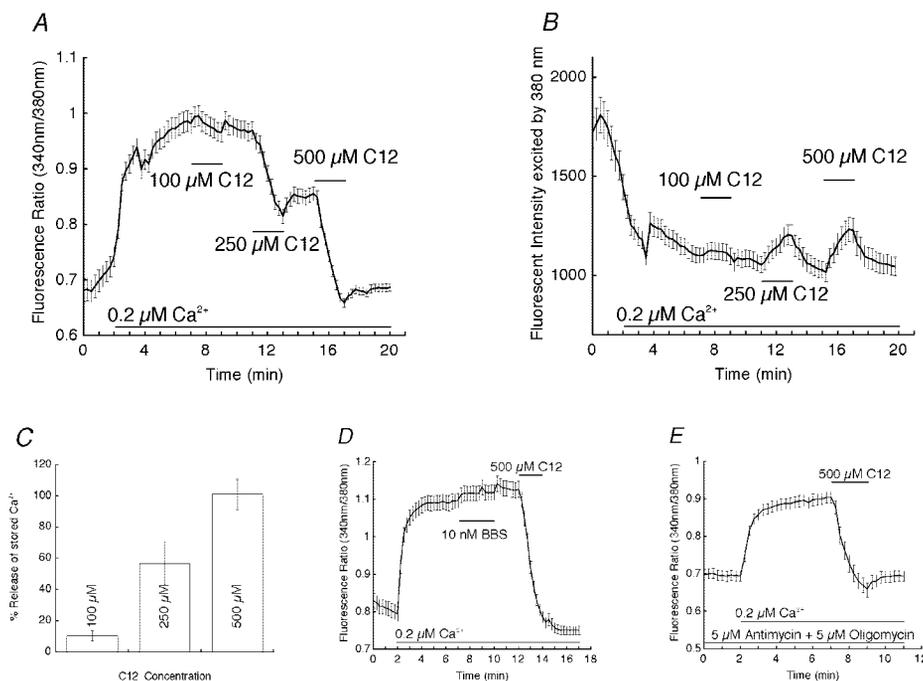
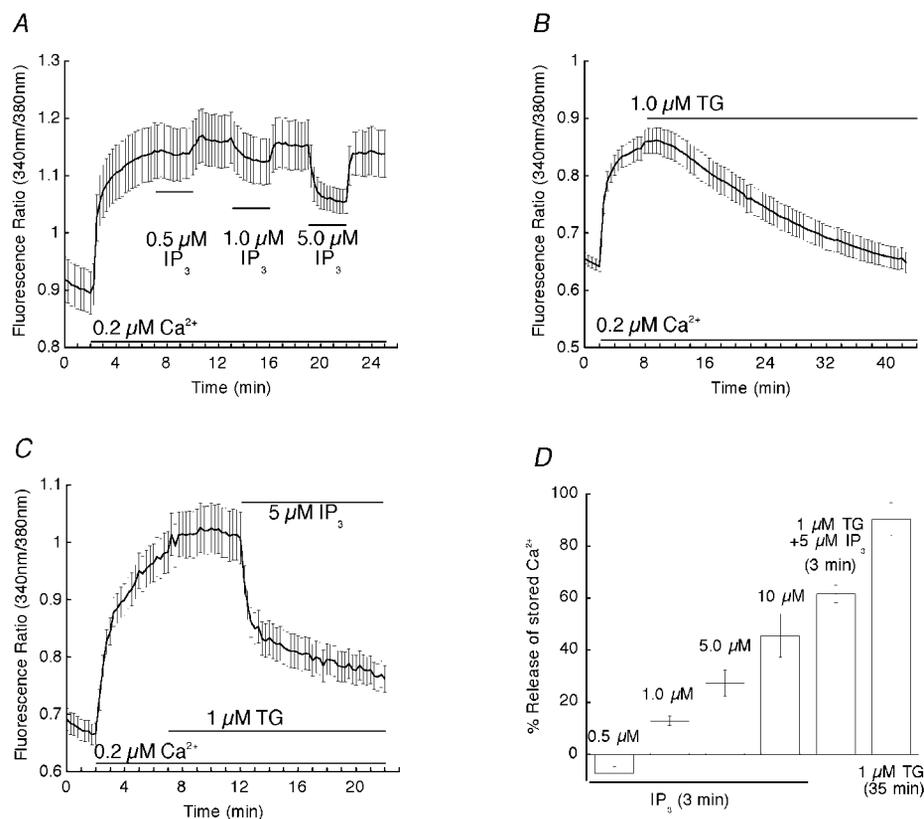
Exposing permeabilized cells to  $IP_3$  resulted in a rapid, dose-dependent release of stored  $Ca^{2+}$  (Fig. 4, A and D). By contrast, thapsigargin induced a slow and continuous decrease in the magfura-2 ratio, indicating the existence of a slow efflux of  $Ca^{2+}$  from intracellular stores that is normally masked by sarco/endoplasmic reticulum calcium ATPase-mediated  $Ca^{2+}$  re-uptake (Fig. 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  $\geq 250 \mu M$  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  $500 \mu M$   $C_{12}$  (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

**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  $\mu M$  free  $Ca^{2+}$  for 5 min. **A**,  $Ca^{2+}$ -loaded cells were sequentially exposed to 0.5, 1.0, and 5.0  $\mu M$   $IP_3$  for 3 min. **B**,  $Ca^{2+}$ -loaded cells were exposed to 1.0  $\mu M$  TG for 35 min. **C**,  $Ca^{2+}$ -loaded cells were exposed to 1.0  $\mu M$  TG for 5 min, and exposed to 5.0  $\mu M$   $IP_3$  in the presence of 1.0  $\mu M$  TG. Values are mean fluorescence ratio  $\pm$  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  $\mu M$  TG alone (in 35 min). Values are mean  $\pm$  S.E. obtained from different experiments ( $n = 6$  for 1–5  $\mu M$   $IP_3$ ,  $n = 5$  for 10  $\mu M$   $IP_3$ ,  $n = 9$  for  $IP_3 + TG$ ,  $n = 5$  for TG, respectively).

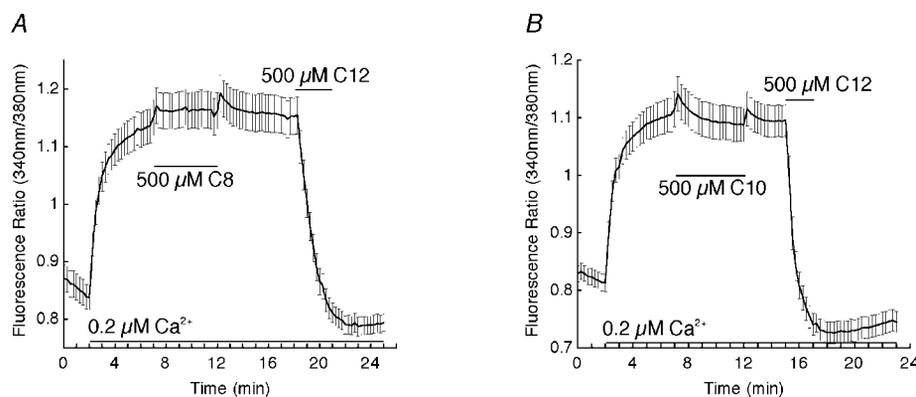


**FIG. 5.  $C_{12}$  induces  $Ca^{2+}$  release from intracellular stores in permeabilized STC-1 cells.** Permeabilized cells were washed with the intracellular buffer containing 1 mM ATP and 0.9 mM free  $Mg^{2+}$  in the absence of  $Ca^{2+}$ , then cells were loaded with 0.2  $\mu M$  free  $Ca^{2+}$  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  $\mu M$   $C_{12}$  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  $\pm$  S.E. obtained from different experiments ( $n = 5$  for 100  $\mu M$ ,  $n = 4$  for 250  $\mu M$ ,  $n = 7$  for 500  $\mu M$   $C_{12}$ , respectively). **D**,  $Ca^{2+}$ -loaded cells were sequentially exposed to 10 nM bombesin (BBS) and 500  $\mu M$   $C_{12}$ . **E**, permeabilized cells were washed and loaded with 0.2  $\mu M$  free  $Ca^{2+}$  in the buffer containing mitochondrial  $Ca^{2+}$  uptake inhibitors (5  $\mu M$  antimycin and 5  $\mu M$  oligomycin).  $Ca^{2+}$ -loaded cells were exposed to 500  $\mu M$   $C_{12}$  in the presence of mitochondria  $Ca^{2+}$  uptake inhibitors. Values in **A**, **B**, and **D** are mean fluorescence ratio or fluorescence intensity  $\pm$  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. 5B). The effect of

$C_{12}$  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

**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  $\mu M$  free  $Ca^{2+}$  for 5 min.  $Ca^{2+}$ -loaded cells were sequentially exposed to: A, 500  $\mu M$   $C_8$  for 5 min and 500  $\mu M$   $C_{12}$  for 2 min; B, 500  $\mu M$   $C_{10}$  for 5 min and 500  $\mu M$   $C_{12}$  for 2 min. Values are mean fluorescence ratio  $\pm$  S.E. of 10–30 cells obtained from a single experiment, and data are representative of at least three different experiments.



cells (Fig. 5D) even though  $C_{12}$  induced a decrease in magfura-2 ratio in the same cell preparations. Mitochondrial  $Ca^{2+}$  uptake inhibitors (23) antimycin (5  $\mu M$ ) and oligomycin (5  $\mu M$ ) did not impair the decrease in magfura-2 ratio induced by  $C_{12}$  (Fig. 5E). As in intact cells (Fig. 1B),  $C_8$  and  $C_{10}$  (500  $\mu M$ ) 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  $\mu M$   $C_{12}$  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. 7A). 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. 7B).

**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  $\mu M$  induced  $Ca^{2+}$  mobilization because of  $Ca^{2+}$  release from intracellular stores (Fig. 8A). 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  $\mu M$   $C_{12}$  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. 8A), 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. 8B 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  $\mu M$   $C_{12}$ . 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  $500 \mu M C_{12}$  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  $\pm$  S.E., and data are representative of three individual experiments.

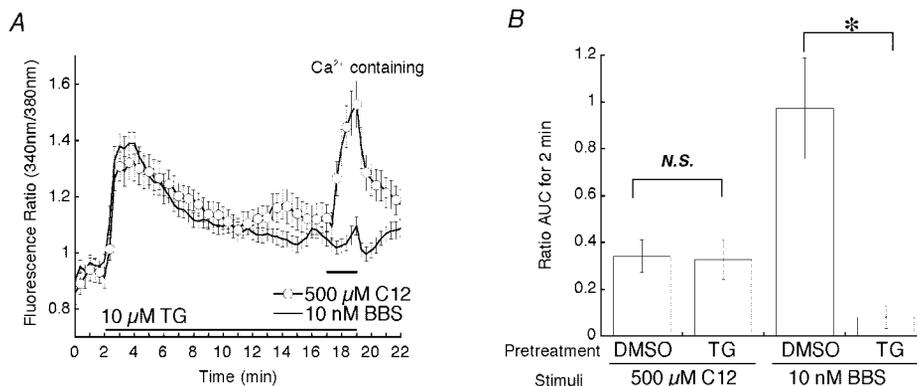
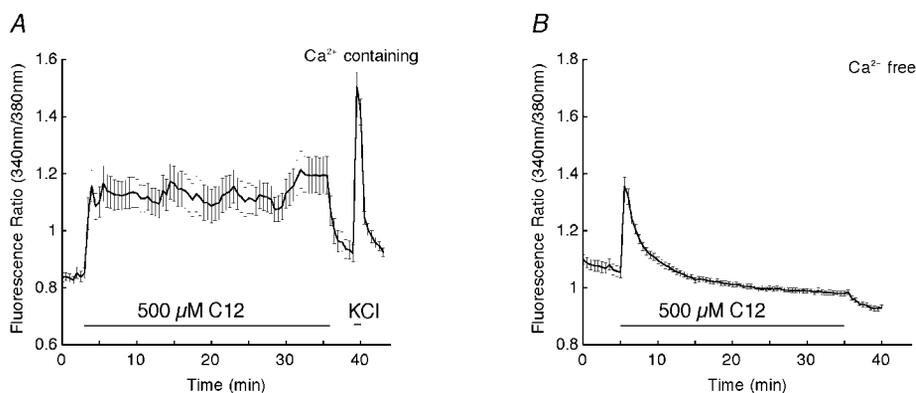


FIG. 8. Changes in  $[Ca^{2+}]_i$  in response to  $C_{12}$  or bombesin after TG treatment in the presence of extracellular  $Ca^{2+}$  in STC-1 cells. In the presence of extracellular  $Ca^{2+}$ , STC-1 cells were exposed to  $10 \mu M$  TG or vehicle (0.1% dimethyl sulfoxide (DMSO)) for 15 min followed by exposure to  $500 \mu M C_{12}$  (open circles) or  $10 nM$  bombesin (BBS; no symbols) for 2 min. A, values are mean fluorescence ratio  $\pm$  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  $500 \mu M C_{12}$  or  $10 nM$  bombesin exposure for 2 min after TG or vehicle treatment ( $n = 4, 7, 4,$  and  $5$  for  $Me_2SO + C_{12}$ ,  $TG + C_{12}$ ,  $Me_2SO +$  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  $500 \mu M C_{12}$  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  $Ca^{2+}$  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

*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 thap-

sigargin, 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 ( $M_r$  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. 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 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.

*Acknowledgments*—We thank Dr. C. M. Townsend, Jr. for providing BON cells, and Dr. P. Padfield for SLO and expert advice on its use.

#### REFERENCES

- Buchan, A. (1999) *Am. J. Physiol.* **277**, G1103–G1107
- Furness, J. B., Kunze, W., and Clerc, N. (1999) *Am. J. Physiol.* **277**, G922–G928
- Raybould, H. E. (1999) *Am. J. Physiol.* **277**, G751–G755
- Hira, T., Hara, H., and Aoyama, Y. (1999) *Biosci. Biotechnol. Biochem.* **63**, 1192–1196
- Nishi, T., Hara, H., Hira, T., and Tomita, F. (2001) *Exp. Biol. Med.* **226**, 1031–1036
- Reimann, F., and Gribble, F. M. (2002) *Diabetes* **51**, 2757–2763
- Gribble, F. M., Williams, L., Simpson, A. K., and Reimann, F. (2003) *Diabetes* **52**, 1147–1154
- McLaughlin, J. T., Grazia Luca, M., Jones, M. N., D'Amato, M., Dockray, G. J., and Thompson, D. G. (1999) *Gastroenterology* **116**, 46–53
- McLaughlin, J. T., Lomax, R. B., Hall, L., Dockray, G. J., Thompson, D. G., and Warhurst, G. (1998) *J. Physiol.* **513**, 11–18
- Benson, R. S., Sidhu, S., Jones, M. N., Case, R. M., and Thompson, D. G. (2002) *J. Physiol.* **538**, 121–131
- Kazmi, S., Sidhu, S. S., Donohoe, T. J., Wickham, M., Jones, M. N., Thompson, D. G., Case, R. M., and Benson, R. S. (2003) *J. Physiol.* **553**, 759–773
- Sidhu, S. S., Thompson, D. G., Warhurst, G., Case, R. M., and Benson, R. S. (2000) *J. Physiol.* **528**, 165–176
- van de Put, F. H., and Elliott, A. C. (1996) *J. Biol. Chem.* **271**, 4999–5006
- Schoenmakers, T. J., Visser, G. J., Flik, G., and Theuvsen, A. P. (1992) *BioTechniques* **12**, 870–874, 876–879
- Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Menezes, A., Zeman, R., and Sabban, E. (1996) *J. Neurochem.* **67**, 2316–2324
- Karlsson, S., Sundler, F., and Ahren, B. (2001) *Biochem. Biophys. Res. Commun.* **280**, 610–614
- Hoening, M., and Sharp, G. W. (1986) *Endocrinology* **119**, 2502–2507
- Chang, C. H., Chey, W. Y., Erway, B., Coy, D. H., and Chang, T. M. (1998) *Am. J. Physiol.* **275**, G192–G202
- Chang, C. H., Chey, W. Y., Sun, Q., Leiter, A., and Chang, T. M. (1994) *Biochim. Biophys. Acta* **1221**, 339–347
- Prpic, V., Basavappa, S., Liddle, R. A., and Mangel, A. W. (1994) *Biochem. Biophys. Res. Commun.* **201**, 1483–1489
- Hertelendy, F., Molnar, M., and Jamaluddin, M. (1992) *Mol. Cell. Endocrinol.* **83**, 173–181
- van de Put, F. H., and Elliott, A. C. (1997) *J. Biol. Chem.* **272**, 27764–27770
- Hofer, A. M., and Machen, T. E. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2598–2602
- Padfield, P. J., and Panesar, N. (1995) *Am. J. Physiol.* **269**, G647–G652
- Padfield, P. J., Panesar, N., Henderson, P., and Baldassare, J. J. (1996) *Biochem. J.* **314**, 123–128
- Pozzan, T., Magalhaes, P., and Rizzuto, R. (2000) *Cell Calcium* **28**, 279–283
- Sanders, K. M. (2001) *J. Appl. Physiol.* **91**, 1438–1449