Bile Acids Induce \( \text{Ca}^{2+} \) Release from Both the Endoplasmic Reticulum and Acidic Intracellular Calcium Stores through Activation of Inositol Trisphosphate Receptors and Ryanodine Receptors*

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Gallstones can cause acute pancreatitis, an often fatal disease in which the pancreas digests itself. This is probably because of biliary reflux into the pancreatic duct and subsequent bile acid action on the acinar cells. Because \( \text{Ca}^{2+} \) toxicity is important for the cellular damage in pancreatitis, we have studied the mechanisms by which the bile acid tauroliothocholic acid 3-sulfate (TLC-S) liberates \( \text{Ca}^{2+} \). Using two-photon plasma membrane permeabilization and measurement of \( [\text{Ca}^{2+}] \) inside intracellular stores at the cell base (dominated by ER) and near the apex (dominated by secretory granules), we have characterized the \( \text{Ca}^{2+} \) release pathways. Inhibition of inositol trisphosphate receptors (IP$_3$Rs), by caffeine and 2-APB, reduced \( \text{Ca}^{2+} \) release from both the ER and an acidic pool in the granular area. Inhibition of ryanodine receptors (RyRs) by ruthenium red (RR) also reduced TLC-S induced liberation from both stores. Combined inhibition of IP$_3$Rs and RyRs abolished \( \text{Ca}^{2+} \) release. RyR activation depends on receptors for nicotinic acid adenine dinucleotide phosphate (NAADP), because inactivation by a high NAADP concentration inhibited release from both stores, whereas a cyclic ADPR-ribose antagonist had no effect. Bile acid-elicited intracellular \( \text{Ca}^{2+} \) liberation from both the ER and the apical acidic stores depends on both RyRs and IP$_3$Rs.

Bile acids, hydrophobic derivatives of cholesterol, have been suggested as a possible cause of acute pancreatitis (1). Many patients with acute pancreatitis have gallstones, blocking the ampulla of Vater, which may allow reflux of bile into the pancreatic duct system, leading to inflammation. It has been proposed that bile acids can trigger acute pancreatitis (2–4), and they have also been postulated to be tumor promoters (5), although the mechanism of action is not clear (6). Bile salts are known to induce severe experimental pancreatitis (7–9). Recent research points mainly toward abnormal calcium signaling as a possible cause of acute pancreatitis (10–13) while casting doubt on the originally proposed ionophore-like mechanism of action of bile acids (14, 15). Previous work has shown that application of bile acids can cause an increase in the levels of cytosolic \( [\text{Ca}^{2+}] \) in both hepatocytes (16, 17) and pancreatic acinar cells (10). Specifically, bile acids activate calcium entry into the cell and cause depletion of internal calcium stores (12). Other effects not linked to calcium signaling (18) have also been observed, including an increase in the intracellular Na$^+$ concentration (19) and depolarization of the inner mitochondrial membrane (20, 21).

\( \text{Ca}^{2+} \) signaling has been extensively studied in pancreatic acinar cells (22, 23) and their organelles (24–26). They are highly polarized, with distinct basal and apical poles. The secretory granules are confined to the apical region (27), which is surrounded by a perigranular Golgi apparatus and a mitochondrial belt (28, 29). All \( \text{Ca}^{2+} \) signals start in the apical pole, and those elicited by low agonist concentrations are mostly confined to this part of the cell. Nevertheless, a major part of the \( \text{Ca}^{2+} \) released into the cytosol is recruited from the basal ER, which has extensions with a high IP$_3$ receptor concentration in the apical region (22). The apical \( \text{Ca}^{2+} \) signals are sufficient for stimulation of both enzyme and fluid secretion (22, 30).

Although the involvement of the ER in stimulant-evoked \( \text{Ca}^{2+} \) release is beyond doubt, there are other relevant \( \text{Ca}^{2+} \) stores. Recently we have reported a new, sensitive, and function-preserving method of acinar cell permeabilization at the apical membrane using a two-photon laser beam (31). This technique produces a stably permeabilized cell without disrupting intracellular organelles, based on pore formation in the cell membrane at just one location. Using this technology, we have, in addition to the well established ER \( \text{Ca}^{2+} \) store, found a smaller intracellular \( \text{Ca}^{2+} \) store, acidic in nature, most likely located in the secretory granules. While it is known that bile acids cause \( \text{Ca}^{2+} \) release from the ER (12, 13) and depolarization of mitochondria (20), the involvement of other organelles (32) has not been investigated. It has been shown previously that functional IP$_3$Rs are needed for bile acid-induced \( \text{Ca}^{2+} \) release from the ER (10, 33), but the possible involvement of

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2 The abbreviations used are: ER, endoplasmic reticulum; NAADP, nicotinic acid adenine dinucleotide phosphate; ROI, region of interest; TLC-S, tauroliothocholic acid 3-sulfate; IP$_3$R, inositol trisphosphate receptor; RyR, ryanodine receptor; RR, ruthenium red; 2-APB, 2-aminoethoxydiphenylborate; TG, thapsigargin; CICR, calcium-induced calcium release.
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RESULTS

Mechanisms of TLC-S-induced Ca\(^{2+}\) Release from the ER—Bile acids have been shown to release Ca\(^{2+}\) into the cytoplasm of pancreatic acinar cells, and this process was inhibited by the IP\(_R\) antagonist caffeine. It was therefore proposed that bile acids activate IP\(_R\)s on the ER (10). To study in detail the role internal stores play in bile acid-induced Ca\(^{2+}\) release, we measured [Ca\(^{2+}\)]\(_i\) changes in the internal stores using a low-K\(_d\) Ca\(^{2+}\) indicator (Fluo 5N) in permeabilized cells. We used the new and sensitive two-photon technique of cellular permeabilization (31). A typical trace showing the effect of the bile acid TLC-S (200 \(\mu\)M) on Fluo 5N fluorescence (F/F\(_0\)) in the intracellular stores is shown in Fig. 1A (\(n = 16\)). TLC-S elicited a marked decrease in the store [Ca\(^{2+}\)]\(_i\), which was reversible after removal of the bile acid. The mean amplitude of the decrease in store [Ca\(^{2+}\)]\(_i\) elicited by TLC-S (defined as 100 \(\pm\) 8% S.E.) was not very different from that induced by 10 \(\mu\)M IP\(_3\) (93 \(\pm\) 4%) or 10 \(\mu\)M cADPR (86 \(\pm\) 3.3%) (Fig. 1H).

Inhibition of IP\(_R\)s Reduces TLC-S-elicited Ca\(^{2+}\) Release—To test the involvement of IP\(_R\)s and also the possible role of RyRs in the TLC-S-evoked Ca\(^{2+}\) release we have used a pharmacological approach. 2-Aminoethoxydiphenylborate (2-APB) (100 \(\mu\)M), an inhibitor of IP\(_R\)s (36), reduced markedly (89 \(\pm\) 4%) the effect of TLC-S. In 5 of 7 experiments, 2-APB abolished the TLC-S-induced Ca\(^{2+}\) release (Fig. 1, C, panel a), whereas in the remaining two cases there was a substantial reduction in the Ca\(^{2+}\) release response (Fig. 1C, panel b). Another IP\(_R\) antagonist, caffeine, at a concentration of 20 \(\mu\)M (37), inhibited the responses to TLC-S by 87 \(\pm\) 4.1% (Fig. 1Da). In 6 of 11 cases there was a complete block (Fig. 1Da), and in the remaining five cases a marked reduction (Fig. 1D, panel b). On average (Fig. 1H), 2-APB appeared to be a marginally better inhibitor than caffeine, possibly because of sensitization of RyRs by caffeine (38), and we have therefore used 2-APB as an IP\(_R\) inhibitor in the majority of the subsequent experiments.

Inhibition of RyRs Reduces TLC-S-elicited Responses—Ruthenium Red (RR), a known inhibitor of RyRs at a concentration of 10 \(\mu\)M, either completely blocked (\(n = 5\) of 8) or substantially inhibited (\(n = 3\) of 8) bile-induced Ca\(^{2+}\) release (Fig. 1B). On average, RR reduced the Ca\(^{2+}\) responses by 71 \(\pm\) 7.5% (Fig. 1H). This result suggests involvement of RyRs in bile-induced Ca\(^{2+}\) release from internal stores. On average, 2-APB displayed a higher level of inhibition than RR, i.e. 89 \(\pm\) 4% (Fig. 1H).

Simultaneous Block of both IP\(_R\)s and RyRs Abolishes TLC-S- induced Ca\(^{2+}\) Release—We have also tested the responses to TLC-S in the presence of a combination of RyR and IP\(_R\) inhibitors, as shown in Fig. 1E. This combination completely blocked TLC-S-elicited Ca\(^{2+}\) release from internal stores (99 \(\pm\) 1%, Fig. 1H). These data indicate that all Ca\(^{2+}\) release from internal stores induced by the bile acid TLC-S occurs through the two well known intracellular Ca\(^{2+}\) release channels, IP\(_R\)s and RyRs.

The Effect of Inhibiting the NAADP Pathway on TLC-S-elicited Ca\(^{2+}\) Release—It has been shown recently that NAADP-induced Ca\(^{2+}\) release can occur through activation of RyRs (26) (31). Therefore we have tested the possibility that bile acids could interfere with NAADP-induced Ca\(^{2+}\) release through RyRs (34), controlled by cADPR or NAADP, has as yet not been studied.

This study was designed to investigate the effects of bile acids, exemplified by tauroliothocholic acid 3-sulfate (TLC-S), on Ca\(^{2+}\) concentrations in different Ca\(^{2+}\) stores in pancreatic acinar cells. We report that bile acids can release Ca\(^{2+}\) from both the ER and an acidic store in the secretory granule area. In both stores TLC-S interacts with both the IP\(_R\)s and the RyRs. TLC-S opens RyRs through activation of the NAADP, but not the cADPR pathway.

EXPERIMENTAL PROCEDURES

Reagents—Most of the chemicals including bile acids TLC-S and TCDC were obtained from Sigma or Calbiochem. Ruthenium red was from Tocris Biosciences. All fluorescent dyes were purchased from Molecular Probes (Invitrogen), FFP-18 (K\(^{+}\)) salt from TEF Labs.

Isolation of Pancreatic Acinar Cells—CD1 mice were humanely sacrificed in accordance with Schedule 1. The pancreatic acinar cells were isolated from CD1 male mice as described previously using collagenase (Worthington, UK) digestion (30). The solution for cell isolation contained (in mM): NaCl, 140; KCl, 4.7; Hepes-KOH, 10; MgCl\(_2\), 1; glucose, 10; CaCl\(_2\), 1 (pH 7.2). Cells were then incubated with dye as appropriate. All experiments were carried out at room temperature.

\([\text{Ca}^{2+}]_i\) Measurements in Intact Cells—Intact cells were loaded with either 2.5 \(\mu\)M Fluo-4 AM or 5 \(\mu\)M Fura Red, for 30 min at room temperature. Cells were perfused with a Na\(^{+}\)-Hepes-based solution, as above, but with varying concentrations of CaCl\(_2\), and pharmacological agents, as discussed later. Cells were visualized using a Leica SP2 MP dual two-photon confocal microscope, with a \(\times 63\) 1.2NA objective lens. Both dyes were excited with 476-nm wavelength light, at 3–6% laser power, and the emission recorded at 500–600 nm. Generally, a series of images was recorded at 256 \(\times\) 256 pixels resolution and analyzed using Leica (Leica, Mannheim, Germany) software. Fluorescence signals are plotted as F/F\(_0\), with F as fluorescence during the experiment, and F\(_0\), the initial level of fluorescence.

\([\text{Ca}^{2+}]_i\) Measurements in Permeabilized Cells—Cells to be permeabilized were loaded with 5 \(\mu\)M Fluo 5N AM for 40 min at 36.5 \(^\circ\)C and then transferred to poly-L-lysine-coated coverslips in a flow chamber. Cells were first washed with an intracellular solution containing (in mM): KCl, 127; NaCl, 20; Heps-KOH, 10; ATP, 2; MgCl\(_2\), 1; EGTA, 0.1; CaCl\(_2\), 0.075; pH 7.2. Cells were then permeabilized using a two-photon microscope, an invaluable tool in fluorescent optical imaging (35), as described elsewhere (31). To summarize, 1 \(\mu\)M FFP was added to the cells, and a cell was then permeabilized at a single point near the granular region using a high intensity two-photon laser beam, with wavelength 766 nm, from Spectraphysics (8W Millenia femtosecond laser). Permeabilization was confirmed by observing diffusion of Texas Red dextran (10,000) or Alexa Fluor 647 dextran (10,000) into the cytoplasm of permeabilized cells. Cells were then observed using the microscope, as described for intact cells. Fluo 5N AM was excited at 476 nm, and the emission at 500–600 nm collected. Cells were then perfused in solutions based on K\(^{+}\)-Hepes, as above, but with varying CaCl\(_2\) concentrations and pharmacological agents.

Inhibition of IP\(_R\) antagonist caffeine, at a concentration of 20 \(\mu\)M (37), inhibited the responses to TLC-S by 87 \(\pm\) 4.1% (Fig. 1Da). In 6 of 11 cases there was a complete block (Fig. 1Da), and in the remaining five cases there was a marked reduction (Fig. 1D, panel b). On average (Fig. 1H), 2-APB appeared to be a marginally better inhibitor than caffeine, possibly because of sensitization of RyRs by caffeine (38), and we have therefore used 2-APB as an IP\(_R\) inhibitor in the majority of the subsequent experiments.

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Simultaneous Block of both IP\(_R\)s and RyRs Abolishes TLC-S-induced Ca\(^{2+}\) Release—We have also tested the responses to TLC-S in the presence of a combination of RyR and IP\(_R\) inhibitors, as shown in Fig. 1E. This combination completely blocked TLC-S-elicited Ca\(^{2+}\) release from internal stores (99 \(\pm\) 1%, Fig. 1H). These data indicate that all Ca\(^{2+}\) release from internal stores induced by the bile acid TLC-S occurs through the two well known intracellular Ca\(^{2+}\) release channels, IP\(_R\)s and RyRs.

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FIGURE 1. The effect of TLC-S on intracellular Ca\(^{2+}\)-release channels in permeabilized pancreatic acinar cells loaded with Fluo SN AM. A, application of 200 \(\mu\text{M}\) TLC-S induces Ca\(^{2+}\) release from the intracellular stores. B, inhibition of TLC-S-induced Ca\(^{2+}\) release (200 \(\mu\text{M}\) TLC-S) in the presence of 10 \(\mu\text{M}\) ruthenium red. Responses were completely abolished in five cells (upper trace) and dramatically reduced in three cells (lower trace). C, 2-APB (100 \(\mu\text{M}\)) inhibited TLC-S-induced Ca\(^{2+}\) release (200 \(\mu\text{M}\) TLC-S). Responses were completely blocked in 5 of 7 cells (panel a) and almost blocked in 2 of 7 cells (panel b). 2-APB (100 \(\mu\text{M}\)) completely blocked responses to 10 \(\mu\text{M}\) IP\(_3\) (panel b). D, caffeine (20 mM) inhibited TLC-S-induced Ca\(^{2+}\) release (200 \(\mu\text{M}\) TLC-S). Responses were completely blocked in 6 of 11 cells (panel a), and dramatically reduced in 5 of 11 cells (panel b). IP\(_3\)-induced Ca\(^{2+}\) release was abolished by caffeine (panel a). E, mixture of ruthenium red (10 \(\mu\text{M}\)) and 2-APB (100 \(\mu\text{M}\)) blocked Ca\(^{2+}\) responses to both TLC-S (200 \(\mu\text{M}\)) and cADPR (10 \(\mu\text{M}\)). F, presence of 10 \(\mu\text{M}\) NAADP substantially reduced TLC-S-induced Ca\(^{2+}\) release. G, mixture of NAADP (10 \(\mu\text{M}\)) and 2-APB (100 \(\mu\text{M}\)) blocked Ca\(^{2+}\) responses to TLC-S in 6 of 9 cells (upper trace) and in 3 of 9 cells nearly abolished responses. H, summary of responses shown in A–G. Relative Ca\(^{2+}\) responses to 200 \(\mu\text{M}\) TLC-S (100%) comparable with responses to 10 \(\mu\text{M}\) IP\(_3\) or 10 \(\mu\text{M}\) cADPR and did not change in the presence of cyclic ADP-ribose antagonist 8-amino-cADPR (10 \(\mu\text{M}\)) but were much higher than responses to TLC-S in the presence of ruthenium red (10 \(\mu\text{M}\)), 10 \(\mu\text{M}\) NAADP, caffeine (10 mM), 2-APB alone (100 \(\mu\text{M}\)), or 2-APB (100 \(\mu\text{M}\)) together with 10 \(\mu\text{M}\) NAADP or with ruthenium red (10 \(\mu\text{M}\)). Responses to cADPR in the presence of 100 \(\mu\text{M}\) 2-APB and ruthenium red were completely blocked (last column). Bars represent S.E.
RyRs. Cells were preincubated with a high concentration of NAADP, 10 μM, which inactivates NAADP-induced Ca\(^{2+}\) release from internal stores in many cell types (39). The high inactivating NAADP concentration reduced the TLC-S-induced Ca\(^{2+}\) release by 76 ± 5% (Fig. 1F, n = 9), i.e. similar to the degree of inhibition caused by RR (Fig. 1E). In contrast, a known cyclic ADP-ribose antagonist, 8-NH\(_2\)-cyclic ADP-ribose (10 μM), had no effect (n = 6, 97 ± 8% of control, p > 0.7). A mixture of 2-APB (200 μM) and NAADP (10 μM) virtually abolished the TLC-S-induced responses (97 ± 1%) (Fig. 1G, n = 9), very similar to the degree of inhibition caused by the mixture of NAADP and RR (Fig. 1E). Fig. 1H shows a comparison of the magnitude of the responses induced by TLC-S, IP\(_3\), cADPR alone and TLC-S in combination with inhibiting concentrations of 2-APB, RR, caffeine, NAADP, and mixtures of these substances. The mean amplitude of the TLC-S induced decrease in store [Ca\(^{2+}\)] was set as 100 ± 8%, (0% inhibition). IP\(_3\) induced a slightly smaller response (93 ± 4%) and cADPR a slightly smaller response again (86 ± 3.3%). RR inhibited TLC-S responses by 71 ± 8%, NAADP by 76 ± 5%, 2-APB by 89 ± 4%, 2-APB + NAADP by 97 ± 1%, and 2-APB + RR by 99 ± 1%. In comparison, the response to cADPR was completely blocked by RR (100 ± 1% inhibition).

These data indicate that the mechanism of bile-induced Ca\(^{2+}\) release from intracellular stores is more specific than has been suggested previously and is exclusively dependent on two unidentified Ca\(^{2+}\) stores involved in the response to TLC-S? The ER and the mitochondria have so far been considered the main players, but the participation of other organelles has not been excluded. Our work on messenger-induced intracellular Ca\(^{2+}\) release has shown that in addition to the classical ER store, there is an unidentified Ca\(^{2+}\) store, acidic in nature, most likely in the secretory granules (31). We therefore investigated the roles of various potential intracellular Ca\(^{2+}\) stores in the TLC-S-induced Ca\(^{2+}\) release and started with the thapsigargin-sensitive ER store.

As demonstrated previously, thapsigargin (TG) (10 μM) reduced markedly [Ca\(^{2+}\)] in the intracellular stores (31), and when subsequently TLC-S was added to the bath solution, the amplitude of the decrease in store [Ca\(^{2+}\)] was only 57 ± 10% of the mean control response in the absence of TG (apical blue area, Fig. 2A, panels c–e). In none of the 35 experiments carried out did TG eliminate the TLC-S induced reduction in store [Ca\(^{2+}\)], suggesting that although the ER is the major store involved, there is another substantial Ca\(^{2+}\) store responsive to TLC-S. Regional analysis of the bile-induced responses showed that after thapsigargin-induced Ca\(^{2+}\) depletion of the ER only the store located in the secretory granular area released Ca\(^{2+}\) (Fig. 2A, panel c, n = 12), while no changes were observed in the basal part of the cell (predominantly occupied by ER) (Fig. 2A, panel e, n = 12). Comparison of the store [Ca\(^{2+}\)] changes in the basal and secretory granular areas is shown in the inset (Fig. 2A, panel d).

We have tested the hypothesis that the TLC-S-sensitive non-ER Ca\(^{2+}\) store in the secretory granular area depends on acidity for maintaining Ca\(^{2+}\) responses, by application of the ionophore nigericin, which destroys pH gradients across acidic organelle membranes by H\(^+\)/K\(^+\) exchange. Nigericin completely abolished TLC-S-induced Ca\(^{2+}\) release from the secretory granule area (Fig. 2B, n = 7). We also tested the effect of bafilomycin A1, an inhibitor of the vacuolar-type H\(^+\)-ATPase. Whereas preincubation with bafilomycin A1 did not change the bile-induced responses from the ER (Fig. 2D, n = 5), the responses from the secretory granule area were completely blocked (Fig. 2C, n = 7).

Lysosomes are potential candidate organelles for the acidic Ca\(^{2+}\) store in the secretory granule area (40). To investigate this possibility, we have destroyed the lysosomes specifically, as described previously (31), by using Gly-Phe-β-naphthylamide (GPN), a specific substrate for cathepsin C, which accumulates inside lysosomes and leads to their collapse (41). It is best to use GPN in combination with 10 μM of cathepsin inhibitor mixture CI-1 (which blocks most of the cathepsins except the lysosome-specific cathepsin C) to avoid general interference with Ca\(^{2+}\) signaling (31). Preincubation of cells with a mixture of GPN and CI-1 did not affect TLC-S-induced responses, either without or with thapsigargin (Fig. 2E, n = 5, 95 ± 10%, p > 0.7, and Fig. 2F, n = 6, 95 ± 5%, p > 0.8). We conclude that lysosomes are unlikely to be the TLC-S-sensitive acidic Ca\(^{2+}\) store in the secretory granule area. Rotenone and oligomycin were used to inhibit the mitochondria, another organelle which might be involved in the bile-induced responses (20). Oligomycin is an inhibitor of ATP synthase, and rotenone blocks mitochondrial electron transport. Cells were permeabilized, and then treated with 10 μM thapsigargin, 1 μM oligomycin, and 1 μM rotenone. This treatment had little or no effect. The TLC-S-induced responses were largely unchanged in the granular region (Fig. 2G, n = 6, p > 0.4). The amplitude of the TLC-S-elicited decrease in store [Ca\(^{2+}\)] was 104.4 ± 9.5% of the control value (Fig. 3J). These data do not of course exclude that mitochondria play some role in the bile acid-induced Ca\(^{2+}\) responses (20), but these organelles are clearly not the source of the Ca\(^{2+}\) release in the granular pole.

Brefeldin A is known to disrupt the Golgi apparatus, and we therefore preincubated cells with this substance before permeabilization, as described previously (29). Subsequently, the ER was depleted of Ca\(^{2+}\) by thapsigargin. The brefeldin A treatment did not significantly diminish the TLC-S-induced reduction in the granular store [Ca\(^{2+}\)] compared with control (94.9 ± 2.4%, p > 0.7, n = 5, Fig. 2H). By the principle of exclusion, the TLC-S-sensitive acidic Ca\(^{2+}\) store is therefore most likely the secretory granules.

**Inhibition of IP\(_3\)Rs and RyRs in the Acidic Store**—The data presented above show that TLC-S activates both IP\(_3\)Rs and RyRs in the ER. We have tested whether this is also the case in the acidic store using Ca\(^{2+}\) depletion of the ER by thapsigargin and subsequent application of IP\(_3\)R and RyR inhibitors. Because there is no TLC-S-induced Ca\(^{2+}\) release in the basal area after TG application (Fig. 2A, panel e), all measurements and statistics related to the...
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FIGURE 2. TLC-S elicits Ca\textsuperscript{2+} release from both thapsigargin-sensitive and thapsigargin-insensitive intracellular stores in permeabilized pancreatic acinar cells. A, panel a, fluorescent image of pancreatic acinar cell loaded with Fluo 5N AM and permeabilized with two-photon high intensity laser beam. Panel b, transmitted light image of the same cell. Panel c, TLC-S (200 μM) added in the presence of thapsigargin (10 μM) induced further additional Ca\textsuperscript{2+} release from the store in the secretory granule area (blue region of interest/trace). Panel d, averaged traces from the last 200 s of the experiments shown in c and e (dotted boxes) with application of 200 μM TLC-S in the continuous presence of 10 μM thapsigargin. Blue trace, granular area; red trace basal area (n = 12, p < 0.001, asterisk shows the time point at which the amplitudes in the granular and basal areas were compared using a Student’s t test; bars represent S.E.). Panel e, same experiment as in panel c, but with ROI in the basal area (red trace). TLC-S (200 μM) does not induce any detectable Ca\textsuperscript{2+} release in the presence of thapsigargin (10 μM). B, Nigericin (7 μM) blocked TLC-S-induced Ca\textsuperscript{2+} release in continuous presence of thapsigargin (n = 7). C, Bafilomycin A1 (500 nM) abolished TLC-S-induced Ca\textsuperscript{2+} release from the secretory granule area in the presence of thapsigargin (n = 7). D, Bafilomycin A1 (500 nM) failed to block TLC-S-induced Ca\textsuperscript{2+} release the in the absence of thapsigargin (n = 5). E, TLC-S induced release of Ca\textsuperscript{2+} from the secretory granule area did not change in the presence of GPN (50 μM) and CI-1 (10 μM) (n = 5). F, GPN (50 μM) in the presence of CI-1 (10 μM) did not block TLC-S-induced Ca\textsuperscript{2+} release in the presence of thapsigargin (n = 6). G, oligomycin/rotenone mixture did not affect TLC-S-induced Ca\textsuperscript{2+} release from the secretory granule area in the presence of thapsigargin (n = 6). H, Brefeldin A (10 μM) did not block TLC-S-induced Ca\textsuperscript{2+} release from the secretory granule area in the presence of thapsigargin (n = 5). Traces B, C, and E–H were taken from the granule region of permeabilized cells.

Acidic stores presented in this section were done with the region of interest placed in the secretory granule area. 2-APB, an inhibitor of IP\textsubscript{3}Rs (36), substantially reduced the TLC-S-induced responses by 76 ± 3% (Fig. 3A, n = 5). RR, an inhibitor of RyRs (38), also reduced substantially the TLC-S-induced responses by 77 ± 6% (Fig. 3B, n = 6).

Application of both inhibitors together reduced the bile acid-induced responses further, by 90 ± 6% (Fig. 3C, n = 6). These results suggest that TLC-S acts on the acidic Ca\textsuperscript{2+} store by the same mechanisms as in the ER, i.e. by activating both IP\textsubscript{3}Rs and RyRs.

Inhibition of the NAADP Pathway in Acidic Store—Because the NAADP pathway was shown to be involved in the bile acid-induced Ca\textsuperscript{2+} release from the ER (Fig. 1), we have also tested the effect of a high inactivating NAADP concentration on the bile acid-induced Ca\textsuperscript{2+} release from the acidic store. NAADP (10 μM) applied with thapsigargin (to deplete the ER of Ca\textsuperscript{2+}) substantially reduced the TLC-S-induced responses from the acidic store by 81 ± 4% (Fig. 3E, n = 7). The level of inhibition was very similar to the level of inhibition achieved by application of RR, the RyR inhibitor. A mixture of 2-APB and a high concentration of NAADP reduced the TLC-S-induced responses by 87 ± 5% (Fig. 3D, n = 7).

RyRs can be activated by another intracellular messenger, namely cADPR (38, 42). We therefore also tested whether bile acid-induced responses depend on the cADPR pathway. However, a known cADPR receptor antagonist, 8-amino-cADPR (43) did not change the TLC-S-induced Ca\textsuperscript{2+} release from the acidic stores (Fig. 3F, n = 7, 93 ± 4% of control, p > 0.8). 8-amino-cADPR completely blocked cADPR-induced release (Fig. 3G, n = 6), whereas in the absence of the antagonist normal responses from the acidic store were evoked by cADPR (n = 7, not shown), as previously described in detail for this preparation (31).
We have previously shown (31) that the NAADP-induced Ca\(^{2+}\) release from acidic stores is highly dependent on the degree of cytosolic Ca\(^{2+}\) buffering and could be completely blocked by clamping the cytosolic \([Ca^{2+}]\) at the normal resting level. A solution clamping the bath (cytosolic) \([Ca^{2+}]\) at \(-100\) nM, containing 10 mM BAPTA and 2 mM CaCl\(_2\), completely blocked the TLC-S-induced Ca\(^{2+}\) release from the acidic stores (Fig. 3H, \(n = 9\)) and reduced the response from the whole cell (dominated by the ER store) by 36 ± 6% (not shown, \(n = 14\)).

Fig. 3f summarizes the effects of the various pharmacological agents on the TLC-S-induced Ca\(^{2+}\) release from the acidic stores in the secretory granule area. These results suggest that the bile acid-induced Ca\(^{2+}\) release from the acidic stores is highly dependent on NAADP receptor (44, 45)-mediated activation of RyRs, but independent of the cADPR pathway.

**TLC-S-induced Responses in Intact Cells**—To test the relevance of the most important findings observed in the permeabilized cells, we have also performed some experiments on intact cells placed in a Ca\(^{2+}\)-free solution. Fig. 4A shows the comparable TLC-S- and ACh-elicited cytosolic Ca\(^{2+}\) signals, consistent with the findings of Lau et al. (12). Similar results were obtained using another bile acid TCDC (not shown). To inhibit IP\(_3\)Rs in intact cells we have used 20 mM caffeine (38) and found that the responses to TLC-S were either completely blocked (\(n = 19\), Fig. 4B) or substantially reduced (70 ± 10%, \(n = 8\), Fig. 4C). Simultaneous application of caffeine and RR allowed us to block both IP\(_3\)Rs and RyRs, which largely abolished the responses to TLC-S (inhibition by 95 ± 1%, \(n = 18\), Fig. 4D). The comparison of the mean responses in the intact cells shown in Fig. 4E is consistent with the findings in the permeabilized cells (Fig. 1H).

We have also investigated the responsiveness of the acidic store to
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FIGURE 4. Blockers of IP\textsubscript{3}Rs and RyRs inhibit TLC-S-induced cytosolic [Ca\textsuperscript{2+}] increase. A, 200 \mu M TLC-S induces calcium elevation in the cytosol of intact pancreatic acinar cells with a comparable amplitude as 20 \mu M acetylcholine (ACh). B and C, 20 \mu M caffeine inhibits cytosolic calcium responses to TLC-S (complete inhibition in 19 cells of 27 and responses with reduced amplitudes in 8 cells of 27). D, mixture of caffeine (20 \mu M) and ruthenium red (10 \mu M) abolishes responses to TLC-S (200 \mu M). Bars represent S.E. 

Because we have been comparing the first and second responses to TLC-S (200 \mu M) under different conditions, we decided to compare them also under the same conditions. A typical trace with repeated applications of 200 \mu M TLC-S is shown in Fig. 5C (n = 6). Comparison of relative amplitudes of first and second Ca\textsuperscript{2+} responses to 200 \mu M TLC-S application do not show any significant difference (Fig. 5D, p > 0.47, n = 6).

We have also established dose-response curves and compared the first and second responses to TLC-S in intact cells. A typical trace of TLC-S-induced calcium elevations in the cytoplasm of intact pancreatic acinar cells, using different concentrations of TLC-S (25 to 400 \mu M), is shown in Fig. 5E (n = 11). Saturation of the response to TLC-S was achieved at much lower concentration, 100 \mu M, than in permeabilized cells. Comparison of the relative Ca\textsuperscript{2+} responses to different concentrations of TLC-S is shown in Fig. 5F. Repeated stimulation of intact pancreatic acinar cells with 200

granule area (averaged response, Fig. 4G2). Inhibitors of IP\textsubscript{3}Rs (caffeine, not shown) and RyRs (RR, Fig. 4G2, n = 7) reduced the TLC-S-induced responses from the acidic stores to a similar degree to that observed in permeabilized cells (Fig. 3I).

Dose Dependence and Reproducibility of TLC-S-induced Responses—Because TLC-S is present in bile in quite high concentrations (up to 1 mM), we have compared the effects of different concentrations of TLC-S on Ca\textsuperscript{2+} responses in permeabilized pancreatic acinar cells. A typical trace with responses to increasing concentrations of TLC-S (10–400 \mu M) from the internal stores of a permeabilized pancreatic acinar cell is shown in Fig. 5A (n = 10). A summary of the dose dependence (10–1000 \mu M) of the TLC-S effects is presented in Fig. 5B and shows an increase of the amplitude of the response up to a concentration of 600 \mu M, much higher than used throughout the article, but still lower than reported for the concentrations of TLC-S in bile.

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DISCUSSION

Our new data show for the first time that the bile acid TLC-S evokes release of Ca\textsuperscript{2+} from intracellular thapsigargin-insensitive acidic stores in the secretory granule area. This is in addition to the previously demonstrated Ca\textsuperscript{2+} liberation from the ER (10). We show here that the Ca\textsuperscript{2+} release from both types of stores is mediated via both IP\textsubscript{3}Rs and RyRs and demonstrate that the RyR activation involves specifically intracellular NAADP, but not cADPR, receptors and that Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release plays a substantial role. Although, we cannot determine here if the bile acid TLC-S activates IP\textsubscript{3}Rs and RyRs directly or indirectly, our data show that both receptors play a crucial role in Ca\textsuperscript{2+} release from both the ER and the acidic store.

With regard to the nature of the acidic Ca\textsuperscript{2+} store, we have obtained data that would appear to rule out the involvement of the Golgi apparatus using Brefeldin A. Using GPN (31) we also ruled out the possibility of a lysosomal origin of the acidic store. Taken together, our data indicate that the acidic Ca\textsuperscript{2+} store is most likely located in the secretory (zymogen) granules.

Whereas the mitochondria undoubtedly would have an influence on the pattern of Ca\textsuperscript{2+} signal generation (46) including those signals elicited by bile acids in intact cells (10), our data show that mitochondrial Ca\textsuperscript{2+} release is not involved in the bile acid-induced Ca\textsuperscript{2+} liberation from thapsigargin-insensitive stores. The main importance of the mitochondria is undoubtedly the production of ATP, and because bile acids have been shown to depolarize the inner mitochondrial membrane (10) it is possible that reduction in the intracellular level of ATP could play an important role in the bile acid-induced toxic effects on intact cells, as
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recently shown with regard to the toxicity of non-oxidative alcohol metabolites (47). In our present study on permeabilized acinar cells, ATP was constantly available, because it was supplied by the bath solution.

Our findings have important implications for the pathogenesis of bile-induced acute pancreatitis. TLC-S is capable of inducing calcium signals at concentrations of a few tens of micromoles per liter, which is about ten times lower than the concentration of TLC-S in bile (48, 49). Therefore, during bile reflux, even after dilution, the TLC-S concentration will be high enough to induce calcium responses in acinar cells. As a standard protocol in our experiments, we have used a concentration of TLC-S of 200 μM, which is quite low as discussed above and gives very reproducible calcium responses in pancreatic acinar cells.

The crucial initiating stage in pancreatitis is now generally recognized to be activation of precursor enzymes in the zymogen granules and the most attractive mechanistic theory currently under discussion involves Ca^{2+} release from the interior of granules through IP_{3}R and RyR (13), which in turn open Ca^{2+}-activated K\(^{+}\) channels in the granule membrane. This allows Ca^{2+} in the granular matrix to be exchanged for K\(^{+}\) from the cytosol. According to Verdugo and co-workers (50) this would make the chemically inert precursor enzymes, normally packaged in a crystalline form, activable, because K\(^{+}\) (unlike Ca^{2+}) would not be able to support a crystalline configuration and the stone-like electron-dense structure would therefore unravel (13). Our findings demonstrating the involvement of RyR in the bile acid-mediated Ca^{2+} release also fit well in line with the recent demonstration from Gorelick’s group that RyR’s have a special role in the zymogen activation leading to pancreatitis (51, 13).

In conclusion, our new results provide strong evidence in support of an acidic intracellular Ca^{2+} store in the apical area within pancreatic acinar cells. Our work shows for the first time that this store can release Ca^{2+} into the cytosol in response to bile acid stimulation, indicating that an acidic store is involved in both pathological and physiological Ca^{2+} signaling. Another novel aspect of this study is that bile acid-elicited Ca^{2+} release from both the ER and the acidic store can be equally attributed to activation of both IP_{3}Rs and RyRs, because inhibition of either of them causes a substantial decrease in the amplitude of the response, whereas inhibition of both receptors practically abolishes release. The last but not the least interesting finding is that the mechanism of TLC-S-induced Ca^{2+} release through RyRs can be explained solely by NAADP-induced activation (31, 26).

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Bile Acids Induce Ca\(^{2+}\) Release from Both the Endoplasmic Reticulum and Acidic Intracellular Calcium Stores through Activation of Inositol Trisphosphate Receptors and Ryanodine Receptors

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