Pancreatic protease activation by alcohol metabolite depends on Ca^{2+} release via acid store IP_3 receptors

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Toxic alcohol effects on pancreatic acinar cells, causing the often fatal human disease acute pancreatitis, are principally mediated by fatty acid ethyl esters (non-oxidative products of alcohol and fatty acids), emptying internal stores of Ca^{2+}. This excessive Ca^{2+} liberation induces Ca^{2+}-dependent necrosis due to intracellular trypsin activation. Our aim was to identify the specific source of the Ca^{2+} release linked to the fatal intracellular protease activation. In 2-photon permeabilized mouse pancreatic acinar cells, we monitored changes in the Ca^{2+} concentration in the thapsigargin-sensitive endoplasmic reticulum (ER) as well as in a bafilomycin-sensitive acid compartment, localized exclusively in the apical granular pole. We also assessed trypsin activity in the apical granular region. Palmitoleic acid ethyl ester (POAEE) elicited Ca^{2+} release from both the ER as well as the acid pool, but trypsin activation depended predominantly on Ca^{2+} release from the acid pool, that was mainly mediated by functional inositol 1,4,5-trisphosphate receptors (IP_3Rs) of types 2 and 3. POAEE evoked very little Ca^{2+} release and trypsin activation when IP_3Rs of both types 2 and 3 were knocked out. Antibodies against IP_3Rs of types 2 and 3, but not type 1, markedly inhibited POAEE-elicited Ca^{2+} release and trypsin activation. We conclude that Ca^{2+} release through IP_3Rs of types 2 and 3 in the acid granular Ca^{2+} store induces intracellular protease activation, and propose that this is a critical process in the initiation of alcohol-related acute pancreatitis.

calcium | inositol trisphopshate receptors | pancreatitis

The pancreatic acinar cell is potentially dangerous because it produces a range of precursor digestive enzymes (zymogens) that, if inappropriately activated inside the cells, cause autodigestion resulting in the often fatal human disease acute pancreatitis (1, 2).

Exocytotic secretion of zymogens is controlled by local cytosolic Ca^{2+} spikes in the apical granular region, generated by small quantities of Ca^{2+} released from internal stores (3, 4). In contrast, prolonged global cytosolic $[Ca^{2+}]$ elevations—associated with emptying the Ca^{2+} stores—cause intracellular trypsin activation and transform the normally electron dense zymogen granules (ZGs) into empty looking vacuoles (2, 5–7). The vacuoles are post-exocytotic, endocytic structures, and it is in these vacuoles that trypsin activation occurs (8).

The association between alcohol abuse and acute pancreatitis is well known (1, 2, 9), but the exact mechanism by which alcohol initiates the disease is unclear. Hypertriglyceridemia is also a recognized cause of pancreatitis (10) and the presence of high concentrations of fatty acid ethyl esters [FAEEs, non-oxidative products of alcohol and fatty acids (FAs)], particularly in the pancreas, was reported in a postmortem study of subjects intoxicated by alcohol at the time of death (11). FAEEs induce trypsin activation and vacuole formation (12) and also elicit global sustained $[Ca^{2+}]_i$ elevations, due to emptying of intracellular Ca^{2+} stores, causing Ca^{2+}-dependent necrosis (9, 13, 14). Although FAs alone can also—but rather slowly—release intracellular Ca^{2+} and elicit necrosis (13), they do not, unlike FAEEs, primarily liberate Ca^{2+} from internal stores, but act by inhibiting mitochondrial ATP synthesis. The reduced intracellular ATP level prevents Ca^{2+} pump function in both intracellular stores and the plasma membrane (9, 14).

Although the endoplasmic reticulum (ER) is the principal source of Ca^{2+} released from internal stores in response to neurotransmitter or hormonal stimulation (3, 15, 16), Ca^{2+} can also be liberated from acid stores (3, 15, 17–22). Several Ca^{2+}-liberating agents release Ca^{2+} from both thapsigargin (TG)-sensitive and bafilomycin (Baf)-sensitive acidic stores (21, 22).

The purpose of this study was to test the hypothesis that the toxic FAEE action is due to trypsin activation linked to Ca^{2+} release from the acid store. Using 2-photon permeabilized acinar cells (21, 22), we show that palmitoleic acid ethyl ester (POAEE) releases Ca^{2+} from both the TG-sensitive ER and the Baf-sensitive acid store in the granular apical region. POAEE activates trypsin in pathophysiologically relevant concentrations and this activation depends on Ca^{2+} release from the apical acid store, mainly through functional IP_3 receptors (IP_3Rs) of types 2 and 3. Inhibition of IP_3Rs of types 2 and 3, but not type 1, with specific antibodies reduces markedly POAEE-elicited Ca^{2+} release as well as trypsin activation, and there are only very small Ca^{2+} release and trypsin activation responses to POAEE when IP_3Rs of type 2 and 3 are knocked out.

Results

Mechanism by which POAEE Induces Ca^{2+} Release. POAEE released Ca^{2+} from intracellular stores (Fig. 1). Fig. 1*A* shows the action of 100 μM POAEE, which elicits a sustained cytosolic $[Ca^{2+}]$ elevation in intact acinar cells (13, 14). POAEE (100 μM) elicited a marked reduction in $[Ca^{2+}]_{store}$ [$\Delta F/F_0$ = 23.0 ± 2.3% (SEM), n = 9] (Fig. 1 *A* and *F*). The POAEE-elicited reduction in $[Ca^{2+}]_{store}$ was concentration-dependent in the range of 10–100 μM (Fig. 1*E*), which is relevant pathophysiologically (11–13).

The principal Ca^{2+} release channels are IP_3Rs and ryanodine receptors (RyRs) (3, 15, 16), and we investigated whether inhibitors of these channels influence the POAEE-elicited Ca^{2+} liberation (Fig. 1). The IP_3R inhibitor 2-aminoethyldiphenyl borate (2-APB) (23) diminished markedly the POAEE-induced Ca^{2+} release (average response: 8.0 ± 2.6%, n = 5; Fig. 1 *B* and *F*). 2-APB also blocks store-operated Ca^{2+} channels in the plasma membrane (24). Although this is not a major concern in studies on permeabilized cells, we tested another IP_3R inhibitor, heparin, which also reduced markedly the POAEE-induced Ca^{2+} release (7.7 ± 1.5%, n = 6; Fig. 1*F*). We blocked RyRs by preincubation with ruthenium red (RR) and this also reduced the POAEE-induced Ca^{2+} liberation (12.8 ± 1.7%, n = 5; Fig.

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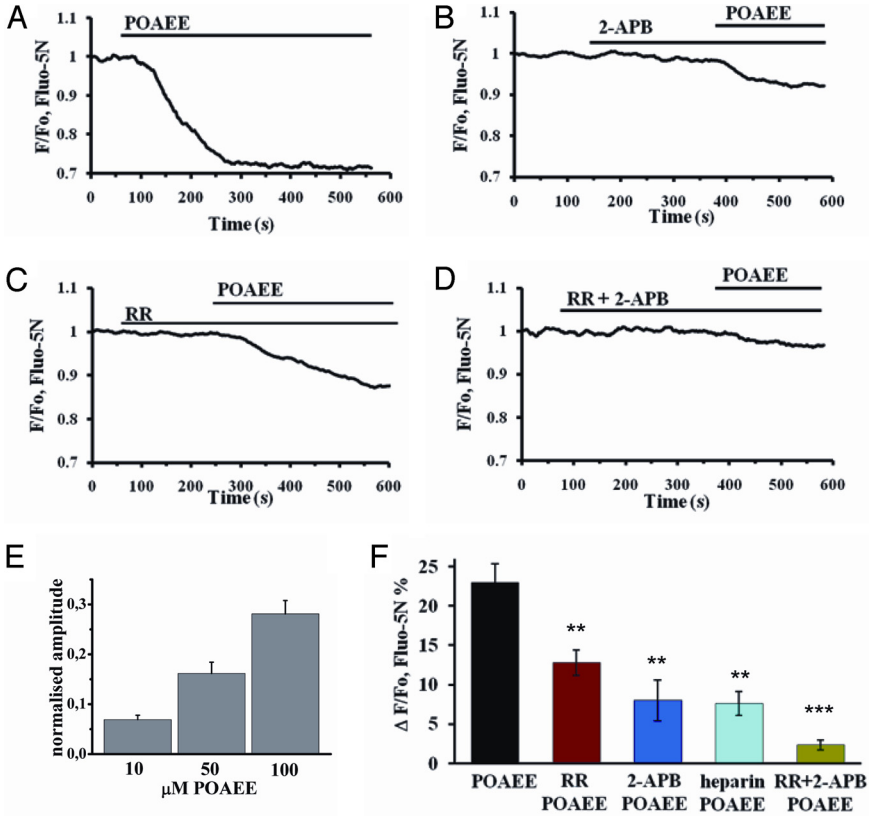

Fig. 1. IP_3Rs and RyRs play major roles in POAEE- induced Ca^{2+} release from intracellular stores. (*A*) POAEE (100 μM) evoked a marked reduction in $[Ca^{2+}]_{store}$ in a Fluo-5N AM loaded permeabilized cell. (*B*) Inhibition of IP_3Rs with 100 μM 2-APB reduced amplitude of POAEE-elicited reduction in $[Ca^{2+}]_{store}$. (*C*) The RyR antagonist ruthenium red (RR) (10 μM) also reduced the POAEE effect. (*D*) Combination of 2-APB (100 μM) and RR (10 μM) very markedly diminished the amplitude of the POAEE-elicited reduction in $[Ca^{2+}]_{store}$. (*E* and *F*) Comparisons of means of averaged amplitudes of POAEE-elicited reductions in $[Ca^{2+}]_{store}$ measured 200 s after POAEE (100 μM in *F*) application. Error bars show S.E.M; *P* values (relating to results summarized in *F*) were calculated with 1-way ANOVA test in comparison to POAEE control (first black column in *F*), **, $P < 0.01$; ***, $P < 0.001$.

1 *C* and *F*), indicating a role for RyRs. Combined application of 2-APB and RR almost abolished the POAEE responses (2.4 ± 0.7%, $n = 8$) (Fig. 1 *D* and *F*).

Localizations and Characteristics of 2 Different Ca^{2+} Stores. Our previous studies of 2-photon permeabilized acinar cells (21, 22) showed the existence of 2 separate Ca^{2+} stores, namely the ER and an acidic store. The ER maintains a high Ca^{2+} concentration by action of a Ca^{2+} pump, which can be specifically inhibited by TG (25) thereby depleting the ER of Ca^{2+} due to leaks in the ER membrane (3, 26). The acidic store does not possess this pump, but depends on a vacuolar-type H^+ pump, which can be inhibited specifically by Baf (27). This store is exclusively present in the apical granular pole of the acinar cells (21, 22).

Fig. 2 illustrates the protocol used to investigate the POAEE action on the acid store. The ER was first emptied of Ca^{2+} by TG. Thereafter POAEE (100 μM) evoked a further reduction in $[Ca^{2+}]_{store}$ in the granular, but not the basal, region (Fig. 2*A*; $n = 5$). Because TG reduced $[Ca^{2+}]_{store}$ in both basal and apical areas, whereas after TG treatment POAEE only reduced $[Ca^{2+}]_{store}$ in the apical granular pole, the functional ER seems to be present throughout the cell (28) whereas the acid TG-insensitive store seems to be confined to the apical part (21). Accumulation of Ca^{2+} into the acid store may depend on a Ca^{2+}-H^+ exchanger (3), because Baf reduces slowly $[Ca^{2+}]_{store}$ in the apical granular pole (21). We re-investigated the action of Baf (100 nM) and found slowly developing reductions in $[Ca^{2+}]_{store}$ restricted to the granular pole ($n = 8$). To speed up the responses, we used a combination of Baf (100 nM) and the Na^+/H^+ antiporter monensin (5 μM). This caused a more acute reduction in $[Ca^{2+}]_{store}$ (14.5% ± 0.9; $n = 6$), which occurred exclusively in the granular region (Fig. S1). The TG-induced reduction in $[Ca^{2+}]_{store}$ in the granular area was 25.4% ± 3.2 ($n = 10$). We tested whether the POAEE-elicited Ca^{2+} release in the granular pole, after emptying the ER elements of Ca^{2+} with TG, was from a Baf-sensitive store. When POAEE was added after TG treatment following a 30-min preincubation with Baf (100 nM), it failed to reduce $[Ca^{2+}]_{store}$ in the granular region (Fig. 2*B*; $n = 6$). POAEE also released Ca^{2+} from ER elements in the apical pole. After preincubation with Baf, but without TG treatment, POAEE elicited a clear (but significantly smaller than without Baf, $P < 0.02$) reduction in $[Ca^{2+}]_{apical\ store}$ (9.5 ± 0.7%, $n = 5$). We conclude that POAEE is capable of releasing Ca^{2+} from both the ER and an acid store in the apical pole.

POAEE-elicited Ca^{2+} Release from the Acid Store Depends on Functional IP_3Rs and RyRs. In these experiments (Fig. S2), we investigated specifically the actions of Ca^{2+} release channel blockers on the effect of POAEE on the acid (non-TG-sensitive) Ca^{2+} stores. In control experiments, after TG treatment, POAEE evoked a marked reduction in $[Ca^{2+}]_{acid\ store}$ (12 ± 0.7%, $n = 5$; Fig. S2*D*). After application of TG + the IP_3R inhibitor 2-APB, POAEE only evoked a very minor reduction in $[Ca^{2+}]_{acid\ store}$ (2.3 ± 0.2%, $n = 5$; Fig. S2 *A* and *D*) and a very similar result was obtained with another IP_3R inhibitor, heparin (2.3 ± 3%, $n = 6$; Fig. S2*D*). RR also inhibited Ca^{2+} release markedly (4.0 ± 0.6%, $n = 4$; Fig. S2*B* and *D*). When TG was combined with both 2-APB and RR, subsequent POAEE stimulation failed to elicit any Ca^{2+} release

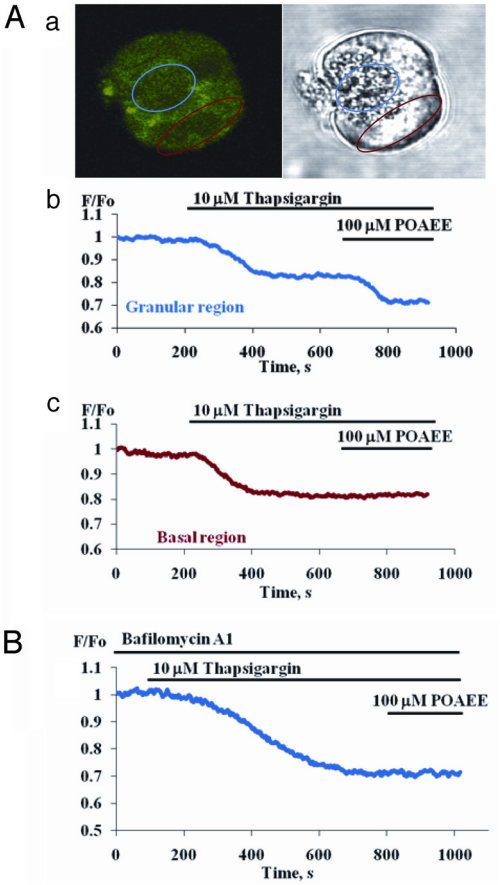

Fig. 2. POAEE releases Ca^{2+} from TG-insensitive, acidic store. (*A*) Panel (*a*) Fluorescent and transmitted light images of a permeabilized cell loaded with Fluo-5N AM. Two regions of interest from which measurements shown in *b* and *c* were obtained are outlined in blue (granular area) and red (baso-lateral area). Panel (*b*) The blue trace shows first that TG reduced $[Ca^{2+}]_{store}$ to a lower stable level. Thereafter POAEE (100 μM) evoked a further reduction in $[Ca^{2+}]_{store}$ in the granular area. Panel (*c*) Red trace shows that POAEE was unable to evoke further Ca^{2+} release in basal area after TG had reduced $[Ca^{2+}]_{store}$. (*B*) After preincubation with Baf (100 nM, 30 min), TG could still reduce $[Ca^{2+}]_{store}$, but thereafter POAEE failed to induce any further Ca^{2+} release in the granular area.

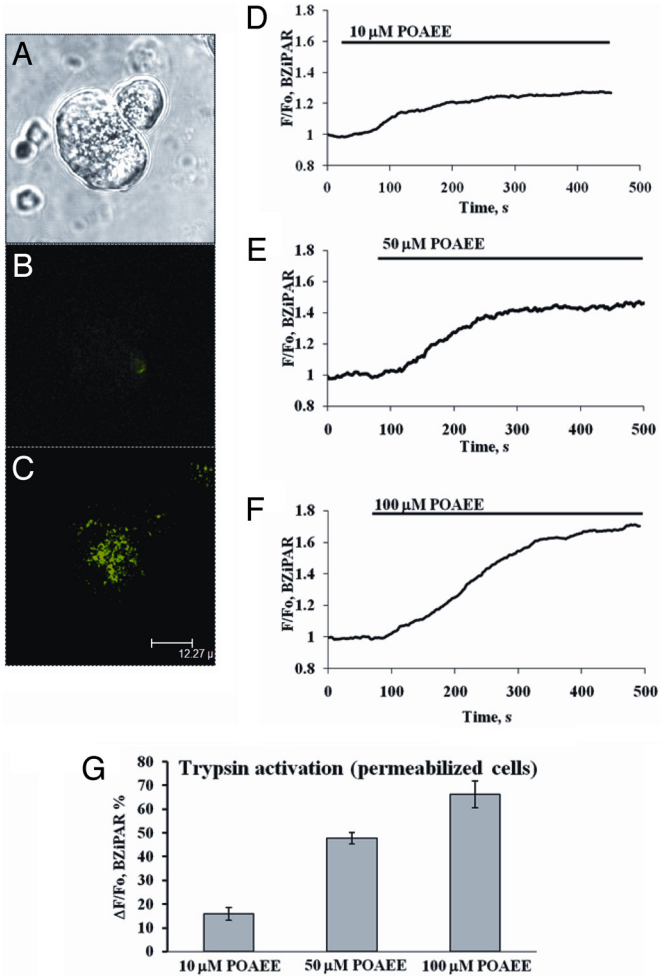

Fig. 3. POAEE activates trypsin in granular area. (*A*) Transmitted light image of 2 cells. Left cell is permeabilized and granular area is clearly visible in the right part [for scale bar (12.27 μm) see (*C*)]. (*B*) Virtual absence of BZiPAR fluorescence in permeabilized, but unstimulated, cell shown in *A*. (*C*) POAEE (100 μM) has evoked trypsin activation in granular region as seen by BZiPAR fluorescence (green). (*D–F*) Fluorescence traces showing time courses of trypsin activation evoked by POAEE (10, 50, and 100 μM). (*G*) Summary of the results of POAEE-induced trypsin activation, at 3 different concentrations (mean ± SEM, n = 5–8 for each column).

(0.1 ± 0.05%, n = 6; Fig. S2 *C* and *D*). This was not due to the acid Ca^{2+} stores being empty, because subsequent addition of a Ca^{2+} ionophore and a protonophore elicited substantial further Ca^{2+} release (Fig. S2*C*) (n = 5). The complete inhibition of Ca^{2+} liberation by blockade of Ca^{2+} release channels indicates that POAEE does not release Ca^{2+} from the acid granular store by causing unspecific membrane permeabilization.

POAEE-elicited Ca^{2+} Release from Acidic Store Does Not Require Phospholipase C Activation. Because the POAEE-elicited Ca^{2+} release from the acidic store is particularly dependent on functional IP_3Rs, we tested whether POAEE acts by stimulating IP_3 production. The most widely used inhibitor of phospholipase C (PLC) is the aminosteroid compound U73122 (29). U73122 is a powerful PLC inhibitor, but other effects—including release of Ca^{2+} from IP_3-sensitive stores—have been noted (29, 30). We used U73122 at a concentration (10 μM) that abolishes cytosolic Ca^{2+} signal generation evoked by muscarinic receptor activation in many systems (29), including pancreatic acinar cells (31). The PLC inhibitor itself caused a reduction in $[Ca^{2+}]_{acid\ store}$ (4.2 ± 0.3%, n = 4) and the subsequent POAEE-elicited reduction in $[Ca^{2+}]_{acid\ store}$ was significantly diminished (6.6 ± 0.9%; n = 9) compared with normal control POAEE responses (12.0 ± 0.7%; n = 8) ($P < 0.0003$) (Fig. S3 *A* and *B*). Importantly, U73122 did not abolish the POAEE-evoked Ca^{2+} release in any of the 8 experiments carried out. The reduced POAEE response in the presence of U73122 is most likely explained by the fact that U73122 had already itself reduced $[Ca^{2+}]$ in the store.

POAEE induces Trypsin Activation. Intra-acinar activation of zymogens is a key event in the initiation of acute pancreatitis (1, 2) and we therefore tested whether POAEE could elicit trypsin activation. We monitored, in real time, trypsin activation using a probe (BZiPAR) that becomes fluorescent when trypsin cleaves the 2 oligopeptide side chains (32). POAEE activated trypsin in the apical granular pole (Fig. 3 *A–G*). Before POAEE application, there was virtually no fluorescence, whereas after POAEE, the substrate had been cleaved preferentially in the granular area (Fig. 3 *B* and *C*). POAEE caused trypsin activation in a concentration-dependent manner, within a range (10–100 μM) (Fig. 3 *D–F*) that is pathophysiologically relevant (11–13). These data are summarized in Fig. 3*G*.

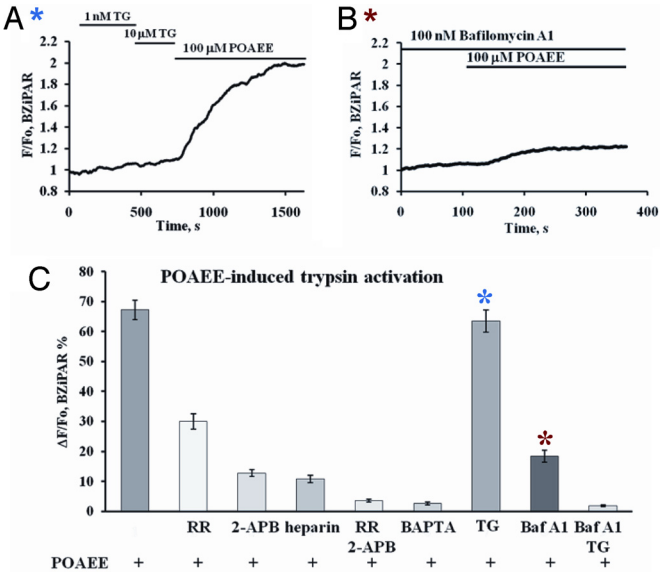

Fig. 4. POAEE-induced trypsin activation is inhibited by agents interfering with Ca^{2+} transport functions. (*A*) Emptying ER Ca^{2+} store by TG did not inhibit POAEE-induced trypsin activation. (*B*) Preincubation with 100 nM Baf reduced very markedly POAEE-induced trypsin activation. (*C*) Summary of results concerning the effects of RR, 2-APB, heparin, BAPTA, TG, and Baf on POAEE-induced trypsin activation (mean ± SEM; n = 5–8 in each case).

Trypsin activation may, at least in part, be mediated by the lysosomal cysteine protease cathepsin B (8, 9, 33). We therefore tested the effect of the cathepsin B inhibitor CA74Me (8, 34) which, at a concentration of 50 μM, markedly reduced POAEE (100 μM)-elicited trypsin activation from the control level of ≈70% (Fig. 3*G*) to 14.8 ± 2.1% (n = 6).

POAEE-induced Trypsin Activation Depends on Ca^{2+} Release from the Acid Store via IP_3Rs. We tested the effects of inhibiting the POAEE-elicited Ca^{2+} release on the ability of POAEE to activate trypsin (Fig. 4 *A–C*). Inhibition of RyRs with 10 μM RR reduced POAEE-elicited trypsin activation (Fig. 4*C*), but stronger inhibitions were observed with the IP_3R inhibitors 2-APB or heparin (Fig. 4*C*). When inhibitors of both IP_3Rs and RyRs were combined, POAEE failed to evoke trypsin activation (Fig. 4*C*). These data indicate that POAEE cannot activate trypsin without releasing Ca^{2+} from internal stores and points to IP_3Rs as the most important elements, although RyRs also play a role.

Depletion of intracellular stores may be important, but a local rise in the cytosolic [Ca^{2+}] could also be significant. To test this, we clamped the cytosolic (bath) [Ca^{2+}] at the physiological resting level by incubating the permeabilized cells in a solution containing a high concentration of a Ca^{2+}/BAPTA (Ca^{2+} chelator) mixture. Under this condition, POAEE failed to induce trypsin activation (Fig. 4*C*).

Finally, we tested whether any particular Ca^{2+} store was of special importance for trypsin activation, using TG and Baf as tools to discriminate between the ER and the acid stores (Fig. 4 *A–C*). Emptying the ER store using TG—using a protocol designed to minimize the risk of Ca^{2+}-induced Ca^{2+} release (Fig. 4*A*) —had virtually no effect on the POAEE-induced trypsin activation, which remained very similar to what was obtained under control conditions (Fig. 4 *A* and *C*). On the other hand, preincubation with Baf, which empties slowly the acid store of Ca^{2+} (21), reduced markedly the subsequent POAEE-induced trypsin activation (Fig. 4 *B* and *C*). Combining TG and Baf abolished the POAEE response (Fig. 4*C*).

Procedures other than POAEE stimulation, which releases Ca^{2+} from the granular store, might also be expected to evoke trypsin activation. As already mentioned, Baf does reduce $[Ca^{2+}]_{acid\ store}$, but only slowly. We therefore tested the effect of Baf (100 nM) and found that trypsin activation did occur (13.7 ± 1.1%, n = 6).

POAEE-induced Ca^{2+} Release Depends Mainly on IP_3Rs of types 2 and 3: Studies with IP_3R Antibodies. The physiologically most important IP_3Rs in the pancreas are types 2 and 3 (35). To test which types are involved in the POAEE-elicited Ca^{2+} release from the critical acid stores, we used antibodies against different IP_3R types (Fig. S4 *A* and *B*). First, we tested whether IP_3-elicited Ca^{2+} release in our preparation was blocked by antibodies against type 2 and 3 IP_3Rs. When both antibodies were combined, IP_3 did not evoke any Ca^{2+} release (Fig. S4*B*), whereas in the presence of an antibody against type 3 alone, there was a markedly diminished IP_3-elicited reduction of [Ca^{2+}] in the acid store (Fig. S4*B*).

Antibodies against type 2 and 3 IP_3Rs markedly reduced the Ca^{2+} release evoked by POAEE (Fig. S4*A*). Further addition of antibodies against type 1 IP_3Rs did not produce any stronger inhibition [Fig. S4*B*, no significant difference (n.s.) between the degree of inhibition produced by antibodies against types 2 and 3 and the extent of inhibition caused by antibodies to all 3 subtypes], suggesting that types 2 and 3 are the main IP_3Rs involved in the POAEE-induced Ca^{2+} release from the acidic stores. Antibodies against type 3 IP_3Rs reduced the Ca^{2+} release responses more than antibodies against type 2 receptors (P < 0.02), but the difference was minor (Fig. S4*B*). Antibodies against type 1 IP_3Rs did not inhibit the POAEE-induced Ca^{2+} release; the response in the presence of the type 1 antibody was not significantly different from control (Fig. S4*B*). Fig. S4*B* summarizes all of the data using single antibodies, combinations of antibodies as well as controls.

POAEE-induced Trypsin Activation Depends on IP_3Rs of types 2 and 3: Studies with IP_3R Antibodies. To investigate which types of IP_3Rs are involved in the POAEE-elicited trypsin activation, we used antibodies against different types of IP_3Rs. Antibodies against type 1 IP_3Rs did not reduce the extent of trypsin activation evoked by POAEE (Fig. S5 *A* and *E*). Antibodies against type 2 IP_3Rs partially inhibited trypsin activation by POAEE (Fig. S5*E*), but antibodies against type 3 IP_3Rs inhibited trypsin activation more markedly (Fig. S5 *B* and *E*). The inhibition by type 3 antibodies was significantly stronger (P < 0.02) than that exerted by the type 2 antibodies.

When antibodies against IP_3Rs of both types 2 and 3 were used together, the POAEE-elicited trypsin activation was reduced to a low level (Fig. S5 *C* and *E*), but no further inhibition was observed by a combination of antibodies against all IP_3R types (1, 2, and 3) (Fig. S5 *D* and *E*). The absence of any significant difference between these 2 data sets suggests that it is mainly IP_3Rs of types 2 and 3 that are involved in the POAEE-induced trypsin activation. Control antibodies did not change significantly trypsin activation induced by POAEE (Fig. S5*E*).

POAEE-elicited Ca^{2+} Release from the Acid Store and Trypsin Activation Depend on Functional IP_3Rs of Types 2 and 3: Knock Out of Type 2 and 3 IP_3Rs. The most direct approach to determining which types of IP_3Rs are involved in POAEE-elicited Ca^{2+} release and trypsin activation is to compare the results from mice in which specific types of IP_3Rs have been knocked out with those from the appropriate wild-type controls (35). As seen in Fig. 5 *A–C*, POAEE evoked a much reduced Ca^{2+} release from the acidic store in acinar cells from $IP_3R2^{-/-}$ mice (5.0 ± 0.4%; n = 7) compared with wild-type controls (11.1 ± 0.6%; n = 6). A stronger reduction in the POAEE-elicited Ca^{2+} release from the TG-insensitive store was observed in acinar cells isolated from mice in which both the type 2 and the type 3 IP_3Rs had been

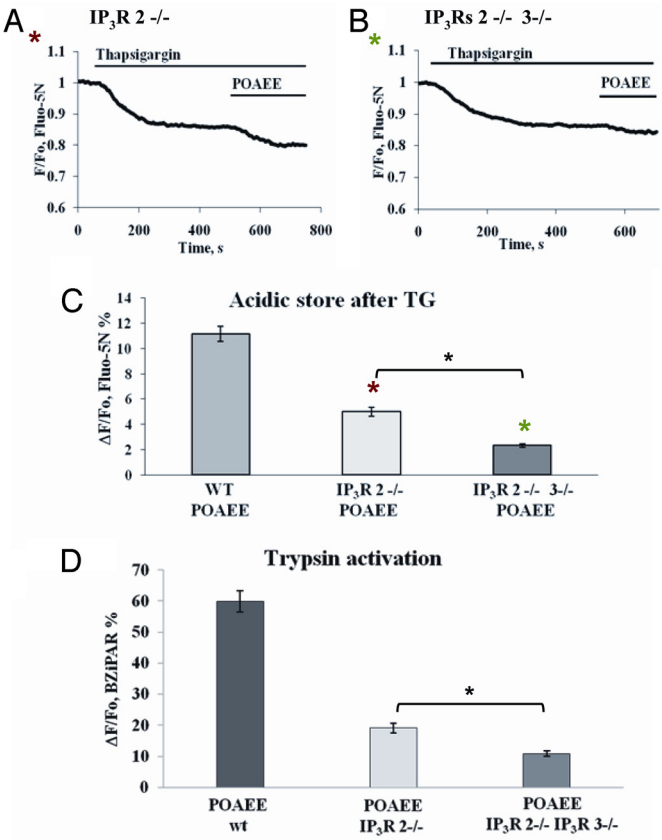

Fig. 5. Inhibition of POAEE-elicited Ca^{2+} release and trypsin activation in pancreatic acinar cells in which the type 2 IP_3R has been knocked out ($IP_3R2^{-/-}$) and in which both types 2 and 3 IP_3Rs have been knocked out ($IP_3R2^{-/-}$,$IP_3R3^{-/-}$). (*A*) Diminished POAEE-elicited reduction in $[Ca^{2+}]_{acid\ store}$ in permeabilized cells from $IP_3R2^{-/-}$ mice. (*B*) POAEE only elicited a tiny reduction in $[Ca^{2+}]_{acid\ store}$ from $IP_3R2^{-/-}$-$3^{-/-}$ double knockout mice. (*C*) Summary of results concerning effects of IP_3R subtypes 2 and 3 knockouts (in comparison with experiments on cells isolated from wild-type mice) on POAEE-elicited reductions in $[Ca^{2+}]_{acid\ store}$ (mean ± SEM, $n = 6–8$ in each case). (*D*) Summary of data concerning effects of knocking out IP_3R subtypes 2 and 3 on POAEE-elicited trypsin activation as compared to control data from wild-type mice (mean ± SEM, $n = 4$ in each case).

knocked out (2.3 ± 0.1%; $n = 8$) (Fig. 5 *B* and *C*). The Ca^{2+} release response from the double KO ($IP_3R2^{-/-}IP_3R3^{-/-}$) mice was significantly smaller ($P < 0.0001$) than from the single KO ($IP_3R2^{-/-}$) mice.

In a separate series of experiments, the POAEE-elicited trypsin activation was tested in permeabilized pancreatic acinar cells from wild type, $IP_3R2^{-/-}$ and $IP_3R2^{-/-}$,$IP_3R3^{-/-}$ mice (Fig. 5*D*). The POAEE-elicited trypsin activation was markedly reduced in the experiments on acinar cells from $IP_3R2^{-/-}$ mice (19.1 ± 0.6%; $n = 4$) as compared to controls (59.9 ± 3.5%; $n = 4$) and even more reduced in the experiments on cells from the double KO ($IP_3R2^{-/-}$,$IP_3R3^{-/-}$) mice (10.9 ± 0.9%, $n = 4$). POAEE-elicited trypsin activation was significantly lower in the double KO experiments compared with the single KOs ($P < 0.004$) (Fig. 5*D*).

Discussion

Our results show that the fatal intracellular trypsin activation, which initiates acute pancreatitis (1, 2), depends mainly on intracellular Ca^{2+} release through IP_3Rs of types 2 and 3 from an acid granular store.

ZGs constitute a major Ca^{2+} store in the apical granular pole with a high $[Ca^{2+}]$ (17, 28). IP_3 releases Ca^{2+} from isolated ZGs, as well as ZGs in intact cells, whereas specific ER Ca^{2+} pump inhibition with TG cannot liberate Ca^{2+} from these organelles (17, 36).

ZGs are not the only acid Ca^{2+} stores. Important stores, from which Ca^{2+} can be mobilized, have also been demonstrated in lysosomes and endosomes (17–21, 36–39). Trypsin activation takes place in acid Baf-sensitive postexocytotic endocytic structures, which are, at least partially, co-localized with lysosomes (8). This may be an important part of the acid Ca^{2+} store involved in the Ca^{2+} release and trypsin activation responses to POAEE characterized in this study. Our result showing that inhibition of the lysosomal enzyme cathepsin B markedly reduces POAEE-elicited trypsin activation is in agreement with this hypothesis.

How could Ca^{2+} release from intracellular stores promote trypsin activation? Our data indicate that POAEE-elicited Ca^{2+} release from the acid granular pool is more important for zymogen activation than Ca^{2+} liberation from the ER. However, our results also show that clamping the cytosolic $[Ca^{2+}]$ at the normal resting level prevents zymogen activation. Most likely, zymogen activation depends both on a reduction in $[Ca^{2+}]_{acid\ store}$ and an increase in $[Ca^{2+}]$ in the apical cytosolic environment. This would agree with the ion exchange concept of Verdugo (19, 39) in which replacement of Ca^{2+} in the matrix of secretory granules by K^+ causes matrix disaggregation. In the case of ZGs or post-exocytotic vacuoles, this would favor toxic enzyme activation. Ca^{2+}-activated opening of K^+ channels in the ZG could play an important role in this process (2, 19, 39).

How does POAEE, and presumably other FAEEs, activate Ca^{2+} release? POAEE stimulation could activate PLC and thereby generate IP_3, but our results with the PLC inhibitor U73122 do not provide evidence for this and furthermore indicate that even if this process did occur it may not be essential for POAEE-evoked Ca^{2+} release. POAEE clearly does not act specifically to open type 2 and 3 IP_3Rs in the acid pool, because Ca^{2+} release can also be activated from the ER and via both IP_3Rs and RyRs. Most likely, the functionally dominant Ca^{2+} release channels in the membranes of the acid stores are mainly IP_3Rs of types 2 and 3, and these molecules would therefore be the principal mediators of the quantitatively important Ca^{2+} release that appears to be chiefly responsible for trypsin activation.

Coffee drinking (caffeine) has some protective effect against alcohol-related pancreatitis (40), and we have previously shown that caffeine reduces POAEE-induced Ca^{2+} signal generation (14). Our finding, that specific inhibition or knock out of type 2 and 3 IP_3Rs very markedly reduces POAEE-elicited trypsin activation, provides fresh evidence indicating that such inhibition could be of potential benefit. Therefore, the inhibitory effect of caffeine on IP_3Rs (41, 42) could be a useful starting point for therapeutic considerations. Unfortunately, other caffeine effects, for example activation of RyRs mediating Ca^{2+} release from the sarcoplasmic reticulum in the heart, potentially causing serious cardiac arrhythmias (43), limit the usefulness of caffeine itself as a drug for pancreatitis treatment.

Our results provide direct evidence at the molecular level demonstrating that reduced IP_3R operation can protect against alcohol-related, and probably also hypertriglyceridemic, pancreatitis. This should encourage development of membrane-permeable agents, possibly related to caffeine, with specific inhibitory actions on IP_3Rs of types 2 and 3.

Materials and Methods

Isolation of Pancreatic Acinar Cells. Single pancreatic acinar cells and clusters of 2 or 3 acinar cells were isolated from the pancreas of adult mice by collagenase digestion and mechanical disruption as described previously (21, 22). We mostly used CD 1 male mice, but in the IP_3R knock-out experiments, control or mutant male or female mice with C57BL/6JJmsSIc origin were used.

All experiments were carried out with freshly isolated cells, attached to the coverslip of the perfusion chamber at room temperature (23 °C).

$[Ca^{2+}]_{store}$ and Trypsin Measurements in Permeabilized Cells. Cells to be permeabilized were loaded with 5–7.5 μM Fluo-5N AM, for 45 min at 36.5 °C, and then transferred to polyL-lysine coated coverslips in a flow chamber. Cells were first washed with an intracellular solution based on K-Hepes, containing (mM): KCl, 127; NaCl, 20; Hepes KOH, 10; ATP, 2; $MgCl_2$, 1; EGTA, 0.1; $CaCl_2$ 0.05; pH 7.2; 291 mosmol/L. Thereafter, cells were permeabilized using a 2-photon microscope, as previously described (21). We used the intracellular K-Hepes-based solution already described, except in the $[Ca^{2+}]$ clamp experiments when 10 mM BAPTA and 2 mM $CaCl_2$ were included. Cells were observed using a Leica SP2 MP dual 2-photon microscope. Fluo-5N AM was excited at 476 nm, and emission at 500–600 nm wavelengths was collected. For trypsin measurements, the trypsin substrate BZiPAR [rhodamine 110, bis (CBZ-L-isoleucyl-L-prolyl-L-arginine amide)] (10 μM) was added to the experimental chamber after permeabilization for the duration of the experiment. Antibodies to IP_3Rs were applied after permeabilization (dilution 1:100) and incubated for 30 min before measurements.

Reagents. Chemicals, unless otherwise indicated, were obtained from Sigma, Calbiochem, Merck. Thapsigargin, ryanodine, and ruthenium red were purchased from Tocris Biosciences. Palmitoleic acid ethyl ester (POAEE) was from MP Biomedicals. All fluorescent dyes including BZiPAR were purchased from Molecular Probes and Invitrogen, and FFP-18 (K^+) salt from TEF Labs. Antibodies against IP_3Rs and control antibodies were from Chemicon International (AB3000, AB9076, and CBL600B) and Insight Biotechnology (sc-28614).

Transgenic Mice. IP_3R2 knockout mice, IP_3R2/IP_3R3 double knockout mice and wild-type control mice were generated in the Laboratory of Developmental Neurobiology (Brain Science Institute, RIKEN, Japan) (35).

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