Ca^{2+} release-activated Ca^{2+} channel blockade as a potential tool in antipancreatitis therapy

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Alcohol-related acute pancreatitis can be mediated by a combination of alcohol and fatty acids (fatty acid ethyl esters) and is initiated by a sustained elevation of the Ca^{2+} concentration inside pancreatic acinar cells ($[Ca^{2+}]_i$), due to excessive release of Ca^{2+} stored inside the cells followed by Ca^{2+} entry from the interstitial fluid. The sustained $[Ca^{2+}]_i$ elevation activates intracellular digestive proenzymes resulting in necrosis and inflammation. We tested the hypothesis that pharmacological blockade of store-operated or Ca^{2+} release-activated Ca^{2+} channels (CRAC) would prevent sustained elevation of $[Ca^{2+}]_i$ and therefore protease activation and necrosis. In isolated mouse pancreatic acinar cells, CRAC channels were activated by blocking Ca^{2+} ATPase pumps in the endoplasmic reticulum with thapsigargin in the absence of external Ca^{2+}. Ca^{2+} entry then occurred upon admission of Ca^{2+} to the extracellular solution. The CRAC channel blocker developed by GlaxoSmithKline, GSK-7975A, inhibited store-operated Ca^{2+} entry in a concentration-dependent manner within the range of 1 to 50 μM (IC_{50} = 3.4 μM), but had little or no effect on the physiological Ca^{2+} spiking evoked by acetylcholine or cholecystokinin. Palmitoleic acid ethyl ester (100 μM), an important mediator of alcohol-related pancreatitis, evoked a sustained elevation of $[Ca^{2+}]_i$, which was markedly reduced by CRAC blockade. Importantly, the palmitoleic acid ethyl ester-induced trypsin and protease activity as well as necrosis were almost abolished by blocking CRAC channels. There is currently no specific treatment of pancreatitis, but our data show that pharmacological CRAC blockade is highly effective against toxic $[Ca^{2+}]_i$ elevation, necrosis, and trypsin/protease activity and therefore has potential to effectively treat pancreatitis.

capacitative Ca^{2+} entry | alcohol metabolite | pancreas | hepatocyte Ca^{2+} entry | AR42J

Acute pancreatitis is a human disease mostly caused by alcohol abuse or complications from biliary disease. In this disease, against which there is currently no effective therapy, digestive proenzymes are prematurely activated inside the acinar cells leading to autodigestion and necrosis (1–3). Intracellular Ca^{2+} plays a critical role in the initiation of this disease process (2–4), but intracellular Ca^{2+} also plays a critical role in the physiological regulation of the normal exocytotic secretion of the digestive proenzymes (5).

The pancreatic acinar cells are capable of generating multiple patterns of cytosolic Ca^{2+} signals depending on the type and concentration of the stimulating agent (5). The physiological Ca^{2+} signals regulating secretion—evoked by the neurotransmitter acetylcholine (ACh) or the hormone cholecystokinin (CCK)—consist of repetitive short-lasting rises in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). These are mostly confined to the apical area, in which the secretory (zymogen) granules (ZGs) are concentrated, by a belt of perigranular mitochondria operating as a firewall against the globalization of the Ca^{2+} signals (6). Rapid Ca^{2+} uptake into the perigranular mitochondria following local cytosolic Ca^{2+} signals also plays a crucial role in activating local mitochondrial ATP production, which is essential for the exocytotic secretion process (5–9). At supraphysiological concentrations of ACh or CCK, or in response to various types of pathological stimulants, sustained global elevations of $[Ca^{2+}]_i$ occur (2, 4). Such signals initiate trypsinogen activation in the apical region of the pancreatic acinar cells as well as vacuole formation (10, 11). Both the trypsinogen activation and the intracellular vacuolization can be prevented by intracellular Ca^{2+} chelation or simply by removal of extracellular Ca^{2+} (10).

Unlike nerve and endocrine as well as muscle cells, exocrine cells do not possess voltage-gated Ca^{2+} channels and the cytosolic Ca^{2+} signals governing pancreatic acinar secretion are primarily generated by release of Ca^{2+} from intracellular stores, principally the endoplasmic reticulum (ER) (5, 12, 13). However, the intracellular Ca^{2+} stores are by definition finite. Ca^{2+} ATPase pumps in the plasma membrane are activated to increase extrusion of Ca^{2+} whenever $[Ca^{2+}]_i$ increases and therefore pancreatic acinar cells would run out of Ca^{2+} in the ER if there were not a mechanism of compensatory Ca^{2+} uptake from the external solution (5). This uptake mechanism is known as store-operated or Ca^{2+} release-activated Ca^{2+} entry (CRAC) and CRAC channels in the plasma membrane have generally been well characterized (14). The molecular nature of these channels (Orai1) is now also known (15) and the link between Ca^{2+} depletion of the ER and opening of the CRAC channels has been established: A reduction in the Ca^{2+} concentration in the ER ($[Ca^{2+}]_{ER}$) causes translocation of a Ca^{2+}-sensing protein, called STIM1, widely distributed in the ER membrane to so-called puncta in the ER close to the plasma membrane, where it can interact with and open Orai1 channels (16–18). This mechanism also operates in the pancreatic acinar cells, because stimulant-elicited release of Ca^{2+} from the ER results in translocation of STIM1 to puncta close to the basolateral plasma membrane, specifically at locations where the ER is devoid of ribosomes and where interaction between STIM1 and Orai1 therefore occurs (19). Despite this, electrophysiological investigations of the currents evoked by Ca^{2+} store depletion have so far failed to provide evidence for the existence in pancreatic acinar cells of Ca^{2+}-selective currents of the CRAC type (20–22) originally discovered in mast cells (23), although activation of nonselective currents were shown (21, 22). Therefore, although the linkage between agonist-evoked Ca^{2+} release from the ER and store-operated Ca^{2+} entry, through STIM1

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and Orai1, is established for the pancreatic acinar cells (19), there is uncertainty about the biophysical nature of the principal Ca^{2+} entry channels. Endocytic Ca^{2+} uptake (24) could also be important and does occur in pancreatic acinar cells (25). It is also the case that there are important intracellular Ca^{2+} stores (acid) outside the ER (4, 26–29). Ca^{2+} release from acid stores—located in ZGs, endosomes, and lysosomes—plays a crucial role in intracellular trypsinogen activation elicited by alcohol and fatty acid ethyl esters in experiments on permeabilized pancreatic acinar cells (28, 29), and trypsinogen activation induced by hyperstimulation occurs in postexocytotic endocytic (and acid) structures (25). In view of the complexities of pancreatic acinar cell Ca^{2+} signaling in physiology and pathology and, in particular, the uncertainty about the nature of the Ca^{2+} entry pathways, it is unknown whether blockade of Ca^{2+}-selective CRAC channels could in principle be effective as therapy against acute pancreatitis. With the availability of relatively specific CRAC channel blockers (30–34) there is now an opportunity to test the hypothesis that one type of Ca^{2+} entry channel is so important that pharmacological blockade could afford effective protection against toxic Ca^{2+} signal generation and its consequences in intact pancreatic acinar cells. Given the current absence of any specific therapy against pancreatitis, a demonstration that pharmacological blockade of CRAC channels could prevent the dangerous necrosis evoked by agents known to initiate pancreatitis, would be a proof of principle that a specific therapy could be developed. In this study we have therefore tested the effect of the CRAC channel blocker developed by GlaxoSmithKline, GSK-7975A (33, 34) on store-operated Ca^{2+} entry, induced by inhibiting specifically the Ca^{2+}ATPase pumps in the ER, as well as on Ca^{2+} signal generation elicited by palmitoleic acid ethyl ester (POAEE), one of the mediators of alcohol-related pancreatitis (26, 35–38). As part of this investigation we also provide a characterization of Ca^{2+}-selective CRAC currents in pancreatic acinar cells. We show that pharmacological CRAC channel blockade prevents the sustained toxic elevation of $[Ca^{2+}]_i$, which is induced by severe depletion of intracellular Ca^{2+} stores or by POAEE. In contrast, such a blockade has little effect on the physiological Ca^{2+} spiking (oscillations) evoked by ACh or CCK and has only minor or no effects on Ca^{2+} entry in hepatocytes and the pancreatic acinar cell line AR42J. Importantly, the CRAC blocker is also effective against the intracellular protease activation and necrosis induced by POAEE. We conclude that pharmacological CRAC blockade has potential for effective treatment of acute pancreatitis.

Results

GSK-7975A Blocks Store-Operated Ca^{2+} Entry in Normal Pancreatic Acinar Cells. Our standard protocol for store-operated Ca^{2+} entry investigations consisted of first emptying the ER store of Ca^{2+} by applying the specific ER Ca^{2+} pump blocker thapsigargin (TG) in the absence of external Ca^{2+}. This caused a transient rise in $[Ca^{2+}]_i$ (Fig. 1*A*). After $[Ca^{2+}]_i$ had returned to near its normal resting level, Ca^{2+} was admitted to the external solution and this resulted in a substantial rise in $[Ca^{2+}]_i$, which was sustained as long as Ca^{2+} was present outside the cell (Fig. 1*A*). When the CRAC blocker GSK-7975A was applied before the admission of Ca^{2+} to the external solution, the subsequent rise in $[Ca^{2+}]_i$ was markedly reduced (Fig. 1 *B–D*). As seen in Fig. 1*D*, the effect of GSK-7975A was maintained after washout of the compound at least for the first 10–15 min. As summarized in Fig. 1*E*, the inhibitory effect was already marked 5 min after start of exposure to GSK-7975A and after 10 min exposure there was very little Ca^{2+} entry. Ten minutes after the start of washout of GSK-7975A, there was still no recovery of Ca^{2+} entry (Fig. 1*E*). Importantly, GSK-7975A had no influence on the thapsigargin-evoked Ca^{2+} mobilization from the ER store (Fig. 1*D*). GSK-7975A also had little or no effect on the Ca^{2+} spiking responses

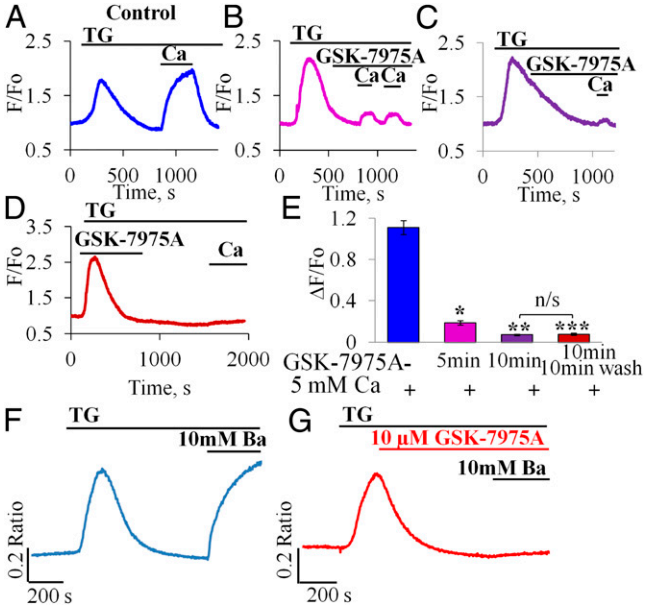

Fig. 1. CRAC channel blocker inhibits Ca^{2+} and Ba^{2+} influx. (*A–C*) Effects of different periods of incubation [5 min, $n = 5$ (*B*) and 10 min, $n = 14$ (*C*)] of pancreatic acinar cells with GSK-7975A (10 μM) compared with control (*A*, $n = 5$) (15 min incubation did not significantly further increase the degree of blockade, $n = 3$). (*D*) Washout of GSK-7975A did not result in recovery of the Ca^{2+} signal within ~10 min. (*E*) Summary of results shown in *A–D*. Mean $[Ca^{2+}]_i$ amplitude change ($\Delta F/F_0$) due to Ca^{2+} influx ($n = 5$, example trace in *A*) was dramatically reduced after 5 min with GSK-7975A ($n = 14$, $^*P < 10^{-6}$, example trace in *B*) and reduced further after 10 min with GSK-7975A ($n = 14$, $^{**}P < 10^{-11}$, example in *C*). Inhibition was effectively irreversible (after washing out for 10 min) as amplitude remained very low ($n = 7$, $^{***}P < 10^{-8}$, example trace in *D*). As seen in *E*, there was no significant difference in averaged amplitudes from experiments of the type shown in *C* and *D* ($P > 0.69$); whereas the averaged amplitudes from the type of experiments shown in *B* and *C* are significantly different ($P < 10^{-6}$). Data presented as mean ± SEM (*F* and *G*) GSK-7975A inhibits Ba^{2+} influx in pancreatic acinar cells. Representative traces of changes in $[Ba^{2+}]_i$ (using Fura-2) due to Ba^{2+} influx in cells exposed to GSK-7975A (10 μM) for 10 min (*G*) compared with control untreated cells (*F*).

to low (physiological) concentrations of the normal stimulants of pancreatic secretion, namely ACh (50 and 100 nM) or CCK (5 pM) (Fig. S1 *A–F*) but reduced the late elevated $[Ca^{2+}]_i$ plateau phase following stimulation with a high concentration of ACh (1 μM) (Fig. S1 *G* and *H*).

Inhibition of Unidirectional Ba^{2+} Influx. Net Ca^{2+} transport across the plasma membrane depends not only on Ca^{2+} influx, but also on Ca^{2+} extrusion and in the pancreatic acinar cells, extrusion is mediated exclusively by the plasma membrane Ca^{2+} pump, activated by a rise in $[Ca^{2+}]_i$ (39). To assess changes in unidirectional Ca^{2+} influx, we used measurements of $[Ba^{2+}]_i$, because Ba^{2+} can easily pass through CRAC channels (40), but cannot be extruded by the plasma membrane Ca^{2+} pump (39, 40). As seen in Fig. 1 *F* and *G*, preincubation with GSK-7975A for 10 min markedly reduced store-operated Ba^{2+} entry into the pancreatic acinar cells. Our data on the inhibition of Ba^{2+} entry by the CRAC blocker are summarized in Fig. S1 *I–K*.

Concentration Dependence of the Acute Effects of GSK-7975A on Ca^{2+} Entry. We also investigated the acute effect of the CRAC blocking agent. As seen in Fig. 2*A*, application of 10 μM GSK-7975A soon after admission of Ca^{2+} to the external solution resulted, after a delay of a few minutes, in a sharp reduction of $[Ca^{2+}]_i$ which, in the continued presence of external Ca^{2+}, fell to

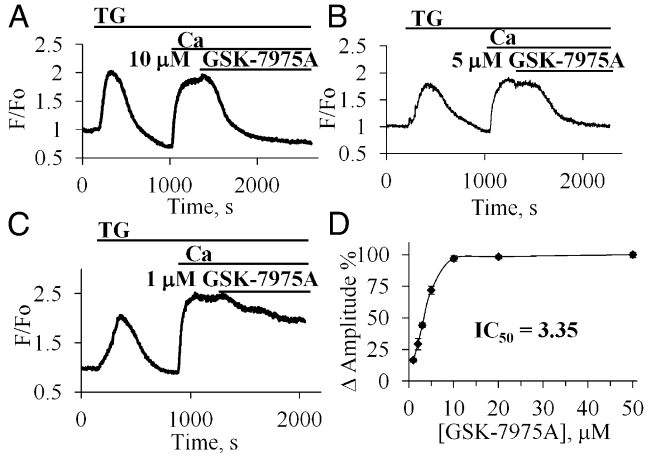

Fig. 2. Concentration dependence of the inhibitory effect of GSK-7975A on the elevated $[Ca^{2+}]_i$ following readmission of external Ca^{2+} after thapsigargin treatment. (*A–C*) Effects of acute application of GSK-7975A in different concentrations [10 μM (*A*), 5 μM (*B*), 1 μM (*C*)] on the elevated $[Ca^{2+}]_i$ plateau in the presence of 5 mM $CaCl_2$. (*D*) Summary of the results of the experiments on the concentration dependence of the inhibitory effect of GSK-7975A normalized to the inhibitory effect of 50 μM (100%).

near the normal resting level within 5–10 min. The relatively slow time course of the GSK-7975A–induced reduction in $[Ca^{2+}]_i$ is not necessarily a reflection of a slow action of the CRAC blocker, but may reflect the time it takes for the plasma membrane Ca^{2+} pump to extrude the excess of Ca^{2+} in the cytosol. We also tested the effects of different CRAC blocker concentrations. As seen in Fig. 2 *A* and *B*, the effects of 5 and 10 μM GSK-7975A were very similar, indicating that the maximal effect had already been attained at 5 μM. A small reduction in $[Ca^{2+}]_i$ was seen at 1 μM (Fig. 2*C*). Fig. 2*D* shows the relationship between CRAC blocker concentration and the degree of reduction in $[Ca^{2+}]_i$. IC_{50} was calculated to be 3.4 μM. The estimated t1/2 was 292.8 ± 88.3 s. Even the highest concentration of GSK-7975A tested (50 μM) did not abolish Ca^{2+} entry into pancreatic acinar cells, at the external $[Ca^{2+}]$ ($[Ca^{2+}]_o$) of 5 mM, but dramatically inhibited it by 87.6 ± 1.9% (n = 10).

Inhibition of Store-Operated Ca^{2+} Current. Application of 2 μM TG activated a slowly developing inward membrane current (Fig. 3 *A*, *i*) with a maximal amplitude of 8.4 ± 0.6 pA (n = 35, or 0.349 pA/pF; Cm = 22.3 ± 1.7 pF) at a membrane potential of −50 mV and $[Ca^{2+}]_o$ = 10 mM. Replacement of extracellular Na^+ by N-methyl-D-glucamine ($NMDG^+$) had little or no effect on the current (Fig. 3 *A*, *i*, n = 8), whereas a reduction in $[Ca^{2+}]_o$ from 10 to 1 mM caused a marked decrease in the maximally developed inward current (n = 7) (Fig. 3 *A*, *ii*). The store-operated inward current was blocked by 100 μM 2-Aminoethoxydiphenyl borate (2-APB) (n = 7) (Fig. 3 *A*, *i*), a well-known (but not specific) blocker of CRAC channels (14). The current–voltage relationship showed clear inward rectification (Fig. 3*B*). We recorded the store-operated Ca^{2+} current in experiments in which we also monitored changes in the Ca^{2+} concentration inside the stores (41). The TG-induced inward current developed with a delay after the reduction in the store Ca^{2+} concentration (Fig. 3*C*) (n = 9). GSK-7975 (10 μM) evoked a marked reduction (by 83.0 ± 4.1%) in the TG-induced inward current in each of the six experiments carried out (Fig. 3*D*).

The Effect of Blocking CRAC Channels on $[Ca^{2+}]_i$ Changes Evoked by Palmitoleic Acid Ethyl Ester. Elevation of $[Ca^{2+}]_i$ in pancreatic acinar cells can be induced by either alcohol or fatty acids (42, 43), but in combination—as fatty acid ethyl esters—they are much more powerful Ca^{2+} releasers (37) and are recognized as important mediators of alcohol-induced pancreatitis (35–38, 42). We therefore tested the effects of blocking CRAC channels on the changes in $[Ca^{2+}]_i$ induced by palmitoleic acid ethyl ester (POAEE). As previously shown, POAEE evokes substantial and sustained elevations of $[Ca^{2+}]_i$ in normal isolated pancreatic acinar cells, initiated by release of Ca^{2+} from the ER (37, 42) as well as acid stores (28). Fig. 4*A* shows the substantial and sustained rise in $[Ca^{2+}]_i$ elicited by 100 μM POAEE as well as the much diminished cytosolic Ca^{2+} signal evoked by the same concentration of POAEE in the presence of GSK-7975A. Fig. 4*B* shows the mean values of all of the data from this series of experiments. It is clear that GSK-7975A very markedly inhibits the $[Ca^{2+}]_i$ elevation normally evoked by 100 μM POAEE.

Inhibition of CRAC Channels Protects Against POAEE-Induced Trypsin and Protease Activation. It has been shown previously that hyperstimulation-induced trypsinogen activation and vacuolization can be prevented by intracellular Ca^{2+} chelation or simply by removal of extracellular Ca^{2+} (10). Preincubation (1 h) of pancreatic acinar cells with POAEE (100 μM) induced a substantial increase of protease activity (from 6.8 ± 1.3% of cells in control to 31.2 ± 1.6% of cells, Fig. 4*C*). Pretreatment of cells with 10 μM GSK-7975A for 10 min before POAEE application reduced protease activity to levels relatively close to control (Fig. 4*C*). Similar levels of inhibition by GSK-7975A were observed in experiments assessing trypsin activity, using the specific trypsin substrate BZIPAR (10, 28, 29). Preincubation of pancreatic acinar cells with POAEE (100 μM for 1 h) induced substantial increase of trypsin activity (from 3.4 ± 0.5% of cells in control to

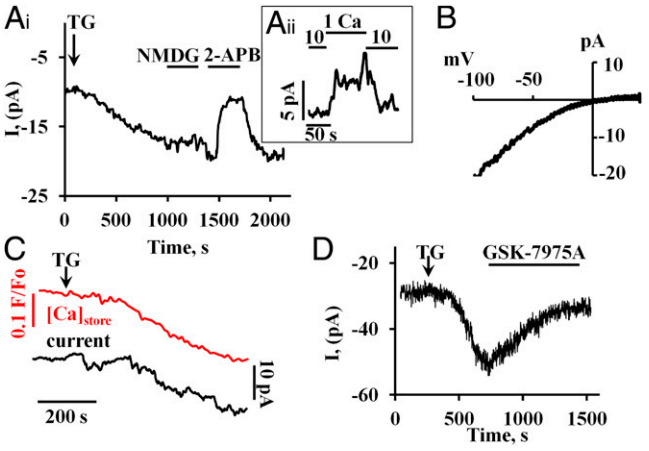

Fig. 3. Store-operated ionic currents, developing after thapsigargin treatment, recorded with the whole cell patch clamp configuration. The individual traces shown were all recorded at a holding potential of −50 mV and with an external $[Ca^{2+}]$ of 10 mM. To prevent activation of the large Ca^{2+}-dependent ion currents in acinar cells (13), patch clamp pipettes were filled with a solution containing a mixture of 10 mM BAPTA and 2 mM Ca^{2+}. (*A*, *i*) Inward current induced by bath application of 2 μM TG to empty the ER Ca^{2+} content. Replacing Na^+ with $NMDG^+$ had little effect on the inward current, but 100 μM of 2-APB practically abolished the current. This effect was rapidly reversible. (*A*, *ii*) Reducing the external Ca^{2+} concentration from 10 to 1 mM (replacement of $CaCl_2$ by $MgCl_2$) reduced reversibly the stable maximal plateau amplitude of the inward current during TG exposure. (*B*) Representative I/V curve obtained using a voltage ramp protocol (0.4 V/s) from −100 mV to 40 mV (difference between ramp registration before and after 2-APB application). (*C*) Simultaneous measurements of changes in the intrastore $[Ca^{2+}]$ and the membrane current following TG application. The *Upper* red trace shows the gradual reduction of the intrastore Ca^{2+} concentration recorded by changes of Fluo-5N fluorescence. The *Lower* black trace shows the development of the inward current. (*D*) GSK-7975 (10 μM) inhibits markedly the inward current evoked by application of 2 μM TG.

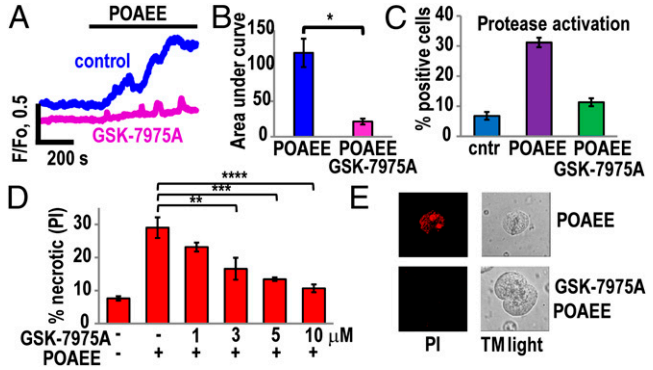

Fig. 4. GSK-7975A dramatically reduces Ca^{2+} overload and necrosis induced by the fatty acid ethyl ester POAEE. (*A*) Representative traces of changes in $[Ca^{2+}]_i$ in response to 100 μM POAEE in the absence ($n = 8$) and presence of GSK-7975A (10 μM) ($n = 12$). (*B*) Quantitative analysis of experiments of the type shown in *A* by comparing the integrated $[Ca^{2+}]_i$ elevation above the baseline (area under the curve) recorded during the first 10 min of POAEE application. Blue bar represents the control (without GSK-7975A), whereas the red bar represents the test results (cells pretreated with 10 μM GSK-7975A for 10 min before application of POAEE). The mean values (±SEM) are significantly different, $P < 0.0002$. (*C*) Pretreatment of cells with 10 μM GSK-7975A for 10 min inhibited by 64% (green bar) protease activation induced by POAEE (100 μM) (purple bar) as measured with generic protease substrate bis-L-aspartic acid amide rhodamine 110 (D2-R110). Blue bar represents the results from control (not exposed to POAEE) cells. The mean values (±SEM) in control and GSK-7975A plus POAEE treatment are not significantly different, $P = 0.05$; $n = 4$, >200 cells in each group. (*D*) POAEE (100 μM)-induced necrosis was dramatically reduced in cells treated with 1 μM, 3 μM, 5 μM, and 10 μM GSK-7975A for 10 min. In the control series of experiments (no POAEE treatment), the level of necrosis was low ($n = 3$ series of experiments with number of tested cells in each group >350). Inhibition of necrosis was significant for 3 μM GSK-7975A (**$P < 0.02$) and highly significant for 5 and 10 μM GSK-7975A (***$P < 0.003$ and ****$P < 0.001$). (*E*) Necrosis was visualized by staining cells with propidium iodide (PI). All experiments were performed in the presence of 1 mM $CaCl_2$.

15 ± 1.1% in the POAEE-treated groups). Pretreatment of cells with GSK-7975A (10 μM for 10 min) before POAEE application substantially reduced trypsinogen activation (6.9 ± 0.7% of cells, $P < 0.0009$, $n = 4$ series of experiments with number of cells >150 in each group).

Blockade of CRAC Channels Protects Against POAEE-Elicited Necrosis. It has previously been shown that POAEE elicits Ca^{2+}-dependent necrosis of pancreatic acinar cells (37, 42). Because GSK-7975A protects against the POAEE-induced $[Ca^{2+}]_i$ rise, as well as against protease activation, we tested whether it would also protect against necrosis. As seen in Fig. 4 *D* and *E*, GSK-7975A did indeed provide a concentration-dependent protection against the development of POAEE-induced necrosis. There was a protective effect already at 3 μM and at 10 μM the percentage of necrotic cells was only slightly higher than the control value (without POAEE) (Fig. 4*D*).

GSK-7975A Protects Against Necrosis Solely by Inhibiting Ca^{2+} Entry. Although GSK-7975A inhibits markedly both Ca^{2+} entry and necrosis, it is theoretically possible that the protection against necrosis could be due to an effect other than on Ca^{2+} entry. To test this point, we exploited the ability of 2-APB—a well-known CRAC blocker (14) (Fig. 3)—to also elicit cytosolic Ca^{2+} signals in pancreatic acinar cells (44). At 100 μM, 2-APB induced substantial rises in $[Ca^{2+}]_i$ and also induced marked necrosis (Fig. S2), but in this situation (where the CRAC channels are already blocked by 2-APB) GSK-7975A was unable to inhibit necrosis (Fig. S2*B*). We also carried out an experiment in which we counteracted the inhibitory effect of GSK-7975A by increasing $[Ca^{2+}]_o$ (Fig. S3) and tested the effect on necrosis. At $[Ca^{2+}]_o = 1$ mM, POAEE evokes a significant increase in $[Ca^{2+}]_i$ (Fig. 4*A*) and a substantial increase in the percentage of necrotic cells (Fig. 4*D*), which is markedly inhibited by GSK-7975A (Fig. 4*D*). Raising $[Ca^{2+}]_o$ to 10 mM in the presence of inhibitor restored the POAEE-induced $[Ca^{2+}]_i$ elevation (Fig. S3*A*) and increased the POAEE-induced rise in the percentage of necrotic cells to the level seen without GSK-7975A at $[Ca^{2+}]_o = 1$ mM (Fig. S3*B*). These experiments (Figs. S2 and S3) indicate that the protective effect of GSK-7975A against necrosis is solely due to inhibition of Ca^{2+} entry.

The Effects of Blocking CRAC Channels on $[Ca^{2+}]_i$ Changes Evoked by Acetylcholine. We tested whether CRAC blockade would also prevent the normal cytosolic Ca^{2+} spiking, elicited by ACh or CCK. As seen in Fig. S1, a low ACh concentration (50 or 100 nM) evoked repetitive $[Ca^{2+}]_i$ spikes and this spike generation was hardly affected, at least for the first ~15 min, by application of 10 μM GSK-7975A (Fig. S1 *A*, *B*, and *D–F*). Likewise, the Ca^{2+} spiking evoked by 5 pM CCK was only affected to a minor degree by CRAC blockade (Fig. S1*C*). A high ACh concentration (1 μM) evoked a large initial rise in $[Ca^{2+}]_i$, followed by a slow decline toward a sustained quasiplateau at a slightly elevated $[Ca^{2+}]_i$ (Fig. S1*G*). In this case, blockade of CRAC channels markedly reduced or even eliminated the sustained plateau phase and during continued exposure to the high (1 μM) ACh concentration, $[Ca^{2+}]_i$ returned to the prestimulation resting level (Fig. S1 *G* and *H*). Because CRAC blockade markedly reduced the sustained phase of Ca^{2+} entry, during prolonged supramaximal stimulation, we tested to what an extent this would have an effect on the intracellular Ca^{2+} stores. A 1-h preincubation of the acinar cells with 10 μM GSK-7975A had very little effect on the rise in $[Ca^{2+}]_i$ in response to a supramaximal ACh stimulus (10 μM) in the presence of thapsigargin (Fig. S4 *A* and *B*). To assess the potential effect of CRAC blockade on Ca^{2+} store reloading, we compared the magnitudes of the $[Ca^{2+}]_i$ response to a second stimulation with a high concentration of ACh (10 μM), after a long period of rest, under three different conditions, namely control, absence of external Ca^{2+}, and in the presence of both Ca^{2+} and the CRAC blocker (Fig. S4*C*). In the pancreatic acinar cells, full reloading of virtually empty ER stores is a very slow process (45, 46) and, as seen by comparing Fig. S4 *A* with *C*, a second supramaximal ACh stimulus, even after a substantial interval during control conditions, only evoked a much diminished response. Even in the absence of external Ca^{2+}, there is a tiny rise in $[Ca^{2+}]_i$ following the second ACh application, most likely due to a small reuptake of Ca^{2+} into the ER before all of the released Ca^{2+} had been extruded across the cell membrane. In the presence of 10 μM GSK-7975A with external Ca^{2+}, there is a significant reduction in the amplitude of the second response to ACh compared with the control situation, but this reduced response is nevertheless significantly larger than the one seen under Ca^{2+}-free conditions (Fig. S4 *C* and *D*). This is consistent with our finding that store-operated Ca^{2+} entry is not abolished by 10 μM GSK-7975A, although very markedly diminished (Fig. 1).

Effect of CRAC Blockade in Other Cell Types. We have also tested whether CRAC channel blockade in other cell types would have effects on store-operated Ca^{2+} entry. In freshly isolated mouse hepatocytes, using the classical thapsigargin protocol for examining store-operated Ca^{2+} entry, we only found a relatively modest inhibitory effect of GSK-7975A ($P < 0.042$, Fig. S5 *A–C*) compared with what was observed in pancreatic acinar cells (Fig. 1). We also tested the pancreatic acinar cell line AR42J, which has a neuronal phenotype (47). As seen in Fig. S6, GSK-7975A had little effect on store-operated Ca^{2+} entry in these cells, in

marked contrast to what we show for the real pancreatic acinar cells (Fig. 1).

Discussion

The results presented here indicate that blockade of store-operated Ca^{2+}- selective entry channels effectively prevents an important mediator of alcohol-related pancreatitis, POAEE, from evoking sustained elevation of $[Ca^{2+}]_i$, protease activation, and pancreatic acinar cell necrosis. This indicates that pharmacological CRAC blockade has therapeutic potential as a unique rational treatment against severe acute pancreatitis, a life-threatening human disease that at the moment is untreatable. Our result, showing that a CRAC channel blocker markedly reduces the sustained $[Ca^{2+}]_i$ elevation that normally follows depletion of the Ca^{2+} store in the ER (Figs. 1 and 2), provides fresh evidence for the importance of store-operated Ca^{2+} channels in this phase of Ca^{2+} signaling, which has already been identified as crucial for the initiation of acinar cell injury (10). Although it has been known for 40 years that cytosolic Ca^{2+} signals in pancreatic acinar cells are initiated by release from internal stores (5), it has also been recognized for a long time that following the initial release from the intracellular stores there is an important Ca^{2+} entry phase that is essential for refilling the stores and indeed for stimulant-evoked enzyme secretion (48–50). Previous investigations have demonstrated that the most important mediators of acinar cell damage, namely alcohol, fatty acids, fatty acid ethyl esters, and bile acids all primarily release Ca^{2+} from various internal stores (28, 29, 37, 42, 43, 51), but that this initial phase is followed by store-operated Ca^{2+} entry, which plays a crucial role in the destruction of the cells (2, 4, 5, 10). Our electrophysiological data (Fig. 3) show that in the pancreatic acinar cells this store-operated inward current is relatively insensitive to removal of external Na^+, but sensitive to changes in the external Ca^{2+} concentration. It is therefore not a Transient receptor potential (TRP)-type non-selective cation current, but a Ca^{2+}-selective CRAC-type current, consistent with the very marked current inhibition evoked by GSK-7975A (Fig. 3*D*), a relatively selective CRAC channel blocker with almost no inhibitory effect on TRP-channel currents (with the exception of those mediated by TRPV6) (33). This agent has recently been shown to block Ca^{2+} currents through CRAC channels in human lung mast cells, T cells, and platelets (31–34). The CRAC channel is emerging as a potentially important therapeutic target in a number of human diseases (52, 53) and could also be important for pancreatitis (54). We have therefore used GSK-7975A (33, 34), to inhibit store-operated Ca^{2+} entry in pancreatic acinar cells. GSK-7975A markedly inhibited Ca^{2+} and Ba^{2+} entry elicited by releasing Ca^{2+} from the ER (Figs. 1–3) as well as the late phase Ca^{2+} entry in response to stimulation with a high ACh concentration (Fig. S1). These results indicate therapeutic potential for curbing excessive Ca^{2+} entry in the early phase of pancreatitis.

We have specifically investigated one pathophysiologically relevant situation, namely the acinar cell injury initiated by POAEE, one of the known mediators of alcohol-related pancreatitis (35–37, 42). These data (Fig. 4) show not only that GSK-7975A markedly reduces the POAEE-evoked $[Ca^{2+}]_i$ elevation, but—even more importantly—also markedly inhibits the extent of the protease activation and necrosis induced by this fatty acid ethyl ester. The GSK-7975A CRAC channel blocker did not inhibit completely the unidirectional Ba^{2+} inflow evoked by arresting the ER Ca^{2+} pump (Fig. S1 *I–K*) and this inability to abolish store-operated Ca^{2+} entry (also reflected in Fig. 1 *A–E*) may be the reason that GSK-7975A has so little effect on the repetitive Ca^{2+} spiking elicited by a low ACh concentration or a physiological CCK concentration (Fig. S1). Although Ca^{2+} spikes are due to release of Ca^{2+} from intracellular stores, the cell should eventually run out of Ca^{2+} in the absence of Ca^{2+} entry because every time $[Ca^{2+}]_i$ goes up there will be activation of the plasma membrane Ca^{2+} pump and therefore loss of Ca^{2+} to the external environment (5). However, because the loss of Ca^{2+} during short spikes of the type shown in Fig. S1 is relatively small (5), even a severely reduced level of Ca^{2+} entry may be sufficient to prevent depletion of the intracellular stores. This point is also relevant with regard to another potential concern, namely the apparent irreversibility of the effect of GSK-7975A, at least for ~10 min following start of washout (Fig. 1). Because of the incompleteness of the CRAC blockade in the acinar cells (Fig. 1 and Fig. S1 *I* and *J*), this may turn out not to be a serious problem for potential therapeutic use. Resting pancreatic acinar cells appeared to have unchanged levels of Ca^{2+} in the ER store after 1 h of incubation with GSK-7975A (Fig. S4 *A* and *B*). Although reloading of the intracellular stores after strong stimulation with ACh was reduced by the CRAC blocker, there was still a significant level of store refilling, compared with conditions when there was no Ca^{2+} entry due to absence of external Ca^{2+} (Fig. S4 *C* and *D*).

CRAC channels are widely distributed in many different cell types and effective CRAC blockade might therefore also have effects on other organ systems. However, the effects are likely to be strongest in those cell types (like the pancreatic acinar cells) in which other types of Ca^{2+} entry pathways are not quantitatively important, unlike the many types of electrically excitable cells. Mast cells and T cells, for example, will be affected by CRAC blockade (31, 34, 52, 53), but this will be advantageous in the treatment of acute pancreatitis, where the initial damage of the acinar cells leading to necrosis is followed by a strong inflammatory response, which is known to cause very significant further damage (1). CRAC blockade would have the most pronounced effect on cells with the most strongly activated Ca^{2+} entry channels, which, in cases of life-threatening severe pancreatitis, would be the pancreatic acinar cells. With regard to organ selectivity, it is interesting that CRAC channel blockade was much less effective in inhibiting store-operated Ca^{2+} entry in hepatocytes and in the neuronal-like AR42J cell line (compare Fig. 1 with Figs. S5 and S6), probably reflecting a more complex situation in these cells (47, 55).

Although CRAC channel inhibition will very effectively reduce cytosolic Ca^{2+} overload, it will not prevent the depletion of intracellular Ca^{2+} stores evoked by POAEE. It could therefore be potentially advantageous to combine CRAC blockade with inhibition of Ca^{2+} release from the internal stores using a synthetic membrane permeable calmodulin activator (29). Overall, our data provide a unique proof of principle that pharmacological CRAC channel inhibition could become an effective tool for reducing the cellular Ca^{2+} overload that is such an important feature of the changes occurring in the initial phase of acute pancreatitis. Specifically, our finding that GSK-7975A markedly inhibits not only the excessive $[Ca^{2+}]_i$ elevation evoked by POAEE, an important mediator of alcohol-related pancreatitis, but also markedly reduces the extent of the associated protease activation and necrosis—although it has little or no effect on the repetitive short-lasting Ca^{2+} spikes evoked by the physiological neurotransmitter ACh—is promising.

Materials and Methods

Full methods are provided in *SI Materials and Methods*. Details about isolation of pancreatic acinar cells were described previously (56). GSK-7975A was provided by GlaxoSmithKline (31). Fluorescent measurements were performed with Fluo-4 or Fura-2 (39), whereas necrosis was assessed with propidium iodide (39) and trypsinogen activation with trypsin fluorescent substrate (10, 29). Hepatocytes were isolated according to a protocol described previously (57). AR42J cell protocols are described in refs. 39, 47.

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