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Bile acids activate ryanodine receptors in pancreatic acinar cells via a direct allosteric mechanism

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ABSTRACT

The earliest critical event of pancreatitis is a long lasting high amplitude rise of intracellular Ca^{2+} concentration of the acinar cell, which can be triggered by high concentration of bile acids. Although, Ca^{2+}-release through ryanodine receptors (RyR) is involved in the process, the significance and the exact mechanism of bile acid's action on RyR has not been fully elucidated yet. Therefore, we aimed to test with various techniques and aspects whether bile acids exert a direct effect on RyR and SERCA pump.

Our data show that taurocholic acid (TCA)-induced Ca^{2+} release in pancreatic acinar cells was significantly reduced by the RyR antagonist dantrolene. Further, we show that TCA enhanced RyR's ^{3}H-ryanodine binding and triggered robust Ca^{2+}-release from RyR-enriched vesicles in the pathologically relevant concentration range. RyR single channel current analysis demonstrated that 200 μM TCA induced a 5-fold increase in the channel's open probability and caused a significant lengthening of the mean open time. TCA also suppressed Ca^{2+}-uptake rate and ATP-ase activity of SERCA-enriched vesicles, but interestingly, failed to decrease Ca^{2+} elimination rate in intact cells.

Overall, our results strongly suggest that TCA opens RyR by an allosteric mechanism, which contribute significantly to bile acid-induced pathologic Ca^{2+}-leak from the endoplasmic reticulum in pancreatic acinar cells.

1. Introduction

Pancreatitis is a painful and potentially fatal inflammatory disease with a high incidence rate of around 13–45/100,000 people/year [1]. Specific medical therapies are not available. The most common cause of pancreatitis are gallstones, which cause reflux of the bile into the pancreatic duct system by occluding the common bile duct [2,3]. Bile acid concentration therefore may reach millimolar levels in the pancreas, which triggers uncontrolled Ca^{2+} release and prolonged elevation of the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in the acinar cell [4]. Impaired Ca^{2+} signaling is considered to be the critical molecular step of acute pancreatitis because it causes premature intraacinar protease activation and acinar cell damage that provokes the inflammation [5,6].

During physiological pancreatic acinar cell stimulation, exocytosis of zymogens is controlled by Ca^{2+} oscillations highly localized to the apical region of the cell [7]. The spatial localization of Ca^{2+} release is explained by high inositol trisphosphate receptor (IP_3R) density in the apical endoplasmic reticulum (ER) (also called Ca^{2+} release trigger zone) [8,9]. In contrast, overstimulation of the pancreatic acinar cell by secretagouges and high concentrations of bile acids induce whole-cell, high amplitude, long lasting, (peak-plateau type) Ca^{2+} signals. The mechanism underlying global Ca^{2+} signals is Ca^{2+} wave propagation, which involves the Ca^{2+} dependent activation of ryanodine receptor (RyR) Ca^{2+} release channels that show higher density in the basolateral-supranuclear region of the acinar cell. Since the ryanodine sensitive compartment is the major source of Ca^{2+} under pathological Ca^{2+} release, the role of RyR in the pathomechanism seems to be essential [4,10,11]. For instance, recent studies showed that Ca^{2+} signals in the basolateral areas induced by supramaximal concentrations of carbachol and that of

Abbreviations: RyR, ryanodine receptor; $[Ca^{2+}]i$, intracellular Ca^{2+} concentration; IP_3R, inositol trisphosphate receptor; ER, endoplasmic reticulum; TC, terminal cisternae; LSR, longitudinal sarcoplasmic reticulum; CICR, Ca^{2+} induced Ca^{2+} release; SERCA, sarco-endoplasmic reticulum Ca^{2+} pump; TLCS, taurolitocholic acid-3-sulphate; TCA, taurocholate; TDC, taurodeoxycholate; CPA, cyclopiazonic acid; RR, ruthenium red; Po, open probability; FWHM, full widths at half maximum; cch, carbachol.

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the subcellular distribution of RyR overlapped with the regions where caerulein-induced early zymogen activation occurred [10]. More importantly, the RyR inhibitor dantrolene diminished the carbachol-induced [Ca^{2+}]i elevation selectively in the basolateral region and reduced the severity of experimental pancreatitis in vivo [11]. These data provide strong evidence that RyR plays a significant role in the pathomechanism of pancreatitis and therefore it can be considered as an important therapeutic target.

The bile acid taurolitocholic acid-3-sulphate (TLCS) was also demonstrated to produce global Ca^{2+} release, which originated in the apical region and spread toward the basal end of the acinar cell [5]. In permeabilized cell assay, bile acid retained its effect when IP_3R or RyR were blocked, but failed when both IP_3R and RyRs were inhibited [12]. Moreover, both dantrolene and ryanodine converted bile acid-induced sustained intracellular [Ca^{2+}] elevations to physiological Ca^{2+} oscillations in intact cells [4]. Although, these data suggest that bile acids trigger excessive Ca^{2+} release by directly opening RyRs too, this possibility remains to be tested, because alternatively, RyR might be activated indirectly via Ca^{2+} induced Ca^{2+} release (CICR) mechanism by the Ca^{2+}, released from acidic vesicles through NAADP receptor Ca^{2+} channels [6,12]. Nevertheless, this question has not been addressed yet. Furthermore, bile acids were reported to inhibit sarco-endoplasmic reticulum Ca^{2+} pump's (SERCA) activity which might result in an increased RyR and IP_3R mediated Ca^{2+} leak and CICR rate [13].

As pictured above, despite the fact that bile acid-induced Ca^{2+} signals in pancreatic acinar cells are intensively studied, the complex interplay between the Ca^{2+} transporters makes the role of RyR and SERCA difficult to evaluate in this process. Therefore, we aimed to test the action of different bile acids explicitly on RyR and SERCA function using the most direct methods available and in the simplest settings possible.

The major components of the bile, cholate and chenodeoxycholate are synthesized by the liver and converted to secondary bile acids including taurocholate, deoxycholate and lithocholates by the intestinal flora. Due to secondary bile acid recycling through the enterohepatic circulation, bile acids occur in taurin-conjugated form. Because taurine conjugated bile acids are the most effective Ca^{2+} mobilizers among bile acids, the major taurin-conjugated bile acid taurocholic acid's (TCA) effect was tested throughout the study. In addition, other secondary bile acids taurodeoxycholate (TDC) and taurolitocholic acid sulphate (TLCS) have also been tested in Ca^{2+} imaging and Ca^{2+} release assays. In order to selectively investigate RyR's or SERCA function, ^3H ryanodine binding assay, Ca^{2+} release measurements, single channel recording, and an enzyme-linked NADH assay were performed using purified microsomes. These experiments all demonstrate that bile acids are potent RyR openers and SERCA inhibitors.

2. Material and methods

2.1. Chemicals

If not specified, chemicals were purchased from Sigma.

2.2. Pancreatic acinar cell isolation

Pancreatic acinar cells were freshly isolated from mouse pancreas as described previously [14]. Briefly, 4 months old NMRI mice of both genders were sacrificed by cervical dislocation and the pancreas was removed. The tissue was injected with F12/DMEM medium containing 100 U/ml collagenase P (Roche), 0.1 mg/ml trypsin inhibitor and 2.5 mg/ml BSA, then incubated in this solution in a 37 °C shaking water bath for 30 min, and continuously gassed with carbogen. The tissue was dissociated by pipetting with a serological pipette 4–6 times, then filtered through mesh #60 (150 μm). Cells were layered on the top of 400 mg/ml BSA and washed through the medium by gentle centrifugation. The pellet was resuspended in saline solution, collected by centrifugation. Acinar cell clumps were gently resuspended in F12/DMEM medium and kept gassed at room temperature until use.

2.3. Intracellular Ca^{2+} imaging

Acinar cell clumps were loaded with 2 μM Fluo-4 AM Ca^{2+} sensitive dye for 30 min at room temperature. Acinar cells were plated on glass coverslips and mounted on a perfusion chamber. After perfusion with Ca^{2+}-free Tyrode's solution containing (in mM): 140 NaCl, 5 KCl, 2 MgCl and 10 HEPES, pH 7.2, taurocholate (1, 2 or 5 mM), tauro deoxycholate (1 mM) or taurolythocholic acid sulphate (0.25 mM) was applied by superfusing the cells for 3 or 12 min at room temperature. At the end of each measurement, 2 μM carbachol was applied to obtain an internal control signal. In some experiments, the loading and imaging media was supplemented with 12 μM dantrolene or 1.8 mM $CaCl_2$.

Fluorescence was monitored using a Zeiss LSM 5 *LIVE* confocal microscope equipped with a 40× objective. Fluo-4 was excited at 488 nm and the emitted light was collected through a 520 nm filter. Fluorescence emission data of single cells was analyzed and F/F0 ratio was calculated after background subtraction using Zeiss ZEN 2009 software. Data were averaged from multiple cells in each experiment then, these values were further averaged to get the mean ± SEM.

2.4. Microsome isolation

Microsomes from rat pancreas were isolated as described earlier [15]. A pancreas was homogenized using a glass homogenizer in 6 ml ice cold DMEM supplemented with Roche protease inhibitor cocktail. The homogenate was transferred into 1.5 ml microcentrifuge tubes and centrifuged at 11,000 × *g* for 15 min The fluffy layer on the top of the pellet was collected and centrifuged at 240,000 × *g* in a sw 55 ti rotor for 30 min at 4 °C. The pellet was resuspended in a small volume of 300 mM sucrose, 10 mM K-PIPES (pH = 7.0), rapidly frozen in liquid nitrogen and stored at −70 °C.

Sarcoplasmic reticulum terminal cisternae (TC)- and longitudinal SR (LSR) vesicles were isolated from 50 g of rabbit skeletal muscle by differential centrifugation as described previously [16]. All the steps were carried out in cold room or on ice and in the presence of protease inhibitors (in μM: 200 pefabloc SC, 0.1 aprotinin, 1 leupeptin, 0.2 pepstatin A, 500 benzamidine). After homogenization in 450 ml buffer (in mM: 100 NaCl, 20 EGTA, 20 Na-HEPES, pH = 7.5), cell debris was pelleted at 3500 × *g*, for 35 min using a tabletop centrifuge equipped with a swing-out rotor. Crude microsomes were collected from the supernatant by centrifugation in a Ti45 rotor at 40,000 × *g*, for 30 min To dissolve the actomyosin content the pellet was resuspended in (in mM) 600 KCl, 10K-Pipes, 250 sucrose, 1 EGTA, 0.9 $CaCl_2$ (pH, 7.0). After incubation for 1 h at 4 °C, the microsome fraction was centrifuged at 109,000 × *g*, for 30 min, the pellet was resuspended and loaded onto a 20–45% linear sucrose gradient (in mM: 105 NaCl, 10 Pipes, 0.1 EGTA, 0.09 $CaCl_2$, pH = 7.0). After spinning overnight at 90,000 × *g* in a SW27 rotor, two visible rings, corresponding to LSR and TC vesicles were collected from the 30–32% and the 36–38% regions of the sucrose gradient, respectively. The microsomes were washed with a buffer, containing (in mM) 475 sucrose, 1 NaCl, 10 Pipes (pH = 7.0) and pelleted by centrifugation at 124,000 × *g* for 60 min in a Ti45 rotor. The pellet was resuspended at a final protein concentration of >20 mg/ml in a solution containing 300 mM sucrose and 10 mM K-PIPES (pH = 7.0). Vesicles were aliquoted and rapidly frozen in liquid nitrogen and stored at −70 °C until further use or were immediately