

TRPM2 channels mediate acetaminophen-induced liver damage

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Acetaminophen (paracetamol) is the most frequently used analgesic and antipyretic drug available over the counter. At the same time, acetaminophen overdose is the most common cause of acute liver failure and the leading cause of chronic liver damage requiring liver transplantation in developed countries. Acetaminophen overdose causes a multitude of interrelated biochemical reactions in hepatocytes including the formation of reactive oxygen species, deregulation of Ca²⁺ homeostasis, covalent modification and oxidation of proteins, lipid peroxidation, and DNA fragmentation. Although an increase in intracellular Ca²⁺ concentration in hepatocytes is a known consequence of acetaminophen overdose, its importance in acetaminophen-induced liver toxicity is not well understood, primarily due to lack of knowledge about the source of the Ca²⁺ rise. Here we report that the channel responsible for Ca²⁺ entry in hepatocytes in acetaminophen overdose is the Transient Receptor Potential Melanostatine 2 (TRPM2) cation channel. We show by whole-cell patch clamping that treatment of hepatocytes with acetaminophen results in activation of a cation current similar to that activated by H₂O₂ or the intracellular application of ADP ribose. siRNA-mediated knockdown of TRPM2 in hepatocytes inhibits activation of the current by either acetaminophen or H₂O₂. In TRPM2 knockout mice, acetaminophen-induced liver damage, assessed by the blood concentration of liver enzymes and liver histology, is significantly diminished compared with wild-type mice. The presented data strongly suggest that TRPM2 channels are essential in the mechanism of acetaminophen-induced hepatocellular death.

Acetaminophen (*N*-acetyl-*p*-aminophenol), when used at prescribed doses, is a safe analgesic and antipyretic drug (1). Its overdose, however, can be life threatening, causing severe liver and kidney damage (2–5). In Western countries, acetaminophen-induced hepatotoxicity is a leading cause of acute liver failure requiring liver transplantation (6). Due to a widespread availability of acetaminophen and potentially lethal consequences of its overdose, the mechanisms of acetaminophen hepatotoxicity have been in focus of a large number of investigations (7). Despite significant progress, the exact pathways of acetaminophen hepatotoxicity that lead to hepatocellular death are still not completely understood. It is clear, however, that acetaminophen toxicity arises from its metabolic activation (8, 9).

In the liver, therapeutic doses of acetaminophen are metabolized by glucuronidation and sulfation into nontoxic compounds (1). Only a small amount of acetaminophen is converted by hepatic cytochrome P450 (CYP)-dependent mixed function oxidases to the reactive intermediate metabolite *N*-acetyl-*p*-arabenzoquinoneimine (NAPQI). The NAPQI generated by a therapeutic dose of acetaminophen is rapidly metabolized to nontoxic products by conjugation with glutathione (GSH) (1, 10). With large doses of acetaminophen, however, hepatic GSH becomes depleted resulting in the accumulation of toxic amounts of NAPQI. Covalent binding of NAPQI to cellular proteins has previously been considered the main cause of liver cell death under these circumstances. Indeed it has been shown that covalent binding precedes hepatocellular death, and treatments that prevent covalent binding also prevent liver necrosis (11). More recently,

however, it has been suggested that, by itself, the covalent binding of NAPQI is not sufficient to induce apoptosis or necrosis. The toxic signal produced by covalent binding undergoes further amplification through the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), deregulation of Ca²⁺ homeostasis, and increased intracellular Ca²⁺, causing oxidant stress in mitochondria and inducing the mitochondrial membrane permeability transition (12, 13). Although widely acknowledged, the role of Ca²⁺ in acetaminophen toxicity is poorly understood and has not been thoroughly investigated. Nonselective Ca²⁺ channels blockers chlorpromazine and verapamil have been shown to attenuate liver injury in mice (14, 15), however, the mechanism of their protective properties in acetaminophen overdose is not clear, and it is not known whether it involves any Ca²⁺-permeable channels on the plasma membrane of hepatocytes.

The only Ca²⁺-selective channel that has been clearly identified in hepatocytes so far is the Ca²⁺ release-activated Ca²⁺ channel activated by the depletion of intracellular Ca²⁺ stores downstream of phospholipase C_β and phospholipase C_γ signaling (16, 17). In addition, a number of Ca²⁺-permeable nonselective cation channels with no clearly defined functions and mostly from the TRP family of channels have been shown to be present in hepatocytes and liver cells (18, 19). One of these channels, Transient Receptor Potential Melanostatine 2 (TRPM2), whose presence in the liver has only been demonstrated on a mRNA level (19), is activated in response to oxidative stress and, potentially, can be involved in acetaminophen-induced Ca²⁺ rise in hepatocytes.

Significance

Acetaminophen overdose is the most common cause of acute liver failure and the leading cause of chronic liver damage requiring liver transplantation in developed countries. There are limited options for early treatment. Acetaminophen liver toxicity leads to the formation of reactive oxygen and nitrogen species which cause an increase in intracellular Ca²⁺ and hepatocellular death. We show that acetaminophen-induced liver toxicity depends on Transient Receptor Potential Melanostatine 2 (TRPM2) cation channels in hepatocytes, which are activated in response to oxidative stress and are responsible for Ca²⁺ overload. Lack of TRPM2 channels in hepatocytes or their pharmacological inhibition protects liver from acetaminophen toxicity. This provides evidence that TRPM2 may present a potential therapeutic target for treatment of oxidative-stress related liver diseases.

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Results

Acetaminophen and H₂O₂ Activate Nonselective Cation Current in Hepatocytes. To investigate the role of Ca²⁺-permeable channels in acetaminophen toxicity in the liver, first we examined the effects of acetaminophen on the free cytoplasmic Ca²⁺ concentration ([Ca²⁺]_{cyt}) in rat hepatocytes. Hepatocytes were incubated with 10–15 mM acetaminophen for 60 min in a bath solution containing 1.3 mM Ca²⁺. Treated hepatocytes were then loaded with Fura-2 acetoxyethyl (AM) in a nominally Ca²⁺-free bath solution, and after washing, were transferred to the microscope stage. After introduction of 1.3 mM Ca²⁺ into the bath, [Ca²⁺]_{cyt} increased to micromolar levels, indicating that acetaminophen activates Ca²⁺ entry across the plasma membrane through Ca²⁺-permeable channels. Control cells showed no change in [Ca²⁺]_{cyt} (Fig. 1A). Ca²⁺ entry activated in hepatocytes by preincubation with acetaminophen was inhibited by 50 μM clotrimazole and 10 μM *N*-(*p*-amylcinnamoyl)anthranilic acid (ACA) (Fig. 1A). Clotrimazole and ACA were previously shown to block heterologously expressed TRPM2 channels (20, 21).

To investigate the nature of the Ca²⁺-permeable channels responsible for acetaminophen-induced Ca²⁺ entry, we used whole-cell patch clamping. After isolation, hepatocytes were cultured for 24–48 h on glass coverslips and treated with or without acetaminophen for 60 min. The average density of baseline current at –100 mV in rat hepatocytes normally varies between 2 and 4 pA/pF and the current-voltage (I-V) plot shows some outward rectification due to Cl[–] conductance (22) (Fig. 1B). Hepatocytes preincubated with 10 mM acetaminophen for 60 min, however, showed significantly larger currents upon establishing whole-cell configuration, with the average current density of 8–10 pA/pF at –100 mV (Fig. 1B). The virtually linear I-V plot, near-zero reversal potential, and sensitivity of the inward current to the replacement of extracellular Na⁺ with the large cation N-methyl-D-glucamine (NMDG⁺) (Fig. 1B), suggested that treatment with acetaminophen resulted in the activation of nonselective cation channels. Similarly to acetaminophen-induced Ca²⁺ entry, this nonselective cation current was blocked by clotrimazole and ACA (Fig. 1B; only clotrimazole is shown, as ACA produced the same level of inhibition). When we increased the treatment time with acetaminophen, hepatocytes became increasingly damaged with extensive membrane blebbing so that they were not amenable to Fura-2 Ca²⁺ measurements and patch clamping. Direct application of acetaminophen to the bath in patch clamping or Ca²⁺ imaging experiments had no acute effect on membrane currents or [Ca²⁺]_{cyt}.

Oxidant stress caused by ROS and RNS formed in the liver in acetaminophen overdose is considered a major mediator of hepatocellular death (23). To determine whether hepatocytes express ROS-sensitive Ca²⁺-permeable channels which can potentially be activated by ROS and RNS generated in hepatocytes treated with acetaminophen, we used Ca²⁺ imaging and patch clamping of isolated rat hepatocytes treated with H₂O₂. Fura-2 experiments indicated a robust rise of [Ca²⁺]_{cyt} in response to incubation with 0.5 mM H₂O₂ for 25–30 min, whereas patch clamping showed activation of a nonselective cation current similar to that activated by acetaminophen (Fig. 1C and D). Both Ca²⁺ entry and the nonselective cation current activated in hepatocytes by incubation with H₂O₂ were inhibited by 50 μM clotrimazole and 10 μM ACA (Fig. 1C and D). As observed for 10–15 mM acetaminophen, 0.5 mM H₂O₂ produced some membrane blebbing in hepatocytes. Cells with damaged membranes were not amenable to patch clamping. Therefore, the amplitude of the cation current activated by incubation with 1 mM H₂O₂ was likely to be underestimated, as only visibly undamaged cells were used. Adding 1 mM H₂O₂ directly to the bath after achieving the whole-cell configuration with control pipette solution did not result in activation of a noticeable current within 10 min of recording or before the seal between the cell and the pipette was lost. However, application of 10 mM H₂O₂ to the bath in such experiments resulted in a relatively rapid development of a large cation current with properties similar to those of the

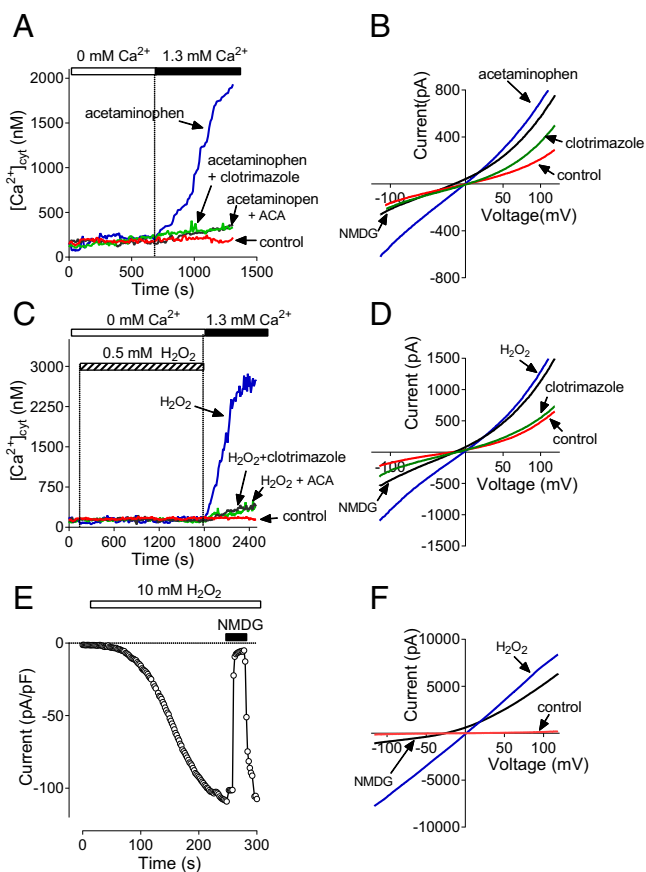


Fig. 1. Acetaminophen and H₂O₂ activate Ca²⁺ entry and a nonselective cation current in rat hepatocytes. (A) Ca²⁺ entry in hepatocytes treated with 10 mM acetaminophen for 60 min under the conditions indicated in A (average data from three separate cell preparations). Both clotrimazole (50 μM) and ACA (10 μM) were applied to the bath 5 min before the addition of Ca²⁺. (B) I-V plots of membrane currents measured in control hepatocytes, and hepatocytes treated with 10 mM acetaminophen for 60 min in control bath solution, after replacement of 140 mM NaCl in the bath solution with 140 mM NMDG Cl and after the addition of 50 μM clotrimazole to the bath (*n* = 22 for each trace). Error bars are omitted for clarity here and all other I-V plots. (C) Ca²⁺ entry in hepatocytes treated with 0.5 mM H₂O₂ (*n* = 3). (D) I-V plots of membrane currents measured in control hepatocytes, and hepatocytes treated with 0.5 mM H₂O₂ in control bath solution, after replacement of 140 mM NaCl in the bath solution with 140 mM NMDG Cl and after the addition of 50 μM clotrimazole to the bath (*n* = 7). (E) Activation of membrane conductance in hepatocytes by 10 mM H₂O₂ applied to the bath. Each data point represents amplitude of the current at –100 mV. (F) I-V plots of membrane currents measured before application of H₂O₂ (control), and after full development of the H₂O₂-activated current in control bath solution (H₂O₂) and after replacement of 140 mM NaCl with 140 mM NMDG Cl (*n* = 5).

currents activated by preincubation of hepatocytes with 0.5 mM H₂O₂ or 10 mM acetaminophen (Fig. 1E and F).

Acetaminophen-Induced Nonselective Cation Current in Hepatocytes Is Mediated by TRPM2 Channels. Although the data presented above demonstrates activation of a nonselective cation current across the plasma membrane and a rise in [Ca²⁺]_{cyt} in hepatocytes in response to acetaminophen treatment, the identity of the channels responsible for this Ca²⁺ entry is not known. One possible candidate that is activated in response to oxidative stress is the TRPM2 (24–26). Evidence that acetaminophen-induced nonselective cation current is mediated by TRPM2 is as follows: (i) the current shows a linear I-V relationship; (ii) it is blocked by clotrimazole and ACA; and (iii) it is activated by H₂O₂ (19, 27).

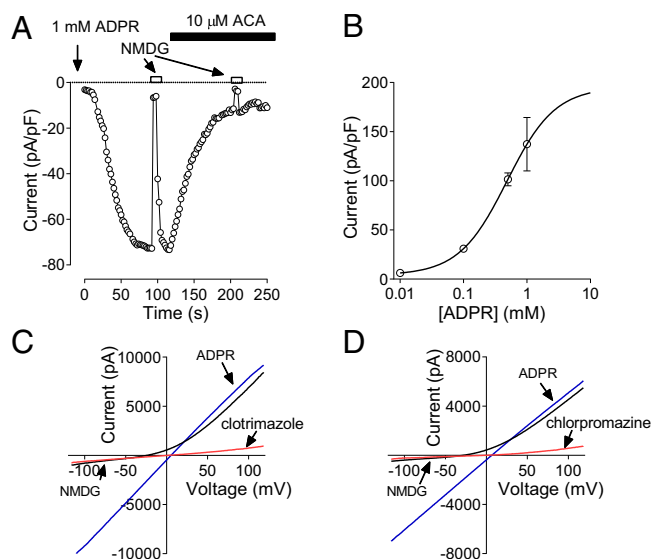


Fig. 2. TRPM2 current in rat hepatocytes. (A) Activation of TRPM2 current in a rat hepatocyte in response to intracellular perfusion with 1 mM ADPR. The current was recorded in response to 100-ms voltage ramps between -120 and 120 mV and applied every 2 s. Current amplitude at -100 mV is plotted against time. Application of $10 \mu\text{M}$ ACA to the bath resulted in a 90% block of the current. (B) The dose–response curve for ADPR. TRPM2 current amplitude at -100 mV is plotted against ADPR concentration in the pipette. The data points are fitted with a Hill equation with slope 1 and EC_{50} of $480 \mu\text{M}$. (C and D) I–V plots of fully developed TRPM2 currents activated by ADPR in control bath solution, after replacement of 140 mM NaCl in the bath solution with 140 mM NMDG Cl, and after application of $50 \mu\text{M}$ clotrimazole or $100 \mu\text{M}$ chlorpromazine to the control bath solution, respectively ($n = 3$).

The hallmark of TRPM2-mediated currents is activation by ADP-ribose (ADPR) and H_2O_2 (28–30). Several splice variants of TRPM2 channel have been reported (31). One of them, with a deletion in the C terminus (TRPM2 ΔC), lacks the ADPR-binding motif and has been shown to be activated by H_2O_2 , but not ADPR (31). To determine the main splice variants of TRPM2 expressed in hepatocytes we used RT-PCR and showed that rat hepatocytes express only long isoform of TRPM2 (LTRPM2) with the ADPR binding motif intact (Table S1 and Fig. S1). To establish if these channels are functional we used whole-cell patch clamping of hepatocytes cultured for 24 h after isolation and a pipette solution supplemented with ADPR. Addition of 1 mM ADPR resulted in activation of a large nonselective cation current (137 ± 27 pA/pF) 1–5 min after establishing the whole-cell configuration (Fig. 2 A and B). Lower concentrations of ADPR produced smaller currents (EC_{50} $480 \mu\text{M}$) with a longer time course of activation. The ADPR-activated current was inhibited by ACA and clotrimazole at the same concentrations as those that inhibited the currents activated by acetaminophen and H_2O_2 (Fig. 2 A and C).

In addition to ACA and clotrimazole we investigated the effects of another broad ion channel and Ca^{2+} -calmodulin inhibitor, chlorpromazine, on an acetaminophen- and ADPR-activated current in hepatocytes. It has previously been shown that chlorpromazine protects against acetaminophen toxicity in mouse liver (14, 32). ADPR- and acetaminophen-activated current in rat hepatocytes was fully blocked by $100 \mu\text{M}$ chlorpromazine (Fig. 2D and Fig. S24), with an EC_{50} of $\sim 5 \mu\text{M}$. The time course of inhibition of the current activated by ADPR was very similar to that of the current induced by acetaminophen (Fig. S2 A and B). To confirm that chlorpromazine blocks TRPM2 channels we used HEK293T cells transfected with TRPM2 cDNA (Fig. S2 C and D). TRPM2 current activated by either acetaminophen or ADPR in transfected HEK293T cells was blocked by chlorpromazine with

similar time courses and concentrations as the currents activated in rat hepatocytes (Fig. S2 E and F).

Acetaminophen overdose has been shown to cause DNA fragmentation in hepatocytes and, as a consequence, activation of poly (ADP-ribose) polymerase (PARP), which generates polyADPR, the main precursor of cytoplasmic ADPR (33). Using antibodies against polyADPR and immunofluorescence we were able to demonstrate an increase in polyADPR production in hepatocytes treated for 45 min by either 10 mM acetaminophen or 1 mM H_2O_2 (Fig. 3A). Incubation with 10 mM acetaminophen overnight induced much stronger polyADPR production. To investigate whether generation of ADPR in hepatocytes in response to H_2O_2 and acetaminophen contributes to activation of Ca^{2+} entry we used PARP inhibitor 3,4-dihydro-5-[4-(1-piperidinyl) butoxy]-1(2H)-isoquinoline (DPO) (34). Measurements of $[\text{Ca}^{2+}]_{\text{cyt}}$ showed that DPO strongly inhibited Ca^{2+} entry in rat hepatocytes, supporting the notion that Ca^{2+} rise induced by H_2O_2 and acetaminophen is mediated by ADPR-activated TRPM2 channels (Fig. 3 B and C).

To confirm that the current activated by ADPR, H_2O_2 , and acetaminophen is mediated by TRPM2 channels we used siRNA-mediated knockdown of TRPM2 in rat hepatocytes. In cells transfected with siRNA against TRPM2, patch clamping showed that membrane currents activated by the application of intracellular ADPR, H_2O_2 , or acetaminophen were each reduced by 65–70% (Fig. 4A). RT-PCR and Western blotting confirmed that by using siRNA-mediated knockdown we reduced TRPM2 expression in primary hepatocytes by about 60% ($57 \pm 5\%$, $n = 3$) within 48 h after transfection (Fig. 4B, Table S2, and Fig. S3). Measurements of $[\text{Ca}^{2+}]_{\text{cyt}}$ using Fura-2AM revealed that siRNA-mediated knockdown of TRPM2 expression resulted in a significant reduction of Ca^{2+} entry in hepatocytes treated by H_2O_2 or acetaminophen (Fig. 4 C and D). These results confirm that the cation current activated by acetaminophen and H_2O_2 in hepatocytes is mediated by TRPM2.

To further test whether H_2O_2 and acetaminophen-activated Ca^{2+} entry is mediated by TRPM2 channels, we conducted measurements of $[\text{Ca}^{2+}]_{\text{cyt}}$ using Fura-2AM and hepatocytes isolated from TRPM2 knockout (KO) and (wild-type) WT mice, and the inhibitors of TRPM2 channel, ACA and clotrimazole (Fig. 5 A–C). RT-PCR and Western blot analysis confirmed that WT mouse

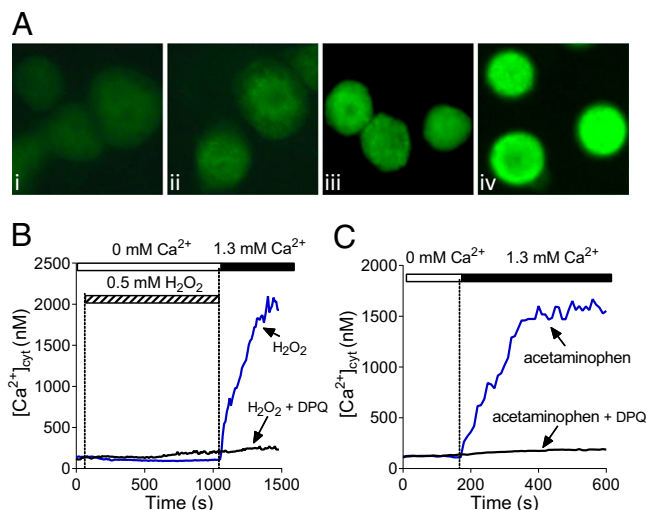


Fig. 3. The role of ADPR in activation of Ca^{2+} entry in hepatocytes. (A) Poly-ADPR-specific immunofluorescence in rat control hepatocytes (A, i) and hepatocytes treated with 10 mM acetaminophen (45 min in A, ii; 16 h in A, iv) or 1 mM H_2O_2 (45 min in A, iii). (B and C) Inhibition of H_2O_2 and acetaminophen-induced Ca^{2+} entry in hepatocytes by PARP inhibitor DPO ($10 \mu\text{M}$). DPO was added to the incubation medium 2 min before the addition of H_2O_2 or acetaminophen ($n = 3$).

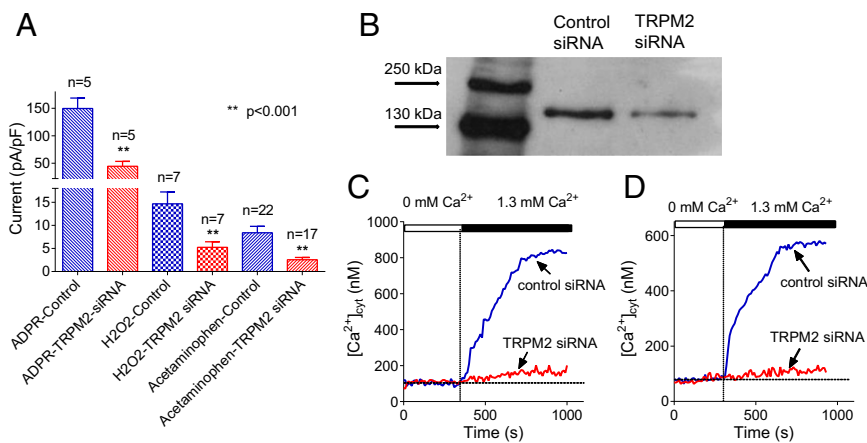


Fig. 4. The effect of TRPM2 knockdown on hepatocyte membrane currents and Ca^{2+} entry. (A) The effect of TRPM2 knockdown on the amplitude of membrane currents activated by intracellular ADPR, H_2O_2 , and acetaminophen. The absolute amplitude of the current measured at -100 mV is shown (mean \pm SEM). (B) Western blot of TRPM2 protein in hepatocytes treated with control siRNA (lane 2) and anti-TRPM2 siRNA (lane 3). (C) The effect of TRPM2 knockdown on Ca^{2+} entry in rat hepatocytes preincubated with 0.5 mM H_2O_2 for 30 min. (D) The effect of TRPM2 knockdown on Ca^{2+} entry in rat hepatocytes preincubated with 10 mM acetaminophen for 60 min.

hepatocytes express the same isoform of TRPM2, LTRPM2, as rat hepatocytes and that TRPM2 protein is absent in TRPM2 KO mouse hepatocytes (Table S3 and Figs. S4 and S5). In response to H_2O_2 or acetaminophen treatment, hepatocytes isolated from TRPM2 WT mice showed Ca^{2+} entry similar to that of rat hepatocytes, which was blocked by ACA and clotrimazole (Fig. 5A and C; compare with Fig. 1A and C). In contrast, hepatocytes isolated from TRPM2 KO mice had a significantly smaller Ca^{2+} entry activated by H_2O_2 and virtually no Ca^{2+} entry in response to the treatment with acetaminophen (Fig. 5B and C). A relatively small Ca^{2+} rise in TRPM2 KO mouse hepatocytes induced by H_2O_2 was blocked by clotrimazole, but not ACA, suggesting the presence of a minor H_2O_2 -activated Ca^{2+} entry pathway not mediated by TRPM2 channels. Results of Ca^{2+} imaging were supported by patch clamping of hepatocytes isolated from TRPM2 WT and KO mice. Treatment of hepatocytes with H_2O_2 or acetaminophen resulted in activation of a significantly larger nonselective cation current in TRPM2 WT hepatocytes compared with hepatocytes from KO animals (Fig. S6A–D).

Ablation of TRPM2 Channels Protects Against Acetaminophen Toxicity in the Liver. Treatment of isolated rat and mouse hepatocytes in culture with acetaminophen causes progressive cell death through oncotic necrosis, thus mimicking the effects of acetaminophen on the intact liver (35). In the next experiment we investigated whether inhibition of TRPM2 channels by ACA affords protection to hepatocytes against high doses of acetaminophen. Indeed, ACA (1 μM) reduced cellular death by $\sim 60\%$ in hepatocytes treated with 10 mM acetaminophen for 16 h compared with hepatocytes treated with acetaminophen alone ($n = 3$; $P < 0.01$) (Fig. S6E).

To establish whether activation of TRPM2 channels plays a role in acetaminophen-induced liver damage in vivo we used TRPM2 KO mice (36). I.p. injection of acetaminophen (500 mg/kg) to WT mice resulted in a large increase in the blood concentrations of the liver enzymes, alanine transaminase (ALT) and

aspartate transaminase (AST) compared with vehicle-injected animals (Fig. 6A), suggesting severe liver damage 24 h postinjection (cf. ref. 37). In TRPM2 Het mice this dose of acetaminophen also caused a large elevation of liver enzymes. However, in TRPM2 KO animals the blood levels of ALT and AST were ~ 5 – 7 times lower than in WT and Het mice (Fig. 6A). Hematoxylin/eosin (H&E) staining of liver sections of acetaminophen-treated TRPM2 WT and Het mice revealed widespread hepatocellular damage (Fig. 6B). This was characterized by areas of necrosis, infiltration by lymphocytes, and hemorrhage, and was prominent in zones 2 and 3 (hepatocytes around the hepatic vein) (Fig. 6B). Liver damage was substantially reduced in TRPM2 KO mice treated with acetaminophen compared with the treated WT and Het mice (Fig. 6B). The area of necrotic damage was much smaller in TRPM2 KO mice and was localized to zone 3. In some sections of livers from acetaminophen-treated TRPM2 WT and Het mice, hemorrhagic necrosis and extravasations of blood were detected along with necrotic damage. Because the prominent and consistent effect of acetaminophen was necrotic damage, the area of necrotic tissue was quantified. The results indicate that the area of necrosis in the livers of TRPM2 KO mice treated with acetaminophen was substantially smaller than in the livers of acetaminophen-treated TRPM2 WT and Het mice (Fig. S6F).

Discussion

In this study we show that hepatocytes express long isoform of TRPM2 channels. These channels function as a cation entry pathway across the hepatocyte plasma membrane and similarly to TRPM2 channels heterologously expressed in HEK293 cells are activated by ADPR included in the patch pipette and H_2O_2 added to the bath solution (24, 38). The important finding of this study is that TRPM2 channels mediate a substantial increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in hepatocytes treated with toxic concentrations of acetaminophen. Furthermore, we show that Ca^{2+} entry through TRPM2 channels plays a significant role in acetaminophen-induced hepatocellular death both in vitro and in vivo.

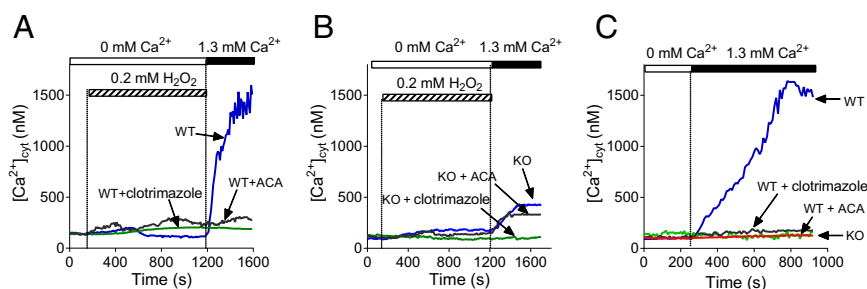


Fig. 5. H_2O_2 - and acetaminophen-activated Ca^{2+} entry is attenuated in TRPM2 KO mouse hepatocytes. (A and B) Ca^{2+} entry in TRPM2 WT (A) and KO (B) mouse hepatocytes activated by the addition of H_2O_2 to the bath solution ($n = 3$). (C) Ca^{2+} entry in TRPM2 WT and KO mice hepatocytes treated with 10 mM acetaminophen for 60 min ($n = 3$). Both clotrimazole (50 μM) and ACA (10 μM) were applied to the bath 5 min before the addition of Ca^{2+} .

(44). The isolated hepatocytes were cultured on glass coverslips at 37 °C in 5% CO₂ in air (vol/vol) in DMEM containing penicillin (100 U/mL), streptomycin (100 µg/mL), and 10% FBS (vol/vol) for 16–72 h before experiments. Isolated mouse hepatocytes were prepared by retrograde perfusion of the liver through the inferior vena cava and cut-open portal vein. The rest of the protocol was similar to that used to prepare isolated rat hepatocytes (44).

Calcium Imaging. Hepatocytes cultured on glass coverslips for 24 h were loaded with Fura2-AM (5 µM) for 30 min, washed, and incubated in Krebs–Ringer–Hepes solution for 10 min in a CO₂ incubator at 37 °C. The fluorescence of Fura-2 was measured using a Nikon TE300 Eclipse microscope equipped with a Sutter DG-4/OF wavelength switcher, Omega XF04 filter set for Fura-2, Photonic Science ISIS-3 intensified CCD camera, and Universal Interface Card MetaFluor software. Fluorescence images were obtained every 20 s using a 20× objective. Fluorescence ratio values (340:380 nm) were transformed to [Ca²⁺]_{cyt} using the equation derived by Grynkiewicz et al. (45).

Patch-Clamp Recording. Membrane currents were measured at room temperature (23 °C) using standard patch clamping in a whole-cell mode and a computer-based EPC-9 patch-clamp amplifier run by PULSE software (HEKA) (22). To monitor the development of membrane currents, voltage

ramps between –120 and +120 mV were applied every 2 s following the achievement of whole-cell configuration. The holding potential was –40 mV. The data were analyzed using PULSEFIT software (HEKA). For current measurements in rat hepatocytes, patch pipettes were pulled from borosilicate glass and fire polished to a resistance between 1.5 and 2.5 MΩ. Mouse hepatocytes were generally less amenable for patch clamping, therefore to increase the probability of forming a gigaohm seal, smaller patch pipettes with a resistance between 3 and 5 MΩ were used. Series resistance was 50–70% compensated.

Statistical Analysis. Data are presented as means ± SEM. Statistical significance was assessed using ANOVA followed by the Bonferroni post hoc test or using unpaired two-tailed *t* test with Welch's correction.

Chemicals, solutions, and methods for RT-PCR, cell transfections, Western blot analysis, cell viability assay, immunofluorescence, in vivo acetaminophen toxicity, blood liver enzymes assay, and histopathology are provided in *SI Materials and Methods*.

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