The ryanodine receptor has been mainly regarded as the Ca\(^{2+}\) release channel from sarcoplasmic reticulum controlling skeletal and cardiac muscle contraction. However, many studies have shown that it is widely expressed, with functions not restricted to muscular contraction. This study examined whether ryanodine receptor plays a role in calcium signaling in the liver. RT-PCR analysis of isolated hepatocytes showed expression of a truncated type 1 ryanodine receptor, but no type 2 or type 3 message was detected. We also detected binding sites for \(^{3}H\)ryanodine in the microsomal cellular fraction and in permeabilized hepatocytes. This binding was displaced by caffeine and dantrolene, but not by ruthenium red, heparin or cyclic ADP-Ribose. Ryanodine, by itself, did not trigger Ca\(^{2+}\) oscillations in either primary cultured hepatocytes or hepatocytes within the intact perfused rat liver. In both preparations, however, ryanodine significantly increased the frequency of the cytosolic free [Ca\(^{2+}\)] oscillations evoked by an \(\alpha_{1}\)-adrenergic receptor agonist. Experiments in permeabilized hepatocytes showed that both ryanodine and cyclic ADP-ribose evoked a slow Ca\(^{2+}\) leak from intracellular stores and were able to increase the Ca\(^{2+}\)-released response to a subthreshold dose of inositol 1,4,5-trisphosphate. Our findings suggest the presence of a novel truncated form of the type 1 ryanodine receptor in rat hepatocytes. Ryanodine modulates the pattern of cytosolic free [Ca\(^{2+}\)] oscillations by increasing oscillation frequency. We propose that the Ca\(^{2+}\) released from ryanodine receptors on the endoplasmic reticulum provides an increased pool of Ca\(^{2+}\) for positive feedback on inositol 1,4,5-trisphosphate receptors.

Two families of channels lead to Ca\(^{2+}\) release from intracellular stores in the endoplasmic reticulum (ER)\(^{2}\)/sarcoplasmic reticulum (SR). One is the inositol 1,4,5-trisphosphate receptor (IP\(_{3}\)R) family. Three different isoforms (types I, II, III) have been characterized and there are also a number of splicing variants. Activation of the IP\(_{3}\)Rs is elicited by IP\(_{3}\) generated by receptor activation of phosphoinositide-specific phospholipase C (PLC). The IP\(_{3}\)Rs are predominantly situated in the ER and play a pivotal role in Ca\(^{2+}\) signaling in almost every cell type (1, 2).

The second family of intracellular Ca\(^{2+}\) channels has been named ryanodine receptor (RyR) because of its high affinity for the plant alkaloid ryanodine that led to its identification and purification (3). Ryanodine has been utilized as a tool to study Ca\(^{2+}\) release from the ER/SR of, at first, muscle cells, and more recently of a wide variety of nonmuscle cells. As with the IP\(_{3}\)R family, the RyR family consists of three isoforms: RyR1 is commonly associated with skeletal muscle cells, RyR2 with cardiac muscle cells and RyR3 is widely distributed with low expression and unclear physiological role (4).

An interesting feature of both channel families is their regulation by Ca\(^{2+}\) itself. Ca\(^{2+}\) can either stimulate or inhibit the channels depending on its concentration. The stimulatory process of Ca\(^{2+}\)-induced Ca\(^{2+}\)-released (CICR) plays a very important role because it is a regenerative mechanism that amplifies the Ca\(^{2+}\) signal and propagates it throughout the cytoplasm (5, 6). In the last few years it has also become clear that cyclic ADP-ribose (cADPR) can induce the opening of RyRs (7).

Hepaticocytes are polarized epithelial cells where Ca\(^{2+}\) signaling plays an essential role in the control of metabolism and function. Hormones, such as vasopressin, angiotensin II, and \(\alpha\)-adrenergic agonists, mobilize internal Ca\(^{2+}\) stores by stimulating the production of the second messenger IP\(_{3}\). At submaximal hormone concentration, these responses manifest as a series of periodic cytosolic free [Ca\(^{2+}\)] \(([\text{Ca}^{2+}]_c)\) spikes (8). The amplitude and rate of rise of each \([\text{Ca}^{2+}]_c\) spike is relatively constant, whereas the frequency of \([\text{Ca}^{2+}]_c\) spikes increases with agonist concentration (9). This frequency-modulated temporal organization has been suggested to be important in the modulation of many cellular processes, such as mitochondrial metabolism (10), glycogen breakdown (11), and gene expression (12).

The RyR has not been identified nor fully characterized in hepatocytes. On one hand there is evidence for \(^{3}H\)ryanodine binding to microsomal fractions (13, 14) and Ca\(^{2+}\) release from internal stores induced by ryanodine treatment (15, 16), but on the other failure to detect RyR message (17, 18), decrease in frequency of \([\text{Ca}^{2+}]_c\), oscillations after ryanodine challenge (19) and insensitivity to uncaging of cADPR (18) are reported.

In the present study, we demonstrate by RT-PCR analysis the presence of a truncated type 1 RyR in rat hepatocytes. Accord-
RyR1 and the responsiveness of neighboring IP3Rs through Ca2+

ing to our results in permeabilized and intact cells, RyRs are
important in amplifying the IP3-induced Ca2+
response. We propose that RyRs play an active role in the Ca2+
signaling of hepatocytes, creating local Ca2+
micromdains that enhance the responsiveness of neighboring IP3Rs through Ca2+
positive feedback.

**EXPERIMENTAL PROCEDURES**

**Primary Hepatocyte Isolation**—Fed, male anesthetized rats (Sprague-Dawley; 200 – 250 g) were used for preparation of isolated hepatocytes by collagenase perfusion of intact liver as described previously (9). For studies with freshly isolated hepatocytes, the cells were stored on ice prior to use. For overnight culture, hepatocytes (7 × 10
5) were plated on collagen-coated glass coverslips in 3 ml of William E medium (WEM) supplemented with 10% of fetal bovine serum, 2 mM glutamine, 10
units/ml penicillin, 100
units/ml streptomycin, 50
μg/ml gentamycin, and 140
μM insulin. After 2–3 h incubation at 37 °C, the cells were washed into the same medium with insulin reduced to 14 nM and maintained in culture overnight. The next day, the cells were washed again and incubated at least 60 min without insulin prior to use.

**Detection of RyR mRNA by RT-PCR**—Rat hepatocytes were isolated as described above and the total RNA was extracted using RNeasy Mini Kit (Qiagen Inc.) and reverse-transcribed using Superscript III (Invitrogen). PCR amplification (Advantage cDNA PCR Kit, BD Biosciences) was used to amplify RyR cDNA. We used the 11 sets of primers shown in Table 1. (Consensus primers, primers for RyR2, RyR3, and primers h-RyR1 were selected from Fitzsimmons et al. (20) and modified according to rat sequences of GenBankTM accession numbers XM_001078539, XM_008338, and XM_001080527. Primers a-RyR1 were selected from Lee et al. (21). Primers b-, c-, d-, e-, f-, g-RyR1 were designed using the program Primer3). The amplification conditions were 94 °C for 30 s, and the respective annealing temperatures reported in Table 1 for 30 s and 68 °C for 10 s/100 bp, for 35–55 cycles. The PCR products were separated by electrophoresis on 1.2% agarose gels or 10% TBE Gel (Invitrogen) and visualized by DNA staining with ethidium bromide. We obtained the same results from two different mRNA preparations and purified using gel-extracting kit (Qiagen) and sequenced using ABI BigDye Terminator chemistry on an ABI 3130xl Genetic Analyzer. As a control, we performed a reaction with the total hepatic RNA in the absence of reverse transcriptase.

**Liver Microsome Preparation**—Liver microsomes were prepared from male Sprague-Dawley rats, as described previously (22, 23). Livers were quickly excised and placed in beakers containing ice-cold sucrose-MOPS-benzamidine buffer (SMB; 0.25 M sucrose, 10 mM MOPS, 2 mM EGTA, 1 mM dithiothreitol, and the following protease inhibitors: 1 mM benzamidine, 1 μg/ml each of aprotinin, leupeptin, and pepstatin and 0.2 mM phenylmethanesulfonyl fluoride, pH 7.0). The livers were weighed, suspended in 5 volumes of ice-cold SMB and then homogenized using 5 up and down strokes of the Dounce homogenizer. The resultant homogenate was centrifuged at 1500 × g for 10 min and 15,000 × g for 15 min (4 °C). The supernatant obtained was centrifuged at 125,000 × g for 45 min (4 °C). The microsomal pellets were homogeneously suspended into 10 ml of ice-cold SMB buffer. The protein concentration of the microsomes was determined using the biuret method.

**[3H]Ryanodine Binding**—Digitonin-permeabilized hepatocytes (2 mg/ml protein) or liver microsomes (1 mg/ml protein) were incubated for 20 min at 37 °C in Mg2+-free intracellular buffer (20 mM Hepes/Tris, 120 mM KCl, 1 mM KH2PO4, and 10 mM NaCl) containing 100 μM Ca2+, 2 mM ATP, 5 mM creatine phosphate (CP), 0.5 units/ml creatine phosphokinase (CPK), 1 μg/ml each of the following protease inhibitors, peptin, and antipain, and the mitochondrial uncouplers, 5 μg/ml oligomycin, 1 μg/ml rotenone, and 5 μg/ml carbonyl cyanide m-chlorophenyl hydrazone (CCCP). Permeabilized cells or liver microsomes were then incubated an additional 20 min in the presence of increasing concentrations of [3H]ryanodine (0.05–48 nM). The unbound [3H]ryanodine was separated from protein-bound [3H]ryanodine by vacuum filtration. All samples were filtered through 0.22-μm pore size Millipore filters and rapidly washed three times with 5 ml of ice-cold washing buffer containing 150 mM KCl and 10 mM Hepes, pH 7.4. Radioactivity retained on the filters was determined by liquid scintillation counting. Nonspecific binding was defined as the amount of [3H]ryanodine bound in the presence of unlabeled ryanodine (100 μM). Specific binding represents the differences between total binding ([3H]ryanodine alone) and nonspecific binding ([3H]ryanodine plus 100 μM unlabeled ryanodine). In parallel experiments, liver microsomes (1 mg/ml protein) were incubated for 20 min with a submaximal concentration of [3H]ryanodine (9 nM) together with unlabeled ryanodine (100 μM) or various known pharmacological modulators of the RyRs. Where indicated, ruthenium red (100 μM), caffeine (5 mM), dantrolene (100 μM), heparin (100 μg/ml) or cADPR (2 μM)

| mRNA | Forward primer | Reverse primer | Expected product size | Annealing T |
|------|----------------|----------------|----------------------|-------------|
| RyR consensus | AACTACTGAGACAAATTTGT | CCCATCCACAGTAGAACA | 741 | 56 |
| RyR2 | GAATGACCAGAATTTGGCATG | TGGTTCTGACTGACCTCATT | 635 | 60 |
| RyR3 | CTCCTCTGACCATCCTCCCT | TCTCCCTGAGGCTAAAGTCCAG | 505 | 60 |
| a-RyR1 | TCTGATTGAGAGCTGGATG | CCACTCTCCAGATTACACCTG | 301 | 58 |
| b-RyR1 | GCTTGCCGACTACATGCAG | CTCGAGGCCGCGAGAGC | 658 | 61 |
| c-RyR1 | GCCGAGACCTCTCTTTCG | AGGCAAGGCCCTTCCCTTC | 479 | 64 |
| d-RyR1 | GCTCCTTCTGACCATCCTACCA | CCAGCGAAACGTACACAC | 682 | 63 |
| e-RyR1 | CACCAATCTCCACTACACACC | CGAGCCCCCTTCCTCAAT | 259 | 65 |
| f-RyR1 | TTTGAGCCGCCGACTGATA | GAAGCCGCTCTTTCTCCTG | 487 | 60 |
| g-RyR1 | GAAGCAAACCGCTGACCC | AGGCTGACGAGACACCTCC | 479 | 62 |
| h-RyR1 | GAAGCTTCTCAGGACAAACACGGG | TCCGTCCTGTTGAGCACTGCGG | 435 | 60 |
were also included in the incubation period followed by vacuum filtration.

Microinjection of Hepatocytes—For microinjection in intact hepatocytes, the cells were plated in WEM containing 10% fetal calf serum and glutamine at 10^5 cells/ml for 1 h at 37 °C in an incubator. Then the plated cells were incubated with 5 μM Indo1/AM for 45 min in the presence of sulfipyrazone (300 μM). Measurements were performed in the Na^+ Hepes buffer containing 2 mM Ca^{2+}. Indo1 fluorescence was acquired with a dichroic beam splitter and a pair of photomultipliers to collect fluorescence simultaneously at 410 nm and 480 nm. The data are presented as the ratio of 410/480 nm fluorescence. The pipettes used to microinject were made with glass from WPI type 1BBL with filament (ref 1B150F-4) diameter 1.5 mm. After touching the cell with the pipette, the different compounds were injected with a pressure of 20 PSI for 40 s using a Picospirter.

Imaging Measurements in Intact and Permeabilized Hepatocytes—For Ca^{2+} measurements in intact hepatocytes, the cells where incubated with 5 μM fura2/AM for 15–25 min in the presence of 100 μM bromosulfophthalein (BSP) and 0.02% of pluronic acid. For Mn^{2+} quenching experiments of compartmentalized fura2, the cells were loaded with fura2/AM for 60 min. The indicator was loaded by incubating the hepatocytes in extracellular medium (ECM) composed of 25 mM HEPES, 121 mM NaCl, 4.7 mM KCl, 1 mM KH_2PO_4, 1.2 mM MgSO_4, 5 mM NaHCO_3, 2 mM CaCl_2, 10 mM glucose, 0.25% bovine serum albumin, pH 7.4. Measurements were performed in the same buffer (CaCl_2 was omitted in Mn^{2+} quenching experiments). Inhibition of the mitochondria was performed 5 min before phenylephrine stimulation by the addition of 5 μM rotenone, 5 μg/ml oligomycin, and 1 μM CCCP.

Ca^{2+} measurements in permeabilized hepatocytes were carried out as described previously (24). Briefly, the intact cells were loaded for 60 min with 6 μM fura2FF/AM at 37 °C in ECM in the presence of 100 μM BSP and 0.02% pluronic acid, washed in Ca^{2+}-free buffer and permeabilized with 15–25 μg/ml digitonin in intracellular medium composed of 10 mM NaCl, 120 mM KCl, 1 mM KH_2PO_4, 20 mM HEPES, 0.2–2 mM ATP, 5 mM CP, 5 units/ml CPK, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin, at pH 7.2. Then the cells were washed in the same buffer without digitonin. Inhibition of the mitochondria was performed after permeabilization with digitonin by incubation for 5 min with 10 μM rotenone and 25 μg/ml oligomycin.

Fura2 fluorescence images (excitation 340 and 380 nm) were acquired at 3-s intervals with a cooled charged-coupled device (CCD) camera under computer control (25). Mn^{2+} quenching of fura2/AM fluorescence was measured at 360 nm, a Ca^{2+}-insensitive excitation wavelength.

Isolation and [Ca^{2+}], Imaging Measurements of Perfused Intact Rat Liver—The livers of fed male anesthetized rats of 200–250 g of body weight were perfused in situ through the portal vein as described in detail previously (26). We used the acetoxymethyl ester of fura2 and Fluo3 as Ca^{2+}-sensitive indicators (5 μM) to load the organ. The Fluo3 fluorescence was monitored with a Bio-Rad MRC-600 laser scanning confocal microscope (488 nm excitation). The fura2 fluorescence was monitored using a cooled CCD camera. The excitation wave-lengths were alternatively 340 nm and 380 nm. We achieved deblurring of the non-confocal images by using a deconvolution algorithm described in Monck et al. (27), as described previously (26).

Statistical Analysis—Results are expressed as means ± S.E. of n observations. Statistical differences of the data were evaluated by paired or unpaired Student’s t test and considered significant at p < 0.05.

RESULTS

RyR mRNA Expression—To determine RyR mRNA expression in rat hepatocytes, we isolated total mRNA and amplified RyR cDNA by RT-PCR. We used both consensus primers and isoform-specific primers. The consensus primer pair led to amplification of the expected band of 741 bp (data not shown). We next used the three isoform-specific pairs of primers. The PCR products amplified with h-RyR1-isoform-specific primers revealed the expected band of 435 bp (Fig. 1A), while the primers for isoforms 2 and 3 did not lead to any RT-PCR products (data not shown). Lane 3 on Fig. 1B shows that the RT negative control does not produce any band, excluding any genomic contamination. We also confirmed the veracity of our results by
extracting the 435-bp band and sequencing the PCR product. The sequencing showed 100% homology with the rat RyR1 sequence (GenBank™ accession number XM_001078539). These primers were designed to amplify a region in the C terminus of the RyR1, which contains the putative pore. We then repeated the PCR analysis with primers designed to amplify a portion of the N-terminal of RyR1 (primers a-RyR1). In these experiments, we did not detect any positive result in liver, even after 55 amplification cycles. On the other hand, these primers gave the expected band of 301 bp in skeletal muscle (Fig. 1A).

To further characterize the RyR message expressed in hepatocytes, we designed a series of primers along the RyR1 protein and used them on both hepatocytes and skeletal muscle cDNA. Every set of primers amplified the expected products in skeletal muscle, while the primers b-, c-, and d-RyR1 did not lead to any band in hepatocytes. On the other hand, the primers e-, f-, and g-RyR1 gave a positive result with hepatocyte cDNA, comparable with that observed with the C-terminal primers (Fig. 1A). We also carried out an experiment in which we combined isolated hepatocytes and skeletal muscle tissue before isolating the total mRNA. This was done to investigate whether some kind of mRNA degradation might be taking place in the processing of the hepatocytes. However, Fig. 1C shows that in the region where we could not detect RyR1 message in hepatocytes, we still have positive results in the hepatocyte/skeletal muscle mixture extract. Therefore, it appears that in hepatocytes the RyR1 message starts around 8500 bp of the full-length 15,220 bp RyR1 protein. Evidence for an even shorter truncated form of the RyR1 has been reported previously (28).

Characterization and cloning of the hepatocyte RyR1 variant will be the subject of a future study.

**Ryanodine Receptor in Liver**

**[3H]ryanodine Binding in Hepatic Microsomes and Permeabilized Hepatocytes**—We examined the binding of [3H]ryanodine to microsomes prepared from rat liver to verify the expression of RyRs at the protein level. Fig. 2A demonstrates that [3H]ryanodine binding to liver microsomes is saturable with a KD of 3 nM and a Bmax of 25 fmol/mg of protein. We repeated the same experiment using permeabilized hepatocytes in order to avoid the possibility of [3H]ryanodine binding to non-parenchyma liver cells, such as Kupffer or endothelial cells (the isolated hepatocyte preparation is >99% hepatocytes). In permeabilized hepatocytes we obtained very similar results with saturable ryanodine binding that yielded a KD of 6 nM and a Bmax of 14 fmol/mg of protein (Fig. 2B). These KD values are in agreement with previous data where the KD was estimated to be between 8 and 12 nM in liver microsomes (13, 14). The Bmax value for the hepatic RyR should be compared with the much higher density of RyR1 in terminal cisternae of skeletal muscle SR, where a value of 2.6 pmol/mg protein has been reported (29).

We also tested the effect of various pharmacological compounds on ryanodine binding to the hepatic RyR. [3H]Ryanodine binding to hepatic microsomes was inhibited by 67% in the presence of caffeine (5 mM), an activator of RyR, and by sodium dantrolene (100 μM), an inhibitor of RyR. Ruthenium red (100 μM), heparin (100 μg/ml), or cADPR (2 μM) did not significantly affect [3H]ryanodine binding (Fig. 2C).

**Microinjection Studies in Single Hepatocytes**—We investigated RyR-mediated Ca2+ release from the ER by microinjec-
Ryanodine Receptor in Liver

Ca\(^{2+}\) pool in the ER of hepatocytes, which is distinct from the IP\(_3\)Rs.

**Effect of Ryanodine on IP\(_3\)-dependent Cytosolic Ca\(^{2+}\) Oscillations**—We examined the effect of ryanodine on [Ca\(^{2+}\)]\(_c\), at the single cell level in freshly isolated and overnight cultured fura2-loaded hepatocytes. It should be noted that relatively high ryanodine concentrations are frequently required in intact cells because of the poor membrane permeability of this agent. Direct application of 1–100 \(\mu M\) ryanodine did not lead to any detectable alteration of the basal Ca\(^{2+}\) and did not trigger any [Ca\(^{2+}\)]\(_c\) oscillations in overnight cultured cells (data not shown). In the fresh preparations, very few cells seemed to respond directly to ryanodine, but the low occurrence of this result lead us to attribute the effect to an autocrine or paracrine factor, such as ATP. In support of this, freshly isolated hepatocytes occasionally show [Ca\(^{2+}\)]\(_c\) oscillations without any hormonal stimulation. Caffeine is frequently utilized as a diagnostic tool to detect the presence of RyRs in cells. Unfortunately, the application of caffeine to intact hepatocytes does not act as classical activator of RyR, but inhibits agonist-induced [Ca\(^{2+}\)]\(_c\) oscillations (data not shown). The molecular mechanism underlying the actions of caffeine on hepatic Ca\(^{2+}\) signals have not been fully delineated (30).

To further investigate whether the RyR has a role in hepatocyte Ca\(^{2+}\) signaling, we analyzed the effect of ryanodine on [Ca\(^{2+}\)]\(_c\), responses generated by receptor agonists. For these experiments ryanodine was added to hepatocyte cultures during continuous exposure to phenylephrine, an IP\(_3\)-forming agonist. Addition of 50–100 \(\mu M\) ryanodine resulted in an overall increase in the frequency of [Ca\(^{2+}\)]\(_c\) oscillations. Fig. 4A shows a representative experiment. We analyzed 135 cells (\(n = 6\) different cell preparations) that responded to phenylephrine and maintained a [Ca\(^{2+}\)]\(_c\) oscillatory pattern after addition of ryanodine. These hepatocytes showed an average basal frequency of 0.55 \pm 0.03 oscillations/min and increased to 0.76 \pm 0.03 after ryanodine administration. We also recorded 10 cells that started to oscillate, 13 cells that showed a sustained [Ca\(^{2+}\)]\(_c\) increase after ryanodine challenge (Fig. 4, B and C), and 10 cells where addition of ryanodine stopped [Ca\(^{2+}\)]\(_c\) oscillations (not shown). We obtained the same results using vasopressin (data not shown) indicating that the ryanodine effect is not agonist-specific, but is an intrinsic property of the Ca\(^{2+}\) signal induced by the phosphoinositide cascade.

We also investigated the possible role of mitochondria in mediating the actions of ryanodine. Mitochondria play a pivotal role in the regulation of Ca\(^{2+}\) feedback on IP\(_3\)Rs (31) and a previous report suggested the presence of RyR in heart mitochondria (32). Our unpublished data show that ryanodine slows down Ca\(^{2+}\) uptake in hepatic mitochondria. Therefore, we tested whether the effect of ryanodine could have been because of slower mitochondrial Ca\(^{2+}\) uptake that would provide an increased pool of Ca\(^{2+}\) for positive feedback on the IP\(_3\)R. Inhibition of mitochondrial Ca\(^{2+}\) uptake with rotenone, oligomycin, and CCCP did not prevent the increase in [Ca\(^{2+}\)]\(_c\) oscillations after ryanodine administration (Fig. 4D), indicating that the effect of ryanodine was not mediated by an effect on mitochondrial Ca\(^{2+}\) uptake.

In summary, the studies described above indicate that ryanodine, by itself is not able to induce [Ca\(^{2+}\)]\(_c\) oscillations but it can enhance IP\(_3\)-dependent [Ca\(^{2+}\)]\(_c\) responses by speeding up the frequency of oscillations. These actions were independent of mitochondria and point to a putative RyR expressed in intracellular membranes. Ryanodine did not affect the speed of either phenylephrine- or vasopressin-induced Ca\(^{2+}\) waves, as previously reported (19).

**Ryanodine and cADPR Release Ca\(^{2+}\) from Endoplasmic Reticulum**—The results described above led us to investigate the direct effect of RyR agonists on ER Ca\(^{2+}\) fluxes. In order to achieve this, hepatocytes were loaded with fura2FF, digitonin-permeabilized in Ca\(^{2+}\)-free intracellular-like buffer and then challenged with agonists to induce Ca\(^{2+}\) release. These protocols allow us to directly monitor the amount of Ca\(^{2+}\) in the ER without any interference from plasma membrane and cytosol. Under these conditions, it was still difficult to measure a direct effect of ryanodine on ER Ca\(^{2+}\)}
stores. Ryanodine never caused a rapid \( \text{Ca}^{2+} \) release from the ER, but low micromolar concentrations (1–5 \( \mu \text{M} \)) induced a slow \( \text{Ca}^{2+} \) release (Fig. 5A). We repeated the experiment except the permeabilized cell preparation was first challenged with a submaximal IP\(_3\) concentration (100 nM) prior to addition of ryanodine. In 73 of 199 hepatocytes analyzed (5 different cell preparations), 1–5 \( \mu \text{M} \) ryanodine induced a more robust \( \text{Ca}^{2+} \) release from the ER as shown in Fig. 5B. Higher concentrations of ryanodine (\( \geq 10 \mu \text{M} \)) did not have any effect, as expected from the bell-shaped activation curve of RyRs by ryanodine. At these ryanodine concentrations, the alkaloid keeps the RyRs in a closed conformation inhibiting their gating. We then performed the same set of experiments using cADPR and obtained very similar results. Direct addition of cADPR does not trigger a rapid \( \text{Ca}^{2+} \) release but rather a slow release of \( \text{Ca}^{2+} \) from ER (Fig. 5C). Prestimulation with a low dose of IP\(_3\) (150 nM) led to cADPR-induced \( \text{Ca}^{2+} \) release from the intracellular store (Fig. 5D) in 79 of the 110 cells analyzed (\( n = 2 \) different cell preparations). The size of the \( \text{Ca}^{2+} \) pool released by cADPR (12.6 \( \pm \) 0.5%) was comparable to the one triggered by ryanodine (7.5 \( \pm \) 0.3%), suggesting that the release is mediated by the same channels in the same \( \text{Ca}^{2+} \) pool. Caffeine application did not affect ER \( \text{Ca}^{2+} \) content.

The permeabilized cell system also allowed us to use heparin to investigate the link between RyRs and IP\(_3\)Rs. After the stimulation with a low concentration of IP\(_3\), we applied heparin in order to block the IP\(_3\)Rs and then we challenged the preparation with ryanodine or cADPR. When added after a low dose of IP\(_3\), heparin partially reverses the IP\(_3\)-induced \( \text{Ca}^{2+} \) release. Under these conditions, the addition of 5 \( \mu \text{M} \) ryanodine (Fig. 5E) or 2 \( \mu \text{M} \) cADPR (data not shown) caused a slow \( \text{Ca}^{2+} \) release from the ER similar to that seen when these compounds were applied in the absence of IP\(_3\) or heparin (Fig. 5, A and C). The subsequent \( \text{Ca}^{2+} \) release induced by 10 \( \mu \text{M} \) IP\(_3\) in the presence of heparin is not surprising, because such high concentrations of IP\(_3\) should overcome the competitive inhibitory effects of heparin on the IP\(_3\)R.

We again tested the possible involvement of mitochondria by repeating the same set of experiments with ryanodine in the presence of rotenone and oligomycin to block mitochondrial function. The results did not show any difference between the two conditions (Fig. 5, F compared with B), further confirming that this effect is mitochondrial-independent.

By examining the images of \( \text{Ca}^{2+} \) release in the permeabilized hepatocyte preparation it was possible to determine the spatial distribution of the responses to cADPR and ryanodine, as described previously for IP\(_3\) (24, 31). In contrast to the findings in pancreatic acinar cells (33), there was no evidence for a localization of RyR \( \text{Ca}^{2+} \) release to the nuclear envelope. Instead, the \( \text{Ca}^{2+} \) release elicited by treatment with cADPR or ryanodine in the presence of submaximal...
IP₃ was distributed throughout the ER network in the cytoplasm.

These data provide further evidence for a role of RyRs in amplifying the IP₃-dependent Ca²⁺ signal, and imply that Ca²⁺ released from RyRs acts through CICR on IP₃Rs leading to substantial Ca²⁺ release. These data in permeabilized cells are consistent with our results in intact cells shown in Fig. 4.

Ryanodine Stimulates Mn²⁺-induced Quenching of Compartmentalized fura2/AM in Intact Hepatocytes—We used the property of Mn²⁺ to quench the fura2 signal within the ER of intact cells (34). By monitoring the dye fluorescence at 360 nm excitation, we can study the gating properties of Ca²⁺ channels located in membranes of intracellular compartments. We loaded the hepatocytes with fura2 for 1 h to facilitate the compartmentalization of the dye, and then added 100 μM Mn²⁺ in Ca²⁺-free extracellular-like medium. We waited until the fluorescence signal reached a new steady state, where all of the cytosolic dye was quenched, prior to adding agonists. The further quenching of the fura2 signal by Mn²⁺ after agonist stimulation reflects the opening of Ca²⁺ channels in the intracellular organelles (34). In most cells, application of phenylephrine (7.5 μM) induced an increase in the rate of quenching that occurred in discrete steps (Fig. 6A). Interestingly, some cells, which did not respond directly to phenylephrine challenge showed this stepwise quenching only after addition of 100 μM ryanodine (Fig. 6B). We also investigated if ryanodine could directly stimulate the rate of Mn²⁺ quenching without pre-application of phenylephrine. In 12 out of 52 cells analyzed, 100 μM ryanodine alone evoked a stepwise quench of compartmentalized fura2, indicating gating of Ca²⁺ channels located on the intracellular stores (Fig. 6C).
The Mn$^{2+}$ permeability properties of RyRs have not been fully characterized (4), but we can assume that their permeability properties are similar to the IP$_3$Rs and thus RyRs, when they are open, should allow Mn$^{2+}$ to diffuse from cytosol to the ER lumen. Our results do not give any quantitative information but tell us that ryanodine induces opening of Ca$^{2+}$ channels in intracellular stores. Based also on previous experiments, we speculate that the effect recorded is mostly due to IP$_3$Rs. We hypothesize that ryanodine induces Ca$^{2+}$ release from the ER providing local Ca$^{2+}$ microdomains for a positive feedback on the IP$_3$Rs that facilitate the stepwise Mn$^{2+}$ quenching recorded.

**Effect of Ryanodine on IP$_3$-dependent Cytosolic Ca$^{2+}$ Oscillations of Intact Perfused Liver—**To evaluate the effect of ryanodine in a more physiological condition, we utilized the perfused intact rat liver preparation. The liver was perfused in situ, loaded with Ca$^{2+}$-sensitive indicator dyes and the Ca$^{2+}$-dependent fluorescence changes were analyzed as described previously (26). In agreement with what we observed in isolated cells, infusing ryanodine alone into the hepatic portal vein of the intact liver did not perturb intracellular Ca$^{2+}$ homeostasis (Fig. 7A). We then assessed the viability of the liver and correct dye loading by challenging the liver with vasopressin, which triggered a series of oscillatory [Ca$^{2+}$]$_c$ waves (Fig. 7A) as reported previously (26, 35). We then investigated whether ryanodine alters the IP$_3$-induced Ca$^{2+}$ responses as we found in the primary hepatocyte cultures. Application of a low dose of phenylephrine (150 nM) induced the typical oscillatory [Ca$^{2+}$]$_c$ signals, which increased in frequency after ryanodine administration (Fig. 7B). In the intact liver, the effect of ryanodine on agonist-evoked [Ca$^{2+}$]$_c$ spikes was much more pronounced compared with the primary cultured hepatocyte; almost every cell in our field of view responded to ryanodine challenge. The 49 hepatocytes examined ($n = 2$ different liver preparations) that responded to phenylephrine showed an average basal frequency of 0.54 ± 0.02 oscillations/min and this increased by 86% after ryanodine administration (Fig. 7C).

**DISCUSSION**

The second messenger IP$_3$ elicits Ca$^{2+}$ signals that control many important processes in hepatocytes, including mitochondrial metabolism (10), glycogen degradation (11) and gene expression (12). This signal is modulated in response to a variety of molecules that mainly act through the well-known phosphoinositide cascade. The essential components of this signaling pathway are the formation of IP$_3$ at plasma membrane, binding of IP$_3$ to IP$_3$Rs and release of Ca$^{2+}$ from the intracellular stores leading to a series of periodic [Ca$^{2+}$]$_c$ spikes. In other tissues, especially excitable cells, another type of Ca$^{2+}$ channel (RyR) is present in the membrane of ER where it releases Ca$^{2+}$ into the cytosol. In the last several years it has become evident that a variety of non-excitable cells express RyRs, including pancreas, lung, spleen, renal cells and T and B lymphocytes (17, 36, 37). Thus far, there is no clear evidence for expression or a role of RyRs in liver, but some studies indirectly suggest its involvement. Our data support the hypothesis of hepatic expression of RyRs and suggest a functional role of these channels along with IP$_3$Rs.

Our PCR analysis in isolated hepatocytes demonstrates the expression of RyR isoform 1, which is typical of skeletal muscle. However, we have identified a truncated form of RyR1, where the first 8500–9000 bp of the N-terminal mRNA sequence are missing, leading to a protein without most of the N-terminal cytosolic domain. We were able to amplify the message for the entire C-terminal of the protein where the pore is situated. The idea of a truncated form of RyR was first introduced by Takeshima et al. (28) who suggested its presence in brain. Our results suggesting an N-terminal truncated RyR1 are consistent with a previous study using PCR analysis of message derived from the whole liver (21). It has been suggested that a modified RyR1 at the N-terminal is also expressed in parotid acinar cells, islets of Langerhans and insulinoma cells. The fact that the truncated form of the RyR would still have the capacity to act as a Ca$^{2+}$ channel is confirmed by functional studies with the C-terminal portion of RyR, which also showed that the pore domain of the channel retains sensitivity to ryanodine and [Ca$^{2+}$]$_c$ (38).

Ryanodine was used to identify and isolate the RyR (3) and it has been previously shown that liver microsomes have ryanodine binding sites (13, 14). We confirmed these observations in liver microsomes and also performed the binding assays in per-
meabilized hepatocytes devoid of other cell types, which gave very similar ryanodine binding results. Nevertheless, pharmacological agents modulated the hepatic ryanodine binding in a different manner compared with skeletal muscle. Caffeine and dantrolene strongly inhibited ryanodine binding in hepatocytes, whereas caffeine enhanced and dantrolene slightly reduced binding in skeletal muscle (13, 29). The incubation with ruthenium red also produced different effects on binding: ryanodine binding in hepatocytes was unchanged while it is potently inhibited in skeletal muscle (39). These observations confirm the presence of a protein with high affinity for ryanodine in the internal membranes of hepatocytes, but with different regulation than skeletal muscle RyR1, most likely because of differences in the N terminus. This is in line with the structure of the channel, since the C-terminal contains the membrane-spanning domain, while the N terminus forms a large cytosolic domain containing most of the regulatory binding sites. It could also explain the caffeine insensitivity of the protein in hepatocytes. In our experiments, caffeine did not release Ca\(^{2+}\) from the ER and, as reported by others (30), it inhibited agonist-induced [Ca\(^{2+}\)]\(_{cytosol}\) oscillations (data not shown). The fact that caffeine lacks the ability to mobilize Ca\(^{2+}\) has been documented in other non-excitatory cell types expressing RyRs (40, 41). Thus, a modification in the N-terminal may change the ability of caffeine to open the channel.

The binding site for ryanodine is in close proximity to the membrane-spanning domain (42, 43), explaining why we can use ryanodine as a diagnostic tool to induce Ca\(^{2+}\) release in hepatocytes. In 45Ca\(^{2+}\) uptake experiments in liver microsomes and populations of permeabilized hepatocytes, ryanodine was reported to release Ca\(^{2+}\) (15, 16). It was also suggested that, in populations of intact hepatocytes, ryanodine causes a small increase in basal [Ca\(^{2+}\)]\(_{cytosol}\) levels (16). In the present study, we applied imaging techniques at the single cell level to monitor the cytosolic and ER lumen free Ca\(^{2+}\) concentrations. Our data show that RyRs play an active role in hepatocytes by modulating the pattern of IP\(_3\)-induced Ca\(^{2+}\) oscillations.

In intact hepatocytes, two known agonists of the RyR, ryanodine, and cADPR, had similar effects on [Ca\(^{2+}\)]\(_{cytosol}\). Microinjection of cADPR in freshly isolated hepatocytes triggered a rapid [Ca\(^{2+}\)]\(_{cytosol}\) spike that was most likely because of recruitment of IP\(_3\)Rs. We tried to test this possibility by using the IP\(_3\)Rs blocker heparin, but these experiments were not practical in intact cells. However, we have shown that heparin drastically attenuates the cADPR and ryanodine effect in permeabilized cells (Fig. 5E). The role of cADPR is likely to be physiologically relevant, since it has been shown that hepatocytes can make cADPR (44), have relevant endogenous levels of cADPR (45) and express the protein CD38 (46, 47), the enzyme responsible for cADPR production. Ryanodine also clearly affected the normal Ca\(^{2+}\) signals induced by the phosphoinositide cascade in intact hepatocytes, leading to an increase of the [Ca\(^{2+}\)]\(_{cytosol}\) oscillatory frequency. Direct application of ryanodine did not trigger the Ca\(^{2+}\) oscillatory machinery. Instead, it was necessary to pre-stimulate the cells with an IP\(_3\)-linked agonist to observe the ryanodine effect. The same synergistic interaction was also striking in the perfused intact liver preparation, a system that is very close to physiological conditions. This phenomenon suggests that RyRs closely interact with IP\(_3\)Rs, to enhance the IP\(_3\)-mediated [Ca\(^{2+}\)]\(_{cytosol}\) response of hepatocytes.

Ryanodine and cADPR clearly facilitated Ca\(^{2+}\) release from the ER in permeabilized cells. The effect of ryanodine on ER Ca\(^{2+}\) was in accordance with its bell-shaped activation curve for RyRs: only concentrations in the low micromolar range were able to trigger Ca\(^{2+}\) release, indicating that this is likely a specific effect of ryanodine on the RyRs. Moreover, we observed the same behavior with cADPR application. These data suggest that hepatic RyRs alone do not mediate a robust Ca\(^{2+}\) release, presumably because of low expression of the channel. However, the RyRs in these cells may give rise to microdomains of high Ca\(^{2+}\) near the ER membrane that can sensitize the IP\(_3\)Rs. This would explain why the addition of ryanodine to intact cells increases the frequency of [Ca\(^{2+}\)]\(_{cytosol}\) spiking without directly releasing a substantial pool of Ca\(^{2+}\). This is the reverse of the postulated relationship between RyRs and IP\(_3\)Rs in heart, where Ca\(^{2+}\) released by the IP\(_3\)R channels is believed to facilitate opening of RyR Ca\(^{2+}\) channels (48).

In summary, we have shown the presence of RyRs in liver and their synergistic interaction with IP\(_3\)Rs. Our studies lead us to propose a model for hepatic [Ca\(^{2+}\)]\(_{cytosol}\) oscillations where these two classes of Ca\(^{2+}\) channels closely influence each other open probability. We suggest that RyRs can create Ca\(^{2+}\) microdomains near the surface of ER membrane, recruiting IP\(_3\)Rs for a further and massive Ca\(^{2+}\) release. This process of CICR lowers the threshold for the generation of an IP\(_3\)-linked Ca\(^{2+}\) spike, thus contributing to the frequency modulation of [Ca\(^{2+}\)]\(_{cytosol}\) oscillations.

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REFERENCES

1. Berridge, M. J. (1993) Nature 361, 315–325
2. Joseph, S. K. (1996) Cell Signal. 8, 1–7
3. Lai, F. A., Erickson, H. P., Rousseau, E., Liu, Q. Y., and Meissner, G. (1988) Nature 331, 315–319
4. Fill, M., and Copello, J. A. (2002) Physiol. Rev. 82, 893–922
5. Galione, A., McDougall, A., Busa, W. B., Willmott, N., Gillot, I., and Whittaker, M. (1993) Science 261, 348–352
6. Wang, S. Q., Song, L. S., Lakatta, E. G., and Cheng, H. (2001) Nature 410, 592–596
7. Galione, A., Lee, H. C., and Busa, W. B. (1991) Science 253, 1143–1146
8. Woods, N. M., Cuthbertson, K. S., and Cobbold, P. H. (1986) Nature 321, 600–602
9. Rooney, T. A., Sass, E. J., and Thomas, A. P. (1989) J. Biol. Chem. 264, 17131–17141
10. Hajnoczky, G., Robb-Gaspers, L. D., Seitz, M. B., and Thomas, A. P. (1995) Cell 82, 415–424
11. Assimacopoulos-Jeannet, F. D., Blackmore, P. F., and Epton, J. H. (1977) J. Biol. Chem. 252, 2662–2669
12. Doolittle, R. E., Xu, K., and Lewis, R. S. (1998) Nature 392, 933–936
13. Shoshan-Barmatz, V., Pressley, T. A., Higham, S., and Kraus-Friedmann, N. (1991) Biochem. J. 276, 41–46
14. Shoshan-Barmatz, V. (1990) FEBS Lett. 263, 317–320
15. Lilly, L. B., and Golland, J. L. (1995) Am. J. Physiol. 268, G1017–G1024
16. Bazotte, R. B., Pereira, B., Higham, S., Shoshan-Barmatz, V., and Kraus-Friedmann, N. (1991) Biochem. Pharmacol. 42, 1799–1803
17. Giannini, G., Conti, A., Mammarella, S., Scrobogna, M., and Sorrentino, V. (1995) J. Cell Biol. 128, 893–904
18. Hirata, K., Pusl, T., O’Neill, A. F., Dranoff, J. A., and Nathanson, M. H. (2002) Gastroenterology 122, 1088–1100
19. Nathanson, M. H., Burgstahler, A. D., and Fallon, M. B. (1994) Am. J. Physiol. 267, G338–G349
20. Fitzsimmons, T. J., Gukovsky, I., McRoberts, J. A., Rodriguez, E., Lai, F. A., and Pandol, S. J. (2000) Biochem. J. 351, 265–271
21. Lee, B. S., Sessanna, S., Laychock, S. G., and Rubin, R. P. (2002) J. Membr. Biol. 189, 181–190
22. Taraschi, T. F., Wu, A., and Rubin, E. (1985) Biochemistry 24, 7096–7101
23. Ellingson, J. S., Janes, N., Taraschi, T. F., and Rubin, E. (1991) Biochim. Biophys. Acta 1062, 199–205
24. Hajnoczky, G., and Thomas, A. P. (1997) EMBO J. 16, 3533–3543
25. Morgan, A. J., and Thomas, A. P. (1999) Methods Mol. Biol. 114, 93–123
26. Robb-Gaspers, L. D., and Thomas, A. P. (1995) J. Biol. Chem. 270, 8102–8107
27. Monck, J. R., Oberhauser, A. F., Keating, T. J., and Fernandez, J. M. (1992) J. Cell Biol. 116, 745–759
28. Takeshima, H., Nishimura, S., Nishi, M., Ikeda, M., and Sugimoto, T. (1993) FEBS Lett. 322, 105–110
29. Pessah, I. N., Stambuk, R. A., and Casida, J. E. (1987) Mol. Pharmacol. 31, 232–238
30. Sanchez-Bueno, A., Marrero, I., and Cobbold, P. H. (1994) Biochem. Biophys. Res. Commun. 198, 728–733
31. Hajnoczky, G., Hager, R., and Thomas, A. P. (1999) J. Biol. Chem. 274, 14157–14162
32. Beutner, G., Sharma, V. K., Giovannucci, D. R., Yule, D. I., and Sheu, S. S. (2001) J. Biol. Chem. 276, 21482–21488
33. Gerasimenko, J., Maruyama, Y., Tepikin, A., Petersen, O. H., and Gerasimenko, O. (2003) Biochem. Soc. Trans. 31, 76–78
34. Hajnoczky, G., Lin, C., and Thomas, A. P. (1994) J. Biol. Chem. 269, 10280–10287
35. Gaspers, L. D., and Thomas, A. P. (2005) Cell Calcium 38, 329–342
36. Tunwell, R. E., and Lai, F. A. (1996) J. Biol. Chem. 271, 29583–29588
37. Sei, Y., Gallagher, K. L., and Basile, A. S. (1999) J. Biol. Chem. 274, 5995–6002
38. Bhat, M. B., Zhao, J., Takeshima, H., and Ma, J. (1997) Biophys. J. 73, 1329–1336
39. Pessah, I. N., Francini, A. O., Scales, D. J., Waterhouse, A. L., and Casida, J. E. (1986) J. Biol. Chem. 261, 8643–8648
40. Zhang, X., Wen, J., Bidasee, K. R., Besch, H. R., Jr., and Rubin, R. P. (1997) Am. J. Physiol. 273, C1306–C1314
41. Hakamata, Y., Nishimura, S., Nakai, J., Nakashima, Y., Kita, T., and Imoto, K. (1994) FEBS Lett. 352, 206–210
42. Callaway, C., Seryshev, A., Wang, J. P., Slavik, K. J., Needleman, D. H., Cantu, C., 3rd, Wu, Y., Jayaraman, T., Marks, A. R., and Hamilton, S. L. (1994) J. Biol. Chem. 269, 15876–15884
43. Witcher, D. R., McPherson, P. S., Kahl, S. D., Lewis, T., Bentley, P., Mullinnix, M. I., Windass, J. D., and Campbell, K. P. (1994) J. Biol. Chem. 269, 13076–13079
44. Rusinko, N., and Lee, H. C. (1989) J. Biol. Chem. 264, 11725–11731
45. Walseth, T. F., Aarhus, R., Zelenikar, R. J., Jr., and Lee, H. C. (1991) Biochim Biophys Acta 1094, 113–120
46. Koguma, T., Takasawa, S., Tohgo, A., Karasawa, T., Furuya, Y., Yonekura, H., and Okamoto, H. (1994) Biochim. Biophys. Acta 1223, 160–162
47. Khoo, K. M., and Chang, C. F. (2000) Arch. Biochem. Biophys. 373, 35–43
48. Mackenzie, L., Roderick, H. L., Proven, A., Conway, S. J., and Bootman, M. D. (2004) Biol. Res. 37, 553–557