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Lipid Rafts Establish Calcium Waves in Hepatocytes

JUN NAGATA^{*,‡}, MATEUS T. GUERRA^{*}, CHRISTINE A. SHUGRUE^{*}, DAWIDSON A. GOMES^{*}, NAOKI NAGATA[‡], and MICHAEL H. NATHANSON^{*}

^{*} Section of Digestive Diseases, Department of Internal Medicine, Yale University School of Medicine, New Haven, Connecticut

[‡] Department of Surgery 1, University of Occupational and Environmental Health, Kitakyushu, Japan

Abstract

Background & Aims—Polarity is critical for hepatocyte function. Ca^{2+} waves are polarized in hepatocytes because the inositol 1,4,5-trisphosphate receptor (InsP3R) is concentrated in the pericanalicular region, but the basis for this localization is unknown. We examined whether pericanalicular localization of the InsP3R and its action to trigger Ca^{2+} waves depends on lipid rafts.

Methods—Experiments were performed using isolated rat hepatocyte couplets and pancreatic acini, plus SkHep1 cells as nonpolarized controls. The cholesterol depleting agent methyl-beta-cyclodextrin (m β CD) was used to disrupt lipid rafts. InsP3R isoforms were examined by immunoblot and immunofluorescence. Ca^{2+} waves were examined by confocal microscopy.

Results—Type II InsP3Rs initially were localized to only some endoplasmic reticulum fractions in hepatocytes, but redistributed into all fractions in m β CD-treated cells. This InsP3R isoform was concentrated in the pericanalicular region, but redistributed throughout the cell after m β CD treatment. Vasopressin-induced Ca^{2+} signals began as apical-to-basal Ca^{2+} waves, and m β CD slowed the wave speed and prolonged the rise time. M β CD had a similar effect on Ca^{2+} waves in acinar cells but did not affect Ca^{2+} signals in SkHep1 cells, suggesting that cholesterol depletion has similar effects among polarized epithelia, but this is not a nonspecific effect of m β CD.

Conclusions—Lipid rafts are responsible for the pericanalicular accumulation of InsP3R in hepatocytes, and for the polarized Ca^{2+} waves that result. Signaling microdomains exist not only in the plasma membrane, but also in the nearby endoplasmic reticulum, which in turn, helps establish and maintain structural and functional polarity.

Cytosolic Ca^{2+} regulates a wide variety of functions in hepatocytes, including glucose release, gene transcription, apoptosis, and bile secretion.^{1–3} Ca^{2+} is able to regulate such a range of cell functions because of the diverse temporal and spatial patterns of Ca^{2+} signals that can be encoded. For example, temporal patterns of Ca^{2+} signals such as Ca^{2+} oscillations encode information in the oscillation frequency, and frequency modulation can differentially regulate expression of transcription factors and cytokines.^{4,5} Similarly, spatial patterns encode information in the form of Ca^{2+} waves, which can regulate fluid and electrolyte secretion and exocytosis.^{6,7} Ca^{2+} signaling in hepatocytes is mediated by inositol 1,4,5-trisphosphate (InsP3),⁸ so Ca^{2+} signaling patterns depend on the expression and subcellular distribution of isoforms of the InsP3 receptor (InsP3R). The type II InsP3R is the predominant isoform in hepatocytes,⁹ and that isoform is expressed most heavily in the pericanalicular region.⁸ However, it is not known why the type II InsP3R accumulates there. Hepatocytes also express

Address requests for reprints to: Michael H. Nathanson, MD, PhD, Digestive Diseases, Room TAC S241D, Yale University School of Medicine, New Haven, Connecticut 06520-8019. Michael.nathanson@yale.edu; fax: (203) 785-4306.

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the type I isoform of the InsP3R, and in neurons, this isoform is drawn to the region of the plasma membrane because it associates with the cytoskeletal linker protein 4.1N.^{10,11} Protein 4.1N belongs to the band 4.1 family of proteins that also includes 4.1R and 4.1G, both of which are cytoskeletal linker proteins expressed widely, including in pancreas and liver.¹² Protein 4.1N is the only 1 of these that is thought to bind to the type I InsP3R, but 4.1N is not expressed in hepatocytes¹³ or in liver cell lines.¹⁴ No binding partners for the type II InsP3R have been identified, but the canalicular membrane of hepatocytes contains lipid rafts,¹⁵ and in other cells lipid rafts can establish signaling microdomains.¹⁶ Therefore, we examined whether lipid rafts are responsible for the pericanalicular accumulation of the type II InsP3R in hepatocytes, and whether this in turn is responsible for shaping Ca²⁺ waves in these cells.

Materials and Methods

Animals and Materials

Male Sprague-Dawley rats obtained from Charles River Laboratories (Wilmington, MA) and weighing 100–250 g were used for all experiments. Animals were housed in temperature- and light-controlled rooms and maintained on a standard diet. Dulbecco's modified Eagle's medium and other tissue culture reagents were from Invitrogen (Basel, Switzerland). ER-Tracker, fluo-4/AM, mag-fluo-4/AM, and rhodamine-conjugated phalloidin were from Molecular Probes (Eugene, OR). Monoclonal antibodies against calreticulin and caveolin-1 were from BD Biosciences (San Jose, CA), antibodies for Na⁺/K⁺ ATPase were from Santa Cruz (Santa Cruz, CA), and for MRP2 were from Abcam (Cambridge, MA). Antibodies specific for InsP3R isoforms I and II were used for immunoblotting and immunofluorescence. Type I InsP3R antibodies from affinity-purified specific rabbit polyclonal antiserum directed against the 19 C-terminal residues of the mouse type I InsP3R⁸ and monoclonal antibodies directed against sarcoplasmic/endoplasmic reticulum (ER) Ca²⁺-ATPase (SERCA) type 2b were commercially obtained from Affinity Bioreagents (Golden, CO). Type II InsP3R antibodies from affinity-purified specific rabbit polyclonal antiserum directed against the 18 C-terminal residues of the rat type II InsP3R⁹ were kindly provided by Richard Wojcikiewicz (State University of New York, Syracuse, NY). Methyl-beta-cyclodextrin (m β CD), filipin, cholesterol, acetylcholine (ACh), and arginine⁸-vasopressin were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals were of the highest quality commercially available.

Isolation of Hepatocytes and Pancreatic Acinar Cells

Isolated rat hepatocyte couplets were prepared in the Cell Isolation Core of the Yale Liver Center as described previously.⁸ This cell preparation was used because hepatocyte couplets retain structural and functional polarity in short-term culture.^{17–19} Briefly, rat livers were perfused with Hanks' A and then Hanks' B medium containing 0.05% collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 0.8 U trypsin inhibitor (Sigma) per unit tryptic activity. Livers were then excised, minced, and passed through serial nylon mesh filters, and the resultant cells were washed. These cells were suspended at a concentration of 1.5×10^5 cells/mL in L-15 medium (GIBCO, Grand Island, NY) containing 50 U penicillin and 50 mg streptomycin/mL, and were plated onto glass coverslips. Cells were incubated at 37°C and used 2–4 hours after plating. Cell viability was measured 2 hours after plating by trypan blue exclusion and exceeded 90%.

Pancreatic acini were isolated as described previously.²⁰ Briefly, fasted rats were anesthetized and killed, and then the pancreas was surgically removed and placed in a Ca²⁺-Mg²⁺-free buffer (pH 7.4). The tissue was finely diced and then transferred to a siliconized flask containing 1 mL digestion buffer. The tissue was shaken, serially filtered through nylon mesh, and then rinsed. Acini were maintained at room temperature and used within 3 hours of isolation.

Hepatocytes or acinar cells were cultured with 5 mmol/L m β CD for 30 minutes at 37°C to disrupt cholesterol-bearing lipid rafts.²¹ Control dishes were treated with culture medium alone. In selected experiments, cells cultured with m β CD were then placed in culture with m β CD preloaded with cholesterol (242 μ g cholesterol/mL) for 90 minutes. This protocol allows depleted cholesterol to be restored to cells,^{22,23} and was performed to determine whether the effects of m β CD were reversible.

Cell Fractionation

OptiPREP gradient (Accurate Chem. & Sci. Corp., Westbury, NY) analysis was performed using a modification of previously published protocols.²¹ Rat hepatocytes in short-term (2–4-hour) culture were homogenized by 20 passages through a 27–30-gauge needle in lysis buffer (250 mmol/L sucrose, 10 mmol/L Tris-HCl, pH 7.4; 1% Triton-X 100 was added in studies to detect caveolin) containing protease inhibitor on ice, and then centrifuged at 500 $\times$ g for 10 minutes to remove cell debris. Supernatants were placed at the top of the tube, and then run through with a discontinuous 0%–45% Optiprep gradient. After centrifugation at 100,000 $\times$ g in a Beckman SW41 ultracentrifuge (Fullerton, CA) overnight at 4°C, 10 serial 1-mL fractions were collected from the top of the gradient.

Immunoblot Analysis

Immunoblots of isolated hepatocytes were prepared as described previously.⁸ Protein concentration was determined,²⁴ and then proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 4%–20% polyacrylamide gel. Gels were subsequently transferred to protein nitrocellulose membranes. The membranes were blocked for 1 hour and incubated at 4°C overnight with the primary antibodies. The antibody against type I InsP3R was used at a dilution of 1:1000, the antibody against type II InsP3R was used at a dilution of 1:100, the antibody against MRP2 was used at a dilution of 1:2000, the antibody against Na⁺/K⁺ ATPase was used at a dilution of 1:100, the antibodies against SERCA 2b and caveolin each were used at a dilution of 1:500, and the antibody against calreticulin was used at a dilution of 1:1000. Membranes were washed, incubated for 1 hour with peroxidase-conjugated antirabbit (1:5000) or antimouse (1:4000) secondary IgG antibody, and revealed by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Confocal Immunofluorescence Microscopy

Confocal immunofluorescence was performed on isolated hepatocytes and on rat liver sections, as described previously.⁸ Cells were fixed with cold acetone and permeabilized with Triton X-100. After blocking steps, specimens were labeled with primary antibodies, rinsed with phosphate-buffered saline, and incubated with secondary antibodies. Cells also were labeled with rhodamine-conjugated phalloidin to facilitate identification of the canalicular membrane.^{17,25} For negative control studies, cells were incubated with secondary antibodies, but primary antibodies were omitted. Specimens were examined with a Zeiss LSM 510 Laser Scanning Confocal Microscope equipped with a krypton/argon mixed gas laser (Thornwood, NY). To ensure specificity of staining, images were obtained using confocal machine settings at which no fluorescence was detectable in negative control samples labeled with secondary antibodies alone. Double-labeled specimens were serially excited at 488 nm and observed at 505–550 nm to detect Alexa 488; then they were excited at 543 nm and observed at >585 nm to detect rhodamine. Triple-labeled specimens were serially excited at 488 nm and observed at 505–550 nm to detect Alexa 488, excited at 543 nm, and observed at >585 nm to detect Alexa 555, and excited at 633 nm and observed at >650 nm to detect Alexa 647. All images were collected using a Zeiss LSM 510 Meta Laser Scanning Confocal Microscope. For filipin/caveolin double-labeling studies, isolated rat hepatocyte couplets were incubated with filipin (0.05 mg/mL) for 60 minutes, then fixed and stained with anticaveolin primary antibody and then Alexa

555 secondary antibody as described above. Filipin fluorescence was determined by 2-photon microscopy using an excitation wavelength of 735 nm and an emission wavelength range of 410–490 nm,²⁶ while Alexa 555 labeling of caveolin antibodies was detected simultaneously using confocal microscopy as above.

Ca²⁺ Measurements

Cytosolic Ca²⁺ (Ca_i²⁺) measurements were performed by time-lapse confocal microscopy.^{27, 28} Hepatocytes were incubated with fluo-4/AM (6 μmol/L) for 30 minutes at 37°C.²⁷ Pancreatic acinar cells were also loaded with fluo-4/AM, but at room temperature.^{20,29} Coverslips containing the dye-loaded cells were transferred to a chamber on the stage of a Zeiss LSM 510 Confocal Microscope, perfused with a HEPES-buffered solution, and observed. Ca_i²⁺ was monitored in these cells by exciting the specimen at 488 nm and detecting emission signals above 515 nm. A krypton-argon laser was used for excitation. Images of apical and basolateral increases in Ca_i²⁺ were obtained at a rate of ~5 frames per second. Neither autofluorescence nor other background signals were detectable at the machine settings used. Changes in fluorescence *F* were normalized by the initial fluorescence (*F*₀) and are expressed as (*F*/*F*₀) × 100%.²⁷ Hepatocytes and SKHep1 cells were observed during perfusion with vasopressin, which causes an InsP3-mediated increase in Ca_i²⁺ in both of these cell types.^{1,30} Similarly, pancreatic acinar cells were stimulated with ACh, which causes an InsP3-mediated increase in Ca_i²⁺ in these cells.²⁰ Apical and basal regions were identified within the cytosol of individual cells, and the Ca_i²⁺ signals in those regions were determined from the recorded images.

ER Imaging

The subcellular distribution of the ER in isolated rat hepatocyte couplets was visualized in 2 different ways, using a low-affinity Ca²⁺ dye to label ER Ca²⁺ stores and using an ER membrane dye.³¹ ER Ca²⁺ stores were labeled by loading cells with mag-fluo-4/AM (6 μmol/L) for 30 minutes at room temperature. Cells were examined using a Bio-Rad MRC 1024 confocal imaging system (Hercules, CA) equipped with a krypton/argon laser. Fluo-4 fluorescence was excited using the 488-nm line of a krypton/argon laser, and emitted fluorescence >515 nm was collected.³¹ The ER membrane was labeled by loading cells with ER-tracker (100 nmol/L) for 30 minutes at room temperature. Cells were examined by 2-photon microscopy using the BioRad MRC 1024 system plus a mode-locked, fsec-pulsed Spectra-Physics Tsunami Titanium:Sapphire laser with a Millennia X pump laser (Mountain View, CA) for 2-photon excitation. ER-Tracker was excited at 790 nm, and 2-photon fluorescence was observed at 500–540 nm using custom-built external detectors.³¹

Statistics

Values listed are mean ± SEM. Statistical comparisons were made using Student *t* test. A value of *P* < .05 was taken as significant.

Results

The Type II InsP3R Is Localized to the Pericanalicular ER in Hepatocytes

The InsP3R is the principal Ca²⁺ release channel in hepatocytes, and the predominant InsP3R isoform in hepatocytes is the type II InsP3R, which is concentrated in the canalicular region.⁸ The InsP3R generally is in the ER membrane,¹ although it has been identified in the plasma membrane as well in some cells.^{32,33} To determine whether the InsP3R in hepatocytes is in the canalicular membrane or the nearby ER, we performed triple-label confocal immunofluorescence studies of rat liver. Sections were stained for type II InsP3R, submembranous actin, and the canalicular membrane transporter MRP2. This revealed that the

type II InsP3R colocalizes with actin beneath the canalicular membrane, rather than with MRP2 on the plasma membrane (Figure 1). This intracellular rather than plasma membrane distribution of the type II InsP3R is consistent with the observation that Ca^{2+} wave speed in hepatocytes is not decreased in Ca^{2+} -free medium.¹⁷ These findings also suggest that the ER and thus ER Ca^{2+} stores would also be found in the canalicular region. To investigate the subcellular distribution of the ER, we labeled isolated rat hepatocyte couplets with the ER membrane dye ER-Tracker, and examined the couplets using 2-photon microscopy (Figure 2A). Second, to determine whether ER Ca^{2+} stores are in the pericanalicular region, couplets were loaded with mag-fluo-4, which preferentially labels ER Ca^{2+} stores owing to its low affinity for Ca^{2+} .³¹ These couplets were examined using confocal microscopy (Figure 2B). These findings demonstrated that the ER and its Ca^{2+} stores are distributed throughout the hepatocyte, including in the pericanalicular region. This is consistent with earlier electron microscopic findings that the ER extends to the pericanalicular region as well.¹⁹ Together, these findings show that the type II InsP3R in hepatocytes is concentrated in the pericanalicular ER, in close association with the canalicular membrane.

The InsP3R Redistributes After Cholesterol Depletion

Lipid rafts within membranes have increased cholesterol content, and cholesterol depletion with $\text{m}\beta\text{CD}$ disrupts the rafts and releases raft-associated proteins.³⁴ The effects of $\text{m}\beta\text{CD}$ on cholesterol depletion and disruption of lipid rafts was examined directly in isolated rat hepatocyte couplets by double labeling the couplets with the fluorescent cholesterol stain filipin plus immunostaining for caveolin (Figure 3). Filipin staining was eliminated and caveolin redistributed from the canalicular membrane to the entire plasma membrane in couplets treated with $\text{m}\beta\text{CD}$. This confirms that $\text{m}\beta\text{CD}$ depletes cholesterol and disrupts lipid rafts in isolated rat hepatocyte couplets. To determine whether the type II InsP3R is associated with lipid rafts, we compared the distribution of the receptor in isolated rat hepatocyte couplets before and after treatment with $\text{m}\beta\text{CD}$ (5 mmol/L) for 30 minutes. The subcellular distribution of InsP3R isoforms was examined by immunoblot of cell fractions and by confocal immunofluorescence. Immunoblot analysis showed that the type II InsP3R initially was localized to only 3 of 5 ER fractions in hepatocytes (membrane fractions 6–8), but redistributed into all 5 ER fractions (fractions 6–10) in cells treated with $\text{m}\beta\text{CD}$ (Figure 4A). This is in contrast to the ER marker calreticulin, which was consistently in all 5 ER fractions. The type I isoform of the InsP3R, which accounts for only a minor fraction of InsP3Rs in hepatocytes⁹ but is distributed throughout the basolateral region,⁸ was in cell membrane fractions 7–10 both before and after cholesterol depletion. Separate blots were performed with different ER and plasma membrane markers, as additional controls. This analysis similarly showed that the type II InsP3R initially was localized to only 3 of 5 ER fractions in hepatocytes (membrane fractions 6–8), but redistributed into all 5 ER fractions (fractions 6–10) in cells treated with $\text{m}\beta\text{CD}$ (Figure 4B). The ER marker SERCA 2b was consistently in all 5 ER fractions, similar to what was observed with calreticulin and consistent with the observation that SERCA 2b staining is detected throughout the cytoplasm in immunofluorescence of liver sections.³⁵ Caveolin, which is known to partition into lipid rafts in the plasma membrane, initially localized to only 2 of 3 plasma membrane fractions but redistributed into all 3 fractions in cells treated with $\text{m}\beta\text{CD}$. Isolated rat hepatocyte couplets also were examined by confocal immunofluorescence to identify the subcellular regions in which the type II InsP3R redistributes. The type II InsP3R was expressed primarily in a pericanalicular distribution under control conditions, but moved into the basolateral region as well after treatment with $\text{m}\beta\text{CD}$ (Figure 5). Line scanning was used to quantify subcellular fluorescence labeling (Figure 6A), and then apical/basolateral fluorescence ratios were calculated so that measurements would be independent of individual fluorescence intensity values, to account for variations in labeling among cells. Under control conditions, the ratio of apical to basolateral fluorescence was 38.7 ± 6.3 . After treatment with $\text{m}\beta\text{CD}$, the apical to basolateral fluorescence ratio decreased to 2.9 ± 0.4 ($P < .0001$, $n = 20$ cells in 10

couplets; Figure 6B). Similar results were obtained when apical and basolateral fluorescence was measured within 2-dimensional regions of interest rather than along 1-dimensional line scans (not shown). In contrast to the redistribution of InsP3R labeling that was observed, the subcellular pattern of actin staining was not affected by treatment with m β CD (Figure 6A); the apical/basolateral fluorescence ratio was 17.8 ± 5.4 under control conditions, while the ratio in the presence of m β CD was 16.3 ± 3.3 ($P = .80$, $n = 20$). In separate experiments, cholesterol was added back to couplets after depletion by m β CD to determine whether the redistribution of type II InsP3R was reversible. The ratio of apical to basolateral fluorescence increased only slightly but significantly (to 8.6 ± 2.4 ; $P < .05$, $n = 10$) in these couplets, relative to what was observed in m β CD-depleted couplets. The observation that the effects of m β CD were not completely reversible in this cell system likely reflects the fact that isolated rat hepatocyte couplets are polarized only because they are incompletely separated during cell isolation, and all couplets gradually lose polarity during the several hours after isolation.¹⁹ Therefore, there is not likely to be a driving force to reestablish aspects of polarity once its loss is hastened by treatment with m β CD. Together, these findings provide evidence that the pericanalicular localization of the type II InsP3R in hepatocytes depends upon the presence of lipid rafts.

Propagation of Ca²⁺ Waves Is Impaired by Cholesterol Depletion

Ca²⁺ waves occur in an apical-to-basal fashion in hepatocytes^{8,17} and other polarized epithelia,^{25,29,36} and this is thought to be the functional correlate of the apical localization of the InsP3R.³⁷ Therefore, we compared Ca²⁺ signaling in isolated rat hepatocyte couplets before and after treatment with m β CD. Couplets were stimulated with vasopressin (10 nmol/L), which induces polarized, apical-to-basal Ca²⁺ waves in hepatocytes.⁸ To quantify the effects of cholesterol depletion on Ca²⁺ signaling, the Ca²⁺ wave speed and the rise time required for the Ca²⁺ signal to increase from 20% to 80% of its peak were determined (Figure 7A–B). Under control conditions, Ca²⁺ waves spread from the apical to the basolateral region in 280 ± 3 milliseconds ($n = 30$ cells in 15 couplets). However, this time interval slowed to 950 ± 6 milliseconds ($P < 10^{-8}$) in hepatocytes treated with m β CD. This corresponded to a Ca²⁺ wave speed of 54 ± 6 μ m/second and a rise time of 0.9 ± 0.04 seconds under control conditions, and a wave speed of 15 ± 1 mmol/L/seconds ($P < 10^{-6}$; Figure 7C) and a rise time of 1.8 ± 0.2 seconds ($P < .01$; Figure 7D) in couplets pretreated with m β CD. These findings demonstrate that redistribution of InsP3R from the apical to the basolateral region is associated with slower initiation and spread of Ca²⁺ waves in hepatocytes.

To understand the generality of these observations, Ca²⁺ signaling was examined in pancreatic acinar cells before and after treatment with m β CD. Like hepatocytes, pancreatic acinar cells are polarized epithelia in which InsP3Rs are concentrated near the apical membrane³⁷ and in which hormone-induced Ca²⁺ signals begin as polarized Ca²⁺ waves.^{7,29} However, acinar cells express all 3 InsP3Rs,^{38,39} and the predominant isoform is the type III InsP3R,^{9,37} which is not expressed in hepatocytes.⁸ Under control conditions, ACh (1 μ mol/L) induced a rapid apical-to-basal Ca²⁺ wave that did not diminish in magnitude as it crossed the cell (Figure 8A–B), as has been reported previously.^{20,29} Ca²⁺ wave speed in these cells was 42.5 ± 3.5 μ m/second ($n = 23$; Figure 8C) and the rise time was 1.0 ± 0.1 seconds (Figure 8D). In cells treated with m β CD, Ca²⁺ wave speed was slowed by over 50%, to 18.4 ± 1.2 μ m/second ($n = 15$, $P < .001$; Figure 8C), while rise time was increased to 1.7 ± 0.2 seconds ($P < .05$; Figure 8D). Both the delayed wave speed and the prolonged rise time were reversed by cholesterol repletion (Figure 8C–D). These findings demonstrate that polarized Ca²⁺ waves depend upon lipid rafts in pancreatic acinar cells, as they do in hepatocytes, and suggest that this is a general feature of polarized epithelia.

To determine whether m β CD has nonspecific effects on InsP3-mediated Ca²⁺ signaling, SKHep1 cells were examined before and after treatment with m β CD. SKHep1 cells were used

because they are a liver-derived epithelial cell line that is not polarized. Furthermore, like hepatocytes,⁸ SKHep1 cells lack ryanodine receptors,³⁰ so that Ca^{2+} signaling depends entirely on InsP3Rs in these cells. The InsP3R was diffusely distributed throughout the cytosol in SKHep1 cells, and this pattern was unchanged after treatment with $\text{m}\beta\text{CD}$ (Figure 9A). Vasopressin-induced Ca^{2+} signaling also was monitored in SKHep1 cells before and after treatment with $\text{m}\beta\text{CD}$ (Figure 9B). The rise time for Ca^{2+} signals in untreated cells was 4.5 ± 0.2 seconds, and was 4.7 ± 0.2 seconds in treated cells ($n = 15$ cells in each group, $P = .52$; Figure 9C). This finding demonstrates that treatment with $\text{m}\beta\text{CD}$ does not induce nonspecific effects on Ca^{2+} signaling pathways.

Discussion

The current work provides evidence that the pericanalicular localization of InsP3Rs in hepatocytes depends upon the presence of detergent-resistant membranes, or lipid rafts. Although the existence of lipid rafts remains controversial,⁴⁰ treatment with $\text{m}\beta\text{CD}$ is a well-accepted approach to disrupt putative rafts, and is based on cholesterol depletion.^{21,40} This was the only method used to disrupt rafts here because the other method commonly used, treatment with fumonisin B1 to remove sphingolipids,²¹ requires pretreatment of cells for 72 hours, which is longer than hepatocytes can remain polarized in isolated cell culture.¹⁹ Disruption of the rafts through cholesterol depletion allowed InsP3Rs to redistribute into the basolateral region, with concomitant slowing of the onset and speed of Ca^{2+} waves. These findings may be of broader significance, because cholesterol depletion had a similar effect on Ca^{2+} waves in pancreatic acinar cells. Although the InsP3R is most concentrated in the apical region in both hepatocytes and pancreatic acinar cells, the receptor is almost exclusively the type II isoform in hepatocytes,⁸ but includes all 3 isoforms in pancreatic acinar cells.^{38,39} Moreover, periapical accumulation of the InsP3R appears to be a general feature of polarized epithelial cells, and has been reported in cholangiocytes,²⁵ salivary acinar, and duct cells,³⁹ and the nonpigmented ciliary epithelium of the eye.³⁶ These observations suggest that lipid rafts may be necessary to localize InsP3Rs to the apical region in most or all polarized epithelia. It is unclear from the current work whether the pericanalicular, type II InsP3Rs in hepatocytes are contained in rafts within a specialized region of ER, or if the InsP3Rs instead are linked to proteins within rafts in the canalicular membrane. Lipid rafts within the ER have been described previously, and can contain proteins such as caveolin-3 and the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase,⁴¹ as well as the cellular prion protein,²¹ which is the precursor protein responsible for transmissible spongiform encephalopathies. However, the efficacy of $\text{m}\beta\text{CD}$ in reducing ER cholesterol is not well established. Moreover, several proteins have been identified that link the InsP3R to plasma membrane proteins. For example, protein 4.1N links the type I InsP3R to syndecan-2.¹¹ However, protein 4.1N is not expressed in liver,¹⁴ and does not bind to the type II or III InsP3R,¹¹ which are the periapical isoforms in hepatocytes,⁸ and cholangiocytes,²⁵ respectively. Members of the Homer family of proteins also bind to the InsP3R and link it to metabotropic glutamate receptors^{42,43} and transient receptor potential channels.⁴⁴ Homer proteins are heavily expressed in brain but only weakly expressed in liver,⁴³ and it is not known which hepatic cell types express Homer. In addition, Homer binds to the type I InsP3R,⁴² but there is no evidence that it binds to either the type II or type III InsP3R. On the other hand, transient receptor potential channels in platelets coimmunoprecipitate with the type II InsP3R.⁴⁵ Therefore, there is precedence for linker proteins drawing InsP3Rs to the region of the plasma membrane, but specific linker proteins for the type II InsP3R in hepatocytes and their binding partners within the canalicular membrane have not yet been identified.

The pericanalicular accumulation of InsP3Rs in polarized epithelia has been called the “trigger zone,” because it defines the subcellular region that triggers the formation of Ca^{2+} waves.^{37,46} There are 3 isoforms of the InsP3R, and no 1 isoform is required to trigger Ca^{2+} waves. For

example, the trigger zone is comprised of type II InsP3Rs in hepatocytes,⁸ type III InsP3Rs in cholangiocytes,²⁵ and all 3 isoforms in pancreatic acinar cells.^{38,39} InsP3 is generated by phospholipase C near plasma membrane hormone receptors, which are often on the basolateral membrane, so it is perhaps counterintuitive that Ca^{2+} signals instead would begin at the apical membrane. Certain biophysical properties of the type II⁴⁷ and III⁴⁸ InsP3Rs may explain why these receptors initiate Ca^{2+} waves at the trigger zone, but an alternative explanation is that clustering of InsP3Rs, which occurs in the apical region, lowers the threshold for Ca^{2+} release.^{49,50} Indeed, the rise time is prolonged and the Ca^{2+} wave speed is reduced in hepatocytes with decreased expression of the type II InsP3R,⁵¹ just as these parameters of Ca^{2+} signaling are reduced merely by redistribution of the receptor. Once initiated in the apical region, Ca^{2+} waves may propagate into and across the basolateral region through 1 of several mechanisms. Pancreatic acinar cells express the ryanodine receptor (RyR) Ca^{2+} release channel in the basolateral region,⁵² and the RyR is activated by Ca^{2+} itself, which results in Ca^{2+} -induced Ca^{2+} release. Therefore, Ca^{2+} waves in pancreatic acinar cells are initiated by InsP3 in the trigger zone, but then propagate across the cell by RyR-mediated Ca^{2+} -induced Ca^{2+} release.^{20,53} Other epithelia, including cholangiocytes, do not express the RyR.²⁵ However, this cell type expresses low levels of InsP3Rs in the basolateral region.²⁵ Because Ca^{2+} can coactivate the InsP3R,⁵⁴ Ca^{2+} waves in cholangiocytes likely propagate by a modified form of Ca^{2+} -induced Ca^{2+} release in which Ca^{2+} released from the apical trigger zone sensitizes basolateral InsP3Rs and thus allows Ca^{2+} waves to form on that basis. The mechanism of Ca^{2+} wave propagation in hepatocytes is less clear. Pharmacologic evidence using RyR inhibitors suggests that the spread of Ca^{2+} waves across hepatocytes depends upon serial activation of apical InsP3Rs, and then basolateral RyRs,¹⁷ similar to what occurs in pancreatic acinar cells.²⁰ However, RT-PCR studies suggest that hepatocytes lack RyRs.⁸ Recently, though, a truncated but functional form of the RyR was detected in hepatocytes.⁵⁵ The subcellular distribution of this novel RyR variant and its role in the formation of Ca^{2+} waves has yet to be determined.

Ca^{2+} signals regulate a number of important processes in the liver, and the spatial location of the signal within the cell determines its specificity. For example, Ca^{2+} signals within the nucleus have different effects on gene transcription than signals within the cytosol,² Ca^{2+} signals generated near mitochondria are more effective in inducing apoptosis,³ and pericanalicular signals are thought to play a role in directing canalicular motility.⁵⁶ The spatial information contained within Ca^{2+} waves is thought to play an essential role in epithelial secretion. Studies in pancreatic acinar cells have shown that the apical-to-basal progression of Ca^{2+} waves serially activates ion channels on the apical, and then basolateral membrane.^{6,7} The result of this coordinated series of events is to direct fluid and electrolyte secretion into the lumen.^{6,7} The apical accumulation of InsP3Rs observed in most polarized epithelia also permits the local Ca^{2+} concentration in the apical region to reach as high as 5–10 $\mu\text{mol/L}$.³² This Ca^{2+} concentration would be toxic elsewhere in the cell but is required to activate synaptotagmins, and thus is necessary for targeting and fusion of vesicles to the apical plasma membrane.⁵⁷ Therefore, apical localization of InsP3Rs is important not only for fluid and electrolyte secretion, but for regulated insertion of transporters into the plasma membrane and for exocytosis as well. What would happen to secretion without this subcellular organization? A clue to this may be found in the little skate, *Leucoraja erinacea*. Hepatocytes in this primitive elasmobranch express a single isoform of the InsP3R, which is distributed uniformly throughout the cytosol.⁵⁸ As a result, Ca^{2+} signals begin at random locations in these cells and no coordinated Ca^{2+} waves are observed.⁵⁸ Moreover, the rate of bile secretion is nearly 2 orders of magnitude slower in skate liver than in mammalian liver.⁵⁸ Another clue to the importance of apical InsP3Rs may be found in studies of cholangiocytes from cholestatic patients and in animal models of cholestasis. After bile duct ligation, rat cholangiocytes lose apical InsP3Rs, and this is associated with selective loss of Ca^{2+} -mediated ductular bicarbonate secretion.⁵⁹ Similarly, expression of apical InsP3Rs is decreased or absent in cholangiocytes from patients with primary biliary cirrhosis, sclerosing cholangitis, common bile duct

obstruction, and biliary atresia, suggesting that this may represent a final common pathway for the development of cholestatic disorders.⁵⁹ Further work will be needed to identify the factors that localize apical InsP3Rs to lipid microdomains in hepatocytes, and to demonstrate the importance of this level of subcellular organization for bile secretion in health and disease.

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Abbreviations used in this paper

ACh	acetylcholine
Ca _i ²⁺	Cytosolic Ca ²⁺
ER	endoplasmic reticulum
F ₀	initial fluorescence
InsP3R	inositol 1,4,5-trisphosphate receptor
MβCD	methyl-beta-cyclodextrin
RyR	ryanodine receptor

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Figure 1.

The type II InsP3R is concentrated in the pericanalicular region of hepatocytes. Confocal immunofluorescence of rat liver shows that the type II InsP3 receptor (*blue*) colocalizes with submembraneous actin (*red*) rather than with the apical plasma membrane protein MRP2 (*green*) in rat hepatocytes.

Figure 2.

The endoplasmic reticulum is distributed throughout the cell in isolated hepatocyte couplets. (A) Isolated rat hepatocyte couplet labeled with the ER membrane dye ER-Tracker visualized by 2-photon microscopy shows that the ER is distributed throughout the apical and basolateral region. (B) Confocal image of an isolated rat hepatocyte couplet loaded with the low-affinity calcium dye mag-fluo-4, which selectively labels ER Ca²⁺ stores. This shows that ER Ca²⁺ stores also are distributed throughout the apical and basolateral region.

Figure 3.

mβCD depletes cholesterol and redistributes caveolin in isolated rat hepatocyte couplets. Isolated rat hepatocyte couplets were double-labeled with the cholesterol dye filipin (*green*) and immunofluorescent staining of caveolin (*red*). Filipin was visualized by 2-photon microscopy while caveolin was visualized simultaneously by confocal microscopy. (A) Under control conditions filipin staining is present in a punctuate pattern throughout each hepatocyte, while caveolin is localized to the plasma membrane and is most concentrated along the canalicular membrane. (B) After treatment with *mβCD* there is little residual filipin staining, and caveolin staining redistributes along the entire plasma membrane.

Results are representative of what was observed in 5 couplets under each condition. (C) Rat liver section double-labeled for caveolin-1 (*green*) and the actin stain phalloidin (*red*) examined by confocal immunofluorescence. Phalloidin staining is specific for filamentous actin, which outlines the plasma membrane in hepatocytes. The staining is most intense along the canalicular membrane, where actin is most concentrated. Areas of colocalization (*yellow*) in the merged image confirm that caveolin is most concentrated along the canalicular membrane of hepatocytes under normal conditions.

Figure 4.

The type II InsP3R is in a cholesterol-dependent fraction of the ER. Membranes were collected from isolated rat hepatocytes, and then separated by density gradient into 10 fractions. (A) Distribution of types I and II InsP3R, along with calreticulin, MRP2, and the Na⁺/K⁺-ATPase. The type II InsP3R is in fractions 6–8 under control conditions but spreads into fractions 9–10 after cells have been treated with mβCD (5 mmol/L) for 30 minutes. The distribution of the type I InsP3R (fractions 7–10) is not affected by treatment with mβCD. Calreticulin is used to identify ER fractions, while MRP2 and Na⁺/K⁺-ATPase are markers for the apical and basolateral plasma membrane, respectively. (B) Distribution of type II InsP3R, along with SERCA 2b and caveolin-1. The type II InsP3R is in fractions 6–8 under control conditions but spreads into fractions 9–10 after cells have been treated with mβCD (5 mmol/L) for 30 minutes. SERCA 2b is used to identify ER fractions, while caveolin is a marker for plasma membrane lipid rafts. Note that caveolin is in (plasma membrane) fractions 3–4 under control conditions but spreads into fraction 5 after treatment with mβCD.

Figure 5.

The type II InsP3R redistributes to the basolateral region after cholesterol depletion. Confocal fluorescence image of an isolated rat hepatocyte couplet labeled with antibody against type II InsP3R receptor (*green*), plus Alexa 647 phalloidin (*red*) to label actin, which is most concentrated along the apical membrane. The type II InsP3R is concentrated in the apical region of hepatocytes under control conditions. After treatment with m β CD, the InsP3R is more diffusely distributed throughout the couplet. The InsP3R receptor was labeled with the same antibody used for immunoblots. No InsP3R labeling was detected in cells stained with secondary antibody alone (not shown).

Figure 6.

Quantification of the redistribution of the type II InsP3R. (A) Line scan analysis was performed to measure fluorescence intensity along the (red) line crossing each couplet (*top panels*). Fluorescence intensity from InsP3R staining (*green tracing*) and actin staining (*red tracing*) along the scan line is graphically depicted in the *bottom panels*. (B) Apical/basolateral fluorescence intensity ratio reveals that basolateral fluorescence labeling of type II InsP3R increases significantly relative to apical fluorescence after treatment with mβCD (* $P < .0001$; $n = 15$ couplets). Fluorescence ratios were used so that measurements would be independent of individual fluorescence intensity values, to account for variations in labeling among cells.

Figure 7.

Cholesterol depletion slows Ca^{2+} waves in hepatocytes. (A) Serial confocal images of an isolated rat hepatocyte couplet loaded with the Ca^{2+} dye fluo-4 and then stimulated with vasopressin (10 nmol/L). Fluorescence was monitored in the apical (A) and basolateral (B) regions. Region of interest is outlined in yellow; serial images demonstrate that a Ca^{2+} wave begins in the apical region of the cell (white arrow in middle panel). This and subsequent Ca^{2+} images are pseudocolored according to the scale shown at bottom. (B) Graphical representation of the fluorescence increase detected in the apical and basolateral region. Note the time lag between the apical increase in Ca^{2+} and the basolateral increase; the Ca^{2+} wave speed is calculated by dividing the distance between the apical and basolateral reference points by the time lag. The rise time is the time required for the Ca^{2+} signal to increase from 20% to 80% of its maximum value. (C) The Ca^{2+} wave speed is slowed in couplets treated with m β CD (* $P < 10^{-6}$, $n = 15$ couplets in each group). (D) The rise time is prolonged in hepatocytes treated with m β CD (* $P < .01$, $n = 15$).

Figure 8.

Cholesterol depletion slows Ca^{2+} waves in pancreatic acinar cells. (A) Transmission image and serial confocal images of a pancreatic acinus loaded with fluo-4 and then stimulated with ACh ($1 \mu\text{mol/L}$). Fluorescence was monitored in the apical (A) and basolateral (B) region of individual acinar cells. Region of interest in a particular cell is outlined in red; serial images demonstrate that a Ca^{2+} wave begins in the apical region of the cell. (B) Graphical representation of the fluorescence increase detected in the apical and basolateral region of a typical acinar cell. As in hepatocytes, the apical increase in Ca^{2+} precedes the basolateral increase, reflecting an apical-to-basal Ca^{2+} wave. (C) Pretreatment with mβCD significantly slows Ca^{2+} wave speed ($*P < .001$, $n = 15$). This effect reverses in acinar cells that have been reloaded with cholesterol ($n = 8$). (D) The rise time of Ca^{2+} signals increases in cells treated with mβCD ($*P < .05$, $n = 15$). This effect reverses in acinar cells that have been reloaded with cholesterol ($n = 8$).

Figure 9. Cholesterol depletion does not affect Ca²⁺ signals in SKHep1 cells. (A) Confocal immunofluorescence shows that the InsP3R does not redistribute in SKHep1 cells after treatment with mβCD. SKHep1 cells were used as a control of mβCD treatment because they are a nonpolarized liver cell line. Cells were triple labeled to reveal actin (red) and the type II (green) and type III (blue) InsP3R; these are the 2 InsP3R isoforms expressed in this cell line. (B) Serial confocal images of an SKHep1 cell loaded with fluo-4 and then stimulated with vasopressin (100 nmol/L). (C) The rise time of vasopressin-induced Ca²⁺ signals is not prolonged by treatment with mβCD ($P = .52$, $n = 15$).