Effect of Ca\(^{2+}\) agonists in the perfused liver: determination via laser scanning confocal microscopy

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Motoyama, Kentaro, Irene E. Karl, M. Wayne Flye, Dale F. Osborne, and Richard S. Hotchkiss. Effect of Ca\(^{2+}\) agonists in the perfused liver: determination via laser scanning confocal microscopy. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R575–R585, 1999.—Ca\(^{2+}\) is a critical intracellular second messenger. A few studies have examined Ca\(^{2+}\) signaling in whole organs. The amplitude and frequency of Ca\(^{2+}\) oscillations encode important cellular information. Using laser scanning confocal microscopy in the perfused rat liver, we investigated the effect of various Ca\(^{2+}\) agonists that act at distinct mechanistic sites on Ca\(^{2+}\) signaling. Lower doses of AVP (0.2–2 nM) caused a single Ca\(^{2+}\) wave that originated in the pericentral vein region and spread centrifugally to the perportal area. Lower doses of AVP (0.2–2 nM) caused multiple Ca\(^{2+}\) waves and Ca\(^{2+}\) oscillations. Perfusion with ATP (1.4–17.5 µM) caused rapid transient elevations in intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) occurring after ATP. Also in contrast to AVP, there was no specific anatomic location within the hepatic lobule that was more susceptible to ATP. Thapsigargin and cyclopiazonic acid did not cause a Ca\(^{2+}\) wave but rather produced a uniform and fairly simultaneous increase in [Ca\(^{2+}\)]i in all hepatocytes in the lobule. Perfusion with 14 µM ryanodine produced a single transient spike in [Ca\(^{2+}\)]i in a small number (<2%) of hepatocytes. Dantrolene, an inhibitor of Ca\(^{2+}\) release, reduced the increased [Ca\(^{2+}\)]i occurring after AVP. Insight into the mechanism of action of these Ca\(^{2+}\)-active compounds on Ca\(^{2+}\) signaling in the intact liver is provided.

hepatocytes: ryanodine; thapsigargin; dantrolene; vasopressin

CALCIUM IS A critical intracellular second messenger that regulates a myriad of cell functions, including hormone secretion, cell motility, muscle contraction, and gene transcription (2). In addition to the role of Ca\(^{2+}\) as a regulator of normal physiological processes, Ca\(^{2+}\) is an important modulator of pathological processes, including inflammation (11) (via its effects on cytokine production and lymphocyte activation) and programmed cell death (4), i.e., apoptosis. Ca\(^{2+}\) homeostasis in an intact organ is more likely to reflect the actual in vivo state, because conditions of cell culture and isolation of cells cause large differences in cell Ca\(^{2+}\) handling (29). Also, the effects of cell-to-cell interaction via gap junctions and paracrine factors are present in intact organs but absent in isolated cells. Furthermore, studies indicate that the amplitude and frequency of Ca\(^{2+}\) oscillations encode important information in the single cell and likely in the whole organ as well (8, 15, 21). Recent advances in fluorescent microscopy have made it possible to examine intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]i) and Ca\(^{2+}\) signaling in the intact perfused organ. Currently, only two studies by Robb-Gaspers and Thomas (21) and Nathanson et al. (15) have been performed in the perfused liver and only a few [Ca\(^{2+}\)]i agonists were examined.

The purpose of this study was to examine Ca\(^{2+}\) signaling in the intact liver using a variety of drugs that act at distinct mechanistic sites. We used laser scanning confocal microscopy to examine the effect of drugs that mobilize Ca\(^{2+}\): either directly, by a receptor-dependent mechanism, or indirectly, by inhibiting reuptake of Ca\(^{2+}\) into the endoplasmic reticulum. Arginine vasopressin (AVP) working through the V1 receptor activates phospholipase C, inducing inositol 1,4,5-triphosphate (IP3) production that diffuses to the IP3 receptor on the endoplasmic reticulum and releases the IP3-sensitive Ca\(^{2+}\) pool (15, 21, 22). In addition to the IP3-sensitive Ca\(^{2+}\) store, a ryanodine-sensitive Ca\(^{2+}\) store is also present in many cell types, including hepatocytes (1). Although ryanodine binding and a ryanodine-sensitive Ca\(^{2+}\) pool have been demonstrated in hepatocytes, the effects of ryanodine have not been investigated in the intact liver. Recently, ATP has been shown to increase [Ca\(^{2+}\)]i in isolated hepatocytes (23). ATP acts to mobilize Ca\(^{2+}\) via a G protein-coupled P2 purinoceptor. Evidence suggests that ATP and other nucleotides may be secreted into the extracellular space and provide a novel paracrine signaling pathway, but its effects in the intact liver are unknown (23). We also examined the effect of drugs that increase [Ca\(^{2+}\)]i by inhibiting reuptake of Ca\(^{2+}\) into the endoplasmic reticulum. Thapsigargin, a tumor-promoting sesquiterpene lactone, increases [Ca\(^{2+}\)]i by a receptor-independent mechanism (28). Thapsigargin irreversibly inhibits the endoplasmic reticulum Ca\(^{2+}\)-ATPase pump, leading to depletion of intracellular Ca\(^{2+}\) stores. Cyclopiazonic acid (CPA) is a tetracyclic acid metabolite of Aspergillus and Penicillium that binds stoichiometrically to sarcoplasmic and endoplasmic reticulum Ca\(^{2+}\)-ATPase, causing inhibition of the pump (4). Previously no studies on the effect of CPA on hepatocytes have been conducted. In contrast to the Ca\(^{2+}\) agonists,
dantrolene reduces [Ca$$^{2+}$$], by inhibiting release of Ca$$^{2+}$$ from the sarcoplasmic and endoplasmic reticulum (5). Although the exact mechanism of action of dantrolene is not known, dantrolene is reported to cause a dose-dependent decrease in binding of ryanodine to hepatic microsomes. Novel observations on the effects of these drugs on Ca$$^{2+}$$ signaling (Ca$$^{2+}$$ waves and Ca$$^{2+}$$ oscillations) in the intact liver and insight into Ca$$^{2+}$$ regulation are the subjects of this report.

EXPERIMENTAL PROCEDURES

Isolated perfused rat liver technique. Male Sprague-Dawley rats (175–300 g; Harlan, Indianapolis, IN) were anesthetized with halothane, and the liver was perfused in situ via the hepatic portal vein with Earle’s balanced salt solution (E-6132; Sigma, St. Louis, MO) according to the methods of Robb-Gaspers and Thomas (21). In brief, the median lobe of the liver was studied and other lobes were tied off and excised. Perfusion flow rate was 4 ml·min$$^{-1}$$·g$$^{-1}$$ wet wt. Additional compounds added to Earle’s perfusion media were (in mM) 1.0 lactate, 0.1 pyruvate, and 120 µM sulfobromophthalein (BSP). A competitive anion transport inhibitor that inhibits cellular extrusion of the fluorescent Ca$$^{2+}$$ indicator, BSP, does not affect liver viability or alter Ca$$^{2+}$$ oscillations when added to isolated hepatocytes (21). Media were filtered with a 0.22-µm filter, and 5% adult bovine serum (Hydnone, Logan, UT) was added. Initially, the liver was perfused in a nonrecirculating mode for 10 min before it was changed to a recirculating system for an additional 15 min of recovery after surgery.

Loading of the liver with indo 1-AM. One milligram of the ratiometric fluorescent dye indo 1 acetoxymethyl ester (indo 1-AM; Molecular Probes, Eugene, OR) was dissolved in 25 µl of a 20% solution of Pluronic F-127 in DMSO (Molecular Probes). We diluted this 25-µl solution with an additional 50 µl of DMSO, and we slowly infused the resultant indo 1-AM solution into 250 ml of Earle’s balanced salt solution while stirring. The liver then was perfused with the fresh solution containing indo 1-AM in a recirculating mode for 45 min. After confirmation of successful loading of the liver with the Ca$$^{2+}$$ indicator via fluorescence microscopy, liver perfusion was changed to a nonrecirculating mode using buffer that did not contain indo 1-AM to wash out excess indo 1-AM. Temperature of the liver was maintained at 34°C, and media were oxygenated with 95% oxygen-5% carbon dioxide and perfused via a peristaltic pump (Cole-Palmer, Vernon Hills, IL).

Fluorescence imaging of [Ca$$^{2+}$$]. Images were obtained using the Nikon RCM 8000 laser scanning confocal microscope system developed by Tsien (31) and as described previously (11). For measurement of [Ca$$^{2+}$$], the indo 1-loaded liver was excited at 351 nM with an argon ion laser (Coherent, Palo Alto, CA). Indo 1 is a dual-emission dye, and when it is excited at 351 nM, two wavelengths are emitted, i.e., a wavelength at 405 nM corresponding to indo 1 bound to Ca$$^{2+}$$ and a wavelength at 480 nM corresponding to free indo 1. The emission bands at 400–440 nM and those >440 nM are separated by dichroic mirrors and long-path filters and detected by parallel photomultipliers. The analog signals are corrected for background and shading, digitized, ratioed, and displayed in color as high-resolution spatial concentration images. Images were stored on a Panasonic optomagnetic disc recorder. For calibration of the indo 1 [Ca$$^{2+}$$] determinations, the intensities of known test solutions of Ca$$^{2+}$$ (Molecular Probes) varying from 0 to 1 mM were determined using the free acid indicator pentapotassium indo 1 (5 µM; Molecular Probes). These Ca$$^{2+}$$ standards also contained K$$^{+}$$ and phosphates to reflect the intracellular milieu. The ratiometric calculations were done using the method described by Grynkiewicz et al. (10).

The liver was positioned on a petri dish with a 45 × 50-mm glass coverslip secured into the base using aquarium sealant (Perfecto Manufacturing, Noblesville, IN). Although the system scanned full-field images of 30 frames/s, the ratiometric images were obtained by averaging sixty-four successive frames to improve image quality (signal-to-noise ratio). A Nikon fluor ×20/0.9 numerical aperture water immersion objective was used for microscopy. The size of the image was 355 × 266 µm and was displayed as 512 × 480 pixels. The optical slice thickness with the ×20 objective was 8.7 µm, with a working distance of 800 µm.

Auto fluorescence. An important point that was critical in accurately determining [Ca$$^{2+}$$] was use of proper acquisition parameters (laser power, neutral density filters, pinhole size, photomultiplier gain) to minimize cell auto fluorescence. Before loading with indo 1-AM, a background image of each liver was obtained. Using the lowest power settings on the argon laser and with the addition of neutral density filters, the background image of the hepatocytes exhibited minimal autofluorescence (Fig. 1, top). Small regions of bright auto-

Fig. 1. Top: background image of liver ($\times$200) obtained before loading with indo 1 acetoxymethyl ester (indo 1-AM). Bottom: image of liver ($\times$200) obtained after loading of indo 1-AM. Identical acquisition parameters were used to obtain background image. Note marked increase in fluorescence in indo 1-AM-loaded liver. Intracellular free Ca$$^{2+}$$ concentration ([Ca$$^{2+}$$]) in nanomoles is determined by reference to color scale bar at right. Same color scale bar is used for all subsequent images. Bottom effect of MnCl$_2$ (100 µM) to quench fluorescence of indo 1. Ten minutes after beginning of infusion of MnCl$_2$, fluorescent image (middle) had markedly diminished and only background auto fluorescence (top) remained.
cence (~0.2–3 µm in diameter) were seen in cells abutting the hepatic plates. (Fig. 1, top). Suematsu et al. (26, 27) noted similar findings in liver undergoing ultraviolet illumination. These investigators reported that these areas were due to vitamin A autofluorescence located in the Ito cells (27). After obtaining background and shading images, we held constant all acquisition parameters affecting fluorescent image intensity (i.e., laser power, neutral density filters, photomultiplier tube gain, and pinhole size), and all observed changes in fluorescent intensity were likely to reflect changes due to the Ca\(^{2+}\) indicator indo 1-AM (Fig. 1, middle). The liver was observed frequently during loading in each experiment, and the pattern of autofluorescence did not change during the course of the experiment and was consistent throughout the liver. To ensure that the autofluorescent pattern of the liver had not changed during the course of the experiments, 100 µM MnCl\(_2\) in Ca\(^{2+}\)-free Hanks' balanced salt solution was infused at the end of selected experiments (n = 8; Fig. 1, bottom). Mn\(^{2+}\) rapidly enters the cell, presumably by Ca\(^{2+}\) channels, and binds to indo 1 with a 20-fold greater affinity than that of Ca\(^{2+}\), causing complete quenching of the fluorescence of indo 1 (24). Significantly, no evidence of phototoxicity or photobleaching was observed during the experiment and images could be obtained almost continuously for 3–5 min without any changes in indo 1 fluorescence.

Pharmacologic protocols. After using fluorescent microscopy to observe that the liver had been loaded with the Ca\(^{2+}\) indicator indo 1-AM, we infused Ca\(^{2+}\)-active drugs via a separate syringe pump into a 20-ml mixing chamber located 10 cm upstream of the liver. This solution entered the liver at 4 ml·min\(^{-1}\)·g\(^{-1}\) wet wt via the portal vein, as stated previously. To avoid potential confounding effects of drugs on intracellular Ca\(^{2+}\) stores, we administered each drug to naive livers, i.e., livers that had not been treated with any other Ca\(^{2+}\) agonist. In addition, the effect of pretreatment of livers with ryanodine, dantrolene, thapsigargin, or CPA on Ca\(^{2+}\) immobilization due to AVP administration was determined.

Statistical analysis. Values expressed are means ± SE. Statistical significance was accepted at the 95% confidence limit.

RESULTS

Basal [Ca\(^{2+}\)]\(_i\) in perfused livers. In addition to obtaining background and shading images on every liver before loading indo 1-AM, we also obtained images of indo 1-loaded livers before drug infusion. After successful loading of indo 1-AM, three to six images (i.e., baseline [Ca\(^{2+}\)]\(_i\)) of different regions of the liver were obtained before evaluation of drug effects. There were no regional differences in the baseline [Ca\(^{2+}\)]\(_i\) in the livers. We collected and stored [Ca\(^{2+}\)]\(_i\) images of the liver, 30 s before the Ca\(^{2+}\) agonist drugs reached the liver. Images were obtained and stored every 4–5 s. In the series of images presented, the baseline image of [Ca\(^{2+}\)]\(_i\) immediately before drug effect is included.

AVP-induced Ca\(^{2+}\) waves and oscillations. The dose-response effect of AVP on [Ca\(^{2+}\)]\(_i\) and Ca\(^{2+}\) mobilization was investigated in the perfused liver. Infusion of a high dose of AVP (20 nM) caused a rapid increase in [Ca\(^{2+}\)]\(_i\) starting at the central vein and moving out centrifugally through the entire field toward the periporal area (Fig. 2). The averaged baseline [Ca\(^{2+}\)]\(_i\) over an entire field of hepatocytes was 153.3 ± 3.1 nM (n = 16
livers) before AVP and increased to a maximal value of 419.5 ± 22.8 nM (n = 16 livers) after 30 s to 2 min of infusion of the drug. The maximum amplitude of the [Ca\textsuperscript{2+}] increase was consistent for all hepatocytes in the lobule; Kupffer cells did not respond to AVP. After stopping of AVP and washing out of the residual drug, [Ca\textsuperscript{2+}] slowly decreased; at 11 min the decrease was ~60%, and at 20 min [Ca\textsuperscript{2+}] was back to pre-AVP levels (Fig. 2). Specifically, [Ca\textsuperscript{2+}] in hepatocytes farthest from the central hepatic vein returned to normal first, whereas [Ca\textsuperscript{2+}] in hepatocytes located around the central vein remained elevated for the longest time (Fig. 2). Although Ca\textsuperscript{2+} wave propagation was usually uniform and homogeneous throughout the lobule, in 3 of the 16 livers treated with AVP, multiple initiation sites of the wave were observed (Fig. 3). The speed of propagation of the Ca\textsuperscript{2+} wave across the hepatic lobule in 16 livers was variable and ranged between 8 and 40 µm/s.

After [Ca\textsuperscript{2+}] in hepatocytes in the lobule had returned to baseline values after 20 nM AVP, the liver was perfused with 2 nM AVP. This [Ca\textsuperscript{2+}] response of the liver was slower compared with the higher concentration of 20 nM AVP. Again, the [Ca\textsuperscript{2+}] rise occurred first in the pericentral region of the hepatic lobule and spread outward centrifugally. However, the centrifugal spread of the [Ca\textsuperscript{2+}] wave was slower, and frequently [Ca\textsuperscript{2+}] had decreased back to baseline values in the pericentral hepatocytes while the [Ca\textsuperscript{2+}] wave was still slowly advancing outward in the peripheral part of the lobule. Also, the [Ca\textsuperscript{2+}] wave failed to progress throughout the entire field of hepatocytes in some instances. In these cases, the Ca\textsuperscript{2+} wave would oscillate such that the wave would begin anew in the pericentral region and spread outward, with the sequence repeating itself after [Ca\textsuperscript{2+}] had returned to baseline in the pericentral hepatocytes of the lobule.

In a subset of livers, infusion of 2.0 nM AVP after a high dose of AVP (20 nM) did not cause a Ca\textsuperscript{2+} wave. Instead of a Ca\textsuperscript{2+} wave, individual hepatocytes or groups of several hepatocytes demonstrated focal oscillations in [Ca\textsuperscript{2+}]. The oscillatory frequency of [Ca\textsuperscript{2+}] in individual hepatocytes was similar although not in the exact same phase (Fig. 4). Not all hepatocytes in the lobule demonstrated an increase in [Ca\textsuperscript{2+}] to the 2.0 nM dose of AVP, and the oscillations in [Ca\textsuperscript{2+}] were restricted to focal groups of cells.

After washout of the 2.0 nM AVP, the liver was allowed to recover and [Ca\textsuperscript{2+}] returned to baseline (141.7 ± 8.0 nM). In three livers, 0.2 nM AVP was infused and no Ca\textsuperscript{2+} wave was observed. Instead, individual hepatocytes in the lobule demonstrated focal oscillatory increases similar to the pattern in Fig. 4. [Ca\textsuperscript{2+}] returned to normal baseline values between the oscillatory spikes. The high-dose AVP (20 nM) was repeated after the AVP dose-response curve in three perfused livers. Although there was a prompt increase in [Ca\textsuperscript{2+}] in the hepatocytes of the livers, [Ca\textsuperscript{2+}] was not as high. Interestingly, shortly after beginning infusion of either 20 or 2.0 nM AVP, a small steady increase

![Fig. 3. [Ca\textsuperscript{2+}] wave due to AVP. Infusion of 20 nM AVP for ~1–2 min caused a [Ca\textsuperscript{2+}] wave that originated at multiple sites in the lobule. Central vein is present at bottom right of each image. This is an example of multiple foci of Ca\textsuperscript{2+} spikes.](image)
in the basal level of [Ca\(^{2+}\)] was observed in most hepatocytes in the lobule (Fig. 4). This increase (~20-40 nM) in [Ca\(^{2+}\)] preceded the initiation of the Ca\(^{2+}\) wave.

To determine if subsequent doses of AVP caused a blunting of the Ca\(^{2+}\) response, the order of administration of AVP was reversed; i.e., AVP was added in increasing concentrations starting at 2 nM, followed by 5 nM, and ending at 20 nM. Resting [Ca\(^{2+}\)] was 141.1 ± 8.0 nM, and infusion of 2 nM AVP caused [Ca\(^{2+}\)] to increase by 72.3 ± 16 to 213.4 ± 23.4 nM (n = 8). After [Ca\(^{2+}\)], had returned toward baseline (150.6 ± 17.9), 5 nM AVP was added and [Ca\(^{2+}\)], increased by 35.0 ± 7.2 to 199.9 ± 25.1 nM. After treatment with 2 nM AVP, [Ca\(^{2+}\)] returned slowly toward baseline, i.e., to 161.2 ± 23.1 nM. Addition of 20 nM AVP caused [Ca\(^{2+}\)] to increase slightly by 21.1 ± 4.0 to 187.3 ± 26.7 nM. The increase in [Ca\(^{2+}\)] of 72.3 ± 16.1 nM due to the initial dose of 2 nM AVP was statistically greater than the 21.4 ± 4.0 nM increase in [Ca\(^{2+}\)] occurring with the highest dose of 20 nM AVP (P < 0.05), thus demonstrating a blunting of effect of AVP. Note that the increase in [Ca\(^{2+}\)], with AVP represents the averaged value for all the hepatocytes in the field of view. Individual hepatocytes had a more marked increase in [Ca\(^{2+}\)] to AVP, whereas some hepatocytes had no response to the drug.

ATP. ATP was infused for 3 min for each concentration, starting at 1.4 µM, followed by 7 µM, and ending at 17.5 µM (n = 6 livers). At 1.4 and 7 µM, ATP caused an increase in [Ca\(^{2+}\)] in ~5–10% of hepatocytes scattered in a random fashion throughout the hepatic lobule (Fig. 5A). The increase in Ca\(^{2+}\) was not sustained and lasted <30 s of the 3-min infusion of ATP. Infusion of 17.5 µM ATP resulted in recruitment of additional numbers of hepatocytes responding with increased [Ca\(^{2+}\)] (Fig. 5B). Note that the hepatocyte response was not a graded response; i.e., the hepatocytes that did respond all exhibited a maximal rise in [Ca\(^{2+}\)]. In contrast to AVP, there was no preferential response of hepatocytes in the pericentral vein region. Also in contrast to AVP, the increase in [Ca\(^{2+}\)] was always brief, with [Ca\(^{2+}\)] returning to baseline in ~30 s despite continuation of the ATP infusion. Only on rare instances did ATP cause oscillations in [Ca\(^{2+}\)]. A bolus injection of ATP (~150 µM) caused the entire hepatic lobule to increase [Ca\(^{2+}\)] to >300 nM, and [Ca\(^{2+}\)] returned quickly to baseline in <1 min.

Thapsigargin. In four livers, 1 µM thapsigargin was infused over a 10-min time period immediately after loading of hepatocytes with the fluorescent Ca\(^{2+}\) indicator. Baseline [Ca\(^{2+}\)] was 151.7 ± 11.4 nM (n = 4). In contrast to the action of AVP, in which the Ca\(^{2+}\) wave began near the central vein, thapsigargin caused a steady progressive increase in [Ca\(^{2+}\)] in all hepatocytes at the same time but no Ca\(^{2+}\) oscillations were observed (Fig. 6). The maximal increase in hepatocyte [Ca\(^{2+}\)] due to thapsigargin was >1,896 nM. After washout of thapsigargin, [Ca\(^{2+}\)] decreased slowly and progressively over the next 15–20 min. In some regions of the hepatic lobule, [Ca\(^{2+}\)] returned to normal, and in others, [Ca\(^{2+}\)] remained elevated in selected hepatocytes located around the central vein.

In the livers in which a high dose (450 nM) of AVP was infused after thapsigargin, there was a marked blunting of the [Ca\(^{2+}\)] response. Lower doses of AVP failed to produce a response. AVP either failed to increase [Ca\(^{2+}\)], or the increase in [Ca\(^{2+}\)], that occurred was small and not sustained over time.

CPA. CPA was infused at 2.1 µM, and [Ca\(^{2+}\)] increased from 146.9 ± 12.9 to 250.4 ± 17.7 nM (n = 7). The increase in [Ca\(^{2+}\)] occurred slowly and progressively over the entire ~7-min infusion period. Similar to thapsigargin but in contrast to AVP, the increase in [Ca\(^{2+}\)] occurred uniformly throughout the entire hepatic lobule and was not more intense in the pericentral vein region. After infusion of CPA was stopped, [Ca\(^{2+}\)] returned to baseline. Prior treatment with CPA did not blunt the hepatocyte [Ca\(^{2+}\)] response to AVP. No oscillations in [Ca\(^{2+}\)] were observed in any livers treated with CPA.
Ryanodine. In six livers, 14 µM ryanodine was infused for a 10-min time period after loading with indo 1-AM. Average baseline value of [Ca\textsuperscript{2+}] was 136.8 ± 8.8 nM. Ryanodine had only a minimal effect on hepatocyte [Ca\textsuperscript{2+}]. The effect of ryanodine consisted of a nonsustained Ca\textsuperscript{2+} spike in single cells, averaging approximately one to three cells per microscopic field. Ryanodine did not cause Ca\textsuperscript{2+} waves or oscillations. When AVP (450 nM) was infused after washout of ryanodine, a rapid sustained maximal Ca\textsuperscript{2+} response to AVP was demonstrated and pretreatment with ryanodine failed to blunt this effect.

Dantrolene. Ten micromolar dantrolene, which inhibits release of Ca\textsuperscript{2+} from sarcoplasmic and endoplasmic reticulum, was effective in causing a significant, rapid decrease in elevated [Ca\textsuperscript{2+}] that occurred in hepatocytes after treatment with high-dose AVP (450 nM; Fig. 7). The decrease in [Ca\textsuperscript{2+}] occurred almost immediately after the beginning of perfusion with dantrolene. However, pretreatment with 10 µM dantrolene did not prevent the maximal increase in [Ca\textsuperscript{2+}] that occurred during administration of high-dose AVP (450 nM). In one of two livers, dantrolene also was effective in decreasing the elevated [Ca\textsuperscript{2+}] that occurred after treatment with 1 µM thapsigargin. The decrease in [Ca\textsuperscript{2+}] that occurred when dantrolene was infused into thapsigargin-treated hepatocytes was not uniform; i.e., only some hepatocytes responded by decreasing [Ca\textsuperscript{2+}] whereas other cells had no decrease.

Dantrolene has been reported to increase autofluorescence in certain cells (17), although this effect is not uniform in all cell types (5, 16). Dantrolene also has

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**Fig. 5. Effect of ATP.** A: 7.5 µM ATP caused a brief, rapid, localized increase in [Ca\textsuperscript{2+}] that did not propagate across hepatic lobule. Each image (×200 magnification) was acquired 3 s apart. B: 17.5 µM ATP caused a similar brief rapid increase in [Ca\textsuperscript{2+}] but occurred in more hepatocytes.
been reported to decrease the fluorescence of fura 2 in bullfrog neurons. We performed a series of studies to determine if the putative decrease in \([\text{Ca}^{2+}]_i\) occurring with dantrolene was due to an unrecognized compounding effect. A solution of 10 µM dantrolene was examined using the argon ion laser and 351 nM ultraviolet illumination, with identical settings used for the perfused liver studies. The dantrolene solution had no apparent autofluorescence properties at these settings. Similarly, infusion of dantrolene into the liver before loading of indo 1-AM had no effect on the background fluorescence of the image.

To determine if dantrolene influenced the fluorescent properties of indo 1, the free acid indicator pentapotassium indo 1 (10 µM) was added to \([\text{Ca}^{2+}]_i\) standard solutions (150 and 602 nM), reflecting the intracellular ionic environments (Molecular Probes). Addition of 5–10 µM dantrolene to these \([\text{Ca}^{2+}]_i\) solutions had no apparent effect on the indo emission spectra examined via laser scanning confocal microscopy.

Evaluation of intracellular compartmentation of indo 1 and liver autofluorescence with MnCl\(_2\). Mn\(^{2+}\) rapidly enters the cell, presumably via \([\text{Ca}^{2+}]_i\) channels (24). Mn\(^{2+}\) binds to indo 1 with a 20-fold greater affinity than that of \([\text{Ca}^{2+}]_i\) and thereby causes complete quenching of the fluorescence of indo 1 free acid. Mn\(^{2+}\) has no effect on the \([\text{Ca}^{2+}]_i\)-insensitive fluorescence of the nondeesterified indo 1-AM (12, 24). MnCl\(_2\) has been used in \([\text{Ca}^{2+}]_i\) experiments for several purposes, including 1) detection of cell autofluorescence, 2) evaluation of fluorescence from incompletely hydrolyzed indo 1-AM, and 3) determination of the contribution of indo 1 fluorescence arising from the noncytosol, i.e., intracellular organelles (21, 24). Although Mn\(^{2+}\) will cause rapid quenching of indo fluorescence, MnCl\(_2\) was not used in the current studies.
ing of cytosolic indo 1, Mn$^{2+}$ may also cause quenching of indo 1 that is located in intracellular organelles, i.e., mitochondria, nucleus, and endoplasmic reticulum.

To evaluate the three points listed above, 100 nM MnCl$_2$ in Ca$^{2+}$-free HEPES buffer was infused at the end of seven experiments. Infusion of MnCl$_2$ caused a rapid loss of fluorescence over an ~8- to 15-min time period (Fig. 1, bottom). At the end of the 15-min time period, fluorescent images had returned to baseline, i.e., before initiation of indo 1 loading. There was no evidence of residual fluorescence in the cytosol or in any organelles.

**DISCUSSION**

The present study evaluating the effect of various Ca$^{2+}$ agonists on Ca$^{2+}$ signaling in the intact liver provides insight into the regulation of intracellular Ca$^{2+}$ in the whole organ. One of the most interesting and intriguing findings was the unique and specific effect of the individual drugs on Ca$^{2+}$ mobilization. For example, both AVP and ATP mobilize Ca$^{2+}$ from IP$_3$-sensitive Ca$^{2+}$ stores (14, 21, 22). AVP acts on V$_{1a}$ receptors to activate phospholipase C, thereby inducing IP$_3$ production. ATP mobilizes Ca$^{2+}$ via a G protein-coupled P$_2$ purinoceptor, which also increases IP$_3$ (23). Despite both drugs causing increases in IP$_3$, the [Ca$^{2+}$]$_i$ response in the liver differed. AVP but not ATP caused a preferential increase in [Ca$^{2+}$]$_i$ in pericentrally located hepatocytes. A 2.0 nM concentration of AVP caused oscillations in [Ca$^{2+}$]$_i$, whereas oscillations were infrequently observed with ATP. An organized Ca$^{2+}$ wave that spread across the entire lobule was observed routinely with AVP, whereas ATP did not cause a Ca$^{2+}$ wave. ATP did cause focal regions of hepatocytes to increase [Ca$^{2+}$]$_i$, but the area did not involve the entire lobule. Also, the increase in [Ca$^{2+}$]$_i$ due to ATP was of much longer duration (min) than that due to ATP (s). Why ATP, which also generates IP$_3$, did not cause a Ca$^{2+}$ wave is also not known.

In liver, hepatocytes are tightly coupled by gap junctions (22, 23). Tordjmann and associates (30) found that second messengers and [Ca$^{2+}$]$_i$ elevation in one hepatocyte cannot trigger Ca$^{2+}$ responses in abutting cells, suggesting that diffusion across gap junctions (although required for coordination) is not sufficient by itself for the propagation of intercellular Ca$^{2+}$ waves and the presence of hormone at the cell surface of each hepatocyte is required for cell-to-cell Ca$^{2+}$ signal propagation (30). They also reported that functional differences between adjacent connected hepatocytes may be the basic mechanism for intercellular propagation of Ca$^{2+}$ waves (30).

Studies by Nathanson et al. (15) have shown an increased number of V$_{1a}$ receptors in hepatocytes located in the pericentral vein region. Therefore, this unique receptor distribution is the most likely rationale for the preferential [Ca$^{2+}$]$_i$ response to AVP of hepatocytes in the pericentral region (15). Robb-Gaspers and Thomas (21), however, noted that perfusion with low doses of vasopressin induced oscillations of hepatocyte Ca$^{2+}$ that were coordinated across entire lobules of the liver by propagation of Ca$^{2+}$ waves along the hepatic plates. At the subcellular level, these periodic Ca$^{2+}$ waves initiated from the sinusoidal domain of cells within the perportal region and spread to the pericentral region. At higher vasopressin doses, a single Ca$^{2+}$ wave was observed and the direction of Ca$^{2+}$ wave propagation was reversed, being initiated in the pericentral region and spreading to the perportal region (21).

Although hepatocytes express ATP receptors (P$_2$) that on stimulation can evoke [Ca$^{2+}$]$_i$ signals, the distribution of ATP receptors within the lobule has not been determined (23). ATP is secreted from hepatocytes into the extracellular space, and it is believed that ATP serves as a novel paracrine signaling pathway (23). Our study in the intact liver confirms the ability of extracellularly administered ATP to cause increased [Ca$^{2+}$]$_i$ in neighboring hepatocytes located throughout the lobule. The increase in [Ca$^{2+}$]$_i$ due to ATP was of much longer duration than that due to ATP (s). This difference in the duration of the [Ca$^{2+}$]$_i$ spike with ATP versus AVP may have important cellular consequences. Because studies indicate that both the amplitude and duration of the agonist-induced increase in [Ca$^{2+}$]$_i$, are important in directing the particular cellular response, differences in the [Ca$^{2+}$]$_i$ signal due to ATP and ATP are partly responsible for the unique effects of the two hormones. Further substantiating the important role of Ca$^{2+}$ oscillations is work by De Koninck and Schulman (8) demonstrating that the frequency of Ca$^{2+}$ oscillations directly encoded the activity of the Ca$^{2+}$- and calmodulin-dependent protein kinase II.

An interesting observation noted with AVP but not as readily apparent with ATP was a small increase in basal [Ca$^{2+}$]$_i$ (~20–40 nM) occurring 30–90 s after addition of AVP (Fig. 4). This increase in [Ca$^{2+}$]$_i$ immediately preceded the initiation of the Ca$^{2+}$ wave and appeared to be present uniformly in all hepatocytes in the lobule. It may be that this increase in [Ca$^{2+}$]$_i$ is a triggering event for initiation of the Ca$^{2+}$ wave, i.e., a Ca$^{2+}$-induced Ca$^{2+}$ wave. This finding has not been previously noted in other studies of the perfused liver (15, 21). Although hepatocytes did not exhibit a basal increase with ATP, they did demonstrate an “all or none” response to the drug. The larger dose of ATP caused recruitment of additional hepatocytes, all of which had a maximal Ca$^{2+}$ response (Fig. 5). There was no increase in [Ca$^{2+}$]$_i$ in the majority of hepatocytes. Therefore, this Ca$^{2+}$ response is similar to neuronal firing in that it is an all or none response.

In the present report, the order of administration of AVP was important in determining the Ca$^{2+}$ response. If high-dose AVP (20 nM) was administered first, the entire hepatic lobule had a brisk sustained rise in [Ca$^{2+}$]$_i$. However, if 20 nM AVP was administered after the 2 and 5 nM AVP doses, the increase in [Ca$^{2+}$]$_i$ was less robust and the Ca$^{2+}$ wave progressed more slowly and on occasion was not sustained despite continued infusion of AVP. One possibility for the differential response is that intracellular Ca$^{2+}$ stores had been partially depleted by the initial dose of AVP, resulting in
a diminished Ca\(^{2+}\) response with subsequent doses of AVP (6). Interestingly, in experiments in which the \(\beta\)-agonist isoproterenol was administered either before or after AVP, the increase in \([\text{Ca}^{2+}]_i\) was greater when the drug was given after AVP (unpublished data), suggesting that intracellular Ca\(^{2+}\) stores may not have been depleted by AVP. Another possible explanation for the decreased response to AVP could be internalization of the \(V_\text{i}\) receptors producing receptor desensitization (16).

Lower concentrations of AVP (2.0 mM) caused oscillations in hepatocyte \([\text{Ca}^{2+}]_i\) (Fig. 4), as reported by Robb-Gaspers and Thomas (21). Both the amplitude (~200 mM) and frequency (~1 cycle/60 s) of the \([\text{Ca}^{2+}]_i\) oscillations reported herein are in close agreement with those reported previously (21). Also consistent with their findings, the individual frequency of the oscillations is similar for individual hepatocytes located at different sites in the hepatic lobule, but the various frequencies are phase shifted, i.e., occur at different time points (Fig. 4) (21).

Ca\(^{2+}\) agonists: Thapsigargin and CPA. Thapsigargin and CPA, which increase \([\text{Ca}^{2+}]_i\) via a receptor and an IP\(_3\)-independent mechanism, did not cause either Ca\(^{2+}\) oscillations or a Ca\(^{2+}\) wave, and the increase in \([\text{Ca}^{2+}]_i\), occurred uniformly and simultaneously throughout the lobule. The absence of the Ca\(^{2+}\) wave with thapsigargin and CPA versus the presence of the Ca\(^{2+}\) wave with AVP may be due to the fact that neither thapsigargin nor CPA generate IP\(_3\), whereas AVP does. Although the exact mechanism of the Ca\(^{2+}\) wave is not known, evidence suggests that diffusion of IP\(_3\) through gap junctions may be involved (2, 6). Although thapsigargin and CPA failed to induce Ca\(^{2+}\) waves, several other findings should be noted. The magnitude of the increase in \([\text{Ca}^{2+}]_i\) with thapsigargin was greater than that with either CPA or AVP, which may be due to a dose-response effect; i.e., higher doses of CPA or AVP may cause an equivalent elevation in \([\text{Ca}^{2+}]_i\). Alternatively, thapsigargin may be more efficient either in emptying Ca\(^{2+}\) stores or in emptying additional non-AVP-responsive Ca\(^{2+}\) stores. Thapsigargin almost completely blocked the ability of AVP to increase \([\text{Ca}^{2+}]_i\).

The different effect of both drugs may be due to the fact that thapsigargin is an irreversible inhibitor, whereas CPA is reversible. Other mechanisms for the increased \([\text{Ca}^{2+}]_i\) may be involved. Because all three drugs cause depletion of endoplasmic reticulum Ca\(^{2+}\) stores, activation of plasma membrane Ca\(^{2+}\) channels and increased influx of extracellular Ca\(^{2+}\) may result (19, 20).

Ryanodine. Several groups have identified separate ryanodine and IP\(_3\) binding sites in hepatic microsomes (13). Studies also show that ryanodine is capable of mobilizing Ca\(^{2+}\) in the hepatocyte from microsomal stores that are distinct from those that can be regulated by IP\(_3\) (1, 13, 16). In our study, 10 \(\mu\)M ryanodine had minimal effects in the isolated liver, and in only a few isolated hepatocytes (2–3 per high-powered field) was there a nonsustained increase in \([\text{Ca}^{2+}]_i\). The dose of ryanodine in the present study (10 \(\mu\)M) was chosen because a dose-response curve with ryanodine on hepatocyte microsomal preparations demonstrated that 10 \(\mu\)M ryanodine caused 80% of the maximum release of Ca\(^{2+}\) (13). It is possible that higher concentrations of ryanodine might have produced a greater effect on \([\text{Ca}^{2+}]_i\).

Bazotte et al. (1), using 200 \(\mu\)M ryanodine in isolated hepatocytes, found a 24% increase in \([\text{Ca}^{2+}]_i\). Nathanson et al. (16) reported that 50 \(\mu\)M ryanodine caused a 15 ± 5 nM increase in hepatocyte \([\text{Ca}^{2+}]_i\), from 113 ± 19 to 128 ± 19 nM. In contrast to thapsigargin, ryanodine failed to prevent the Ca\(^{2+}\) response to AVP in the perfused liver. Studies by Nathanson et al. (16) in isolated hepatocytes also demonstrated that 50 \(\mu\)M ryanodine did not inhibit the increase in \([\text{Ca}^{2+}]_i\), induced by AVP.

Dantrolene. Although the molecular mechanism of action of dantrolene is unknown, it has been shown to strongly inhibit ryanodine but not IP\(_3\) binding to hepatic microsomal preparations. In addition to inhibition of ryanodine binding, there is one report that dantrolene and its analogs block dihydropyridine receptors. If confirmed, this may be another possible mechanism for its Ca\(^{2+}\) antagonist effect (9). Dantrolene also inhibits Ca\(^{2+}\)-induced Ca\(^{2+}\) release in diverse types of cells, including neurons and smooth and skeletal muscle cells (5, 18). Pretreatment with dantrolene failed to prevent the hepatocyte Ca\(^{2+}\) response to our dose of 20 nM AVP, and it did not affect either the speed of the wave or the peak \([\text{Ca}^{2+}]_i\). Nathanson and associates (16) noted that 10 \(\mu\)M dantrolene reduced the peak cytosolic Ca\(^{2+}\) response to 40 nM AVP and ANG II by 50% in isolated hepatocytes. A possible explanation for the different findings in the present study versus the report of Nathanson et al. (16) is the dose of AVP that was used in the two studies. Furthermore, in the current study, dantrolene was used as a pretreatment only and was not present in solution when the AVP was added. However, dantrolene was effective in decreasing the increased \([\text{Ca}^{2+}]_i\) that occurred with AVP. Within 1–2 min after beginning dantrolene, the increased \([\text{Ca}^{2+}]_i\) that resulted from AVP had decreased significantly.

Using fluorescent indicators, dantrolene has been shown to have a number of effects that may complicate measurement of \([\text{Ca}^{2+}]_i\), i.e., intrinsic drug fluorescence, increase in cell autofluorescence, and decrease in fura 2 fluorescence (17). To address the above issues regarding the compounding effects of dantrolene, we conducted a series of studies. Using the identical experimental parameters used for the perfused liver, we determined that dantrolene did not 1) exhibit intrinsic autofluorescence, 2) affect the fluorescent properties of indo 1, or 3) effect hepatocyte autofluorescence. These findings are in agreement with studies by Nathanson and associates (16), who reported that dantrolene had no effect on hepatocyte autofluorescence and that its inhibitory effects on Ca\(^{2+}\) signaling were not due to nonspecific effects.

Indo 1-AM intracellular compartmentation. Another potential problem of Ca\(^{2+}\) indicators is their transport from the cytosol into intracellular organelles such as the nucleus, endoplasmic reticulum, and mitochondria.
If the Ca\(^{2+}\) indicators are sequestered in these organelles, an inaccurate assessment of cytosolic free Ca\(^{2+}\) concentration, i.e., [Ca\(^{2+}\)], will result (12, 24). Ca\(^{2+}\) indicators may give a falsely high value for [Ca\(^{2+}\)] if localized in endoplasmic reticula or mitochondria. Although it may be difficult to determine if the Ca\(^{2+}\) indicator has been taken up by organelles, a heterogeneous appearance of the cell on microscopic examination may be one due (16). No heterogeneous appearance occurred in any of the livers in the current study. In three livers, nuclear labeling with indo 1 appeared to be present, although this finding may have represented a layer of cytoplasm overlying the nucleus. Three-dimensional reconstruction could be used to determine if the nuclei did in fact load indo 1.

Another method that has been used to evaluate organelle loading is examination of the effect of MnCl\(_2\) (12, 24). MnCl\(_2\) presumably entering via Ca\(^{2+}\) channels rapidly displaces Ca\(^{2+}\) from indo 1 and quenches fluorescence. It is presumed that indo 1 located within intracellular organelles will be less accessible to Mn\(^{2+}\) and therefore will not be as readily quenched, resulting in a heterogeneous appearance of the cell during MnCl\(_2\) infusion. In the present study, MnCl\(_2\) produced a rapid uniform and complete loss of fluorescence (excepting fluorescence due to presumed 1to cells) and occurred over ~10–15 min. There was no evidence of sequestration of indo 1 in organelles. This finding does not unequivocally prove that indo 1 was confined to the cytoplasm, because it is possible that ion channels on the intracellular organelles (e.g. endoplasmic reticulum, mitochondria, nucleus) were activated under the conditions of the study, thus enabling Mn\(^{2+}\) to rapidly enter these sites. Nevertheless, the results from studies with MnCl\(_2\) suggest, but do not prove, that indo 1 fluorescence in the perfused liver is confined to the cytoplasm. These findings are in contrast to MnCl\(_2\) studies in the indo 1-AM-perfused rat heart that indicate that >50% of the fluorescence due to indo 1 is noncytosolic (24). It is important to note, however, that if a part of the indo 1 was localized in intracellular organelles as well as the cytoplasm, the Ca\(^{2+}\) response to hormones would be significantly blunted and basal [Ca\(^{2+}\)] might be falsely elevated because of the higher intraorganelle Ca\(^{2+}\) concentration.

In summary, Ca\(^{2+}\) agonists have unique effects both on the site of Ca\(^{2+}\) mobilization within the liver lobule and the nature of the Ca\(^{2+}\) response, i.e., duration of increase in [Ca\(^{2+}\)], and probability of propagation of the Ca\(^{2+}\) wave. The different effects of AVP and ATP on Ca\(^{2+}\) homeostasis in the liver may be contributing to their diverse actions. Dantrolene, a Ca\(^{2+}\) antagonist that inhibits release of Ca\(^{2+}\) from endoplasmic reticulum, failed to blunt the increase in [Ca\(^{2+}\)], due to suprathreshold doses of AVP but did reduce the increased [Ca\(^{2+}\)], occurring after AVP.

Perspectives

A broad implication of the present study is that the liver and potentially other organs respond to different Ca\(^{2+}\) agonists in a unique fashion that is specific for each agent. The [Ca\(^{2+}\)] response to the different agonists varied in regard to the location of responding hepatocytes within the hepatic lobule, the duration of the response, and the ability of the response to propagate across the lobule. Additionally, the [Ca\(^{2+}\)] response to different concentrations of a specific agonist (AVP) varied from oscillatory waves (low concentration of AVP) to a more prolonged elevation lasting many minutes. Interestingly, the amplitude of the [Ca\(^{2+}\)] response of individual responding hepatocytes to many of the Ca\(^{2+}\) agonists (AVP, ATP, ryanodine) was similar. Either no increase in [Ca\(^{2+}\)] occurred, or a maximal response occurred. This study demonstrates the remarkable complexity of the Ca\(^{2+}\) response in the whole organ and individual cell. The broad range of organ and cellular Ca\(^{2+}\) response available demonstrate why Ca\(^{2+}\) is such a pivotal cellular second messenger. Finally, these detailed observations in the intact organ were made possible by recent advances in laser scanning confocal microscopy and water immersion microscope objectives. It is undoubtedly technically possible to perform similar measurements on Ca\(^{2+}\) in vivo, and such findings should follow.

We thank Larry D. Robb-Gaspers for patience and many helpful discussions concerning the methods of liver perfusion and Ca\(^{2+}\) indicator loading.

This work was supported by National Institute of General Medical Sciences Grant GM-44118, National Institute of Allergy and Infectious Diseases Grant AI-28480, and the Alan A. and Edith L. Wolff Foundation.

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Received 10 July 1998; accepted in final form 27 October 1998.

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