THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 264, No. 22, Issue of August 5, pp. 12859-12866, 1989
Printed in U.S.A.

Ca^{2+} Oscillations Induced by Hormonal Stimulation of Individual Fura-2-loaded Hepatocytes*

(Received for publication, December 7, 1988)

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Isolated rat hepatocytes were loaded with the Ca^{2+} indicator fura-2 to measure cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) in individual cells by digital ratio imaging microscopy. Stimulation with 0.1 nM vasopressin, 0.5 μM phenylephrine, or 0.5 μM ATP caused repetitive spikes of high $[Ca^{2+}]_i$ in a high percentage of cells, in agreement with Woods *et al.* (Woods, N. M., Cuthbertson, K. S. R., and Cobbold, P. H. (1986) *Nature* 319, 600–602), but unlike the results of Monck *et al.* (Monck, J. R., Reynolds, E. E., Thomas, A. P., and Williamson, J. R. (1988) *J. Biol. Chem.* 263, 4569–4575). Reduction in extracellular $[Ca^{2+}]$ decreased the frequency but not the amplitude of the spikes, suggesting that the spikes result from dumping of intracellular stores and that the entry of extracellular Ca^{2+} affects only the rate of replenishment of those stores. Membrane depolarization failed to elevate $[Ca^{2+}]_i$ and had an effect similar to removal of extracellular Ca^{2+} in decreasing the frequency of agonist-evoked $[Ca^{2+}]_i$ oscillations or inhibiting them altogether, arguing against any significant role for voltage-operated Ca^{2+} channels.

The concentration of cytosolic free Ca^{2+} is a key parameter in regulating many cellular processes (1, 2). In the liver, stimulants that activate glycogenolysis are known to increase the concentration of cytosolic Ca^{2+}, $[Ca^{2+}]_i$ (3). Woods *et al.* (4, 5) reported that in single isolated hepatocytes microinjected with aequorin, the increase in $[Ca^{2+}]_i$ due to agonist stimulation is a periodic train of spikes. Oscillations in extracellular Ca^{2+} concentration representing periodic releases of Ca^{2+} from hormone-stimulated perfused liver have also been observed (6). In addition, Capiod *et al.* (7) found in guinea pig hepatocytes that norepinephrine elicited cyclic fluctuations of K^+ conductance which could be due to Ca^{2+}-activated K^+ channels. $[Ca^{2+}]_i$ oscillations have also been observed in a wide variety of other cell types (see Refs. 8–10 for reviews). Their prevalence has led to speculations (4, 5, 8, 9) that repetitive $[Ca^{2+}]_i$ spiking may be a general mode of frequency-dependent coding of information roughly analogous to action potentials in excitable cells. However, oscillations are not observed under all conditions of stimulation, and even when oscillations are most prevalent, not every cell in the population participates. In contrast to the pioneering results of Woods *et al.* (4, 5) with aequorin in isolated hepatocytes, Monck *et al.* (12) were hardly able to detect any clear Ca^{2+} oscillations in nominally similar cells by using fura-2. Instead, the responses to vasopressin or phenylephrine consisted of a single spike slowly decaying to a plateau, with at most small ripples on the decaying phase. Monck *et al.* (12) speculated that fura-2 may contribute enough Ca^{2+} buffering to interfere with the feedback mechanisms generating Ca^{2+} oscillations. If so, this effect would suggest that variation of the buffering level might be an interesting way to probe those mechanisms. On the other hand, we have found (10) in the rat embryo fibroblast cell line REF52 that repetitive Ca^{2+} oscillations are readily observable with fura-2 if the cells are simultaneously depolarized and stimulated with mitogens, whereas with either stimulus alone the response consists merely of a single spike. This result prompted us to ask whether the oscillations seen by Woods *et al.* (4, 5) may have resulted from microinjection damage or whether fura-2 buffering of Ca^{2+} really prevents oscillations in hepatocytes. We now report that Ca^{2+} oscillations are induced by phenylephrine, vasopressin, and ATP in fura-2 loaded hepatocytes. Depolarization is not a prerequisite for these oscillations, but rather inhibits them. Moreover, spike frequency but not amplitude is lowered with decreasing extracellular Ca^{2+} concentrations.

EXPERIMENTAL PROCEDURES

Materials—Collagenase (grade II) and gramicidin D were obtained from Boehringer Mannheim. Bovine serum albumin, ATP, glucose, phenylephrine, vasopressin, and nifedipine were purchased from Sigma. Fura-2 acetoxymethyl ester (fura-2/AM)[1] and fura-2 were from Molecular Probes. Acridine orange, rhodamine 123, and Percoll were obtained from Aldrich, Eastman Kodak, and Pharmacia LKB Biotechnology Inc., respectively. The (+) and (−) enantiomers of 202-791 were a gift from Dr. Richard W. Tsien (Yale University). All other reagents were commercial products of the highest available grade of purity.

Preparation of Isolated Hepatocytes—Isolated hepatocytes were prepared by the method of Moldeus *et al.* (13) and the suspension was then centrifuged through a mixture of Ca^{2+}-free and Mg^{2+}-free Hank's solution and Percoll (final density 1.06 g/ml) to eliminate dead cells. Cell viability, judged by trypan blue exclusion, was more than 90%. It should be noted that imaging of fura-2 in single cells provided two further protections against contributions from damaged cells: leaky cells lost their fura-2 and were excluded from the analysis;

* This work was supported by a fellowship from the Science and Technology Agency of Japan (to T. K.), by National Institutes of Health Grants GM31004 and EY04372 (to R. Y. T.), and by a grant from the National Foundation for Cancer Research (to M. T. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: fura-2/AM, fura-2 penta(acetoxymethyl) ester; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Me_2SO, dimethyl sulfoxide; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

also, the cells were continually monitored by simultaneous infrared transmitted illumination viewed by an infrared video camera, and any showing a pathological appearance, for example surface blebs, were likewise omitted. The cells were incubated in modified Krebs-Henseleit buffer containing 12.5 mM HEPES (13), 20 mM glucose, and 1 mM methionine at 37 °C under 95% O_2, 5% CO_2 for 30 min, washed, and resuspended in another modified Krebs-Henseleit buffer containing 20 mM HEPES, 5.5 mM glucose, and no methionine (KH buffer) at 10^6 cells/ml, then stored in a refrigerator (7–8 °C) and used within 6 h.

Fura-2 Loading of Cells—An aliquot of hepatocytes was preincubated in KH buffer for 30–60 min at 37 °C under 95% O_2, 5% CO_2. Fura-2 loading was started by adding 1 ml of the hepatocyte suspension (5×10^4 cells/ml) to the mixture of fura-2/AM (2 μl of 1 mM solution in Me_2SO) and 1 μl of 25% Pluronic F127 in Me_2SO. Loading was carried out for 40 min at room temperature. After loading, intracellular fura-2 concentrations were 250–350 μM. These values were obtained by comparing the fluorescence of fura-2 released from the cells treated with 0.2% Triton X-100 with that of solutions of known concentrations of fura-2. At these loadings, the autofluorescence of unloaded cells was at most 10–20% of the signal from dye-loaded cells. Moreover, the 350:385 nm excitation ratios obtained from unloaded cells showed no change and no oscillations even when adjacent loaded cells in the same field were stimulated to oscillate by addition of agonists. Therefore changes in autofluorescence, for example due to reduced pyridine nucleotides, did not contribute to the fura-2 $[Ca^{2+}]_i$ signals.

Measurement of Intracellular Ca^{2+} in Individual Cells—Fura-2 loaded cells were adhered to glass coverslips lightly coated with polylysine. After 5 min, unattached cells were washed off with KH buffer. Then, 3 ml of the buffer was added and the plastic holders were put into a thermostated holder on the stage of a Zeiss IM-35 microscope. The temperature was kept at 32 °C because intracellular fluorescence of fura-2 decreased rapidly at 37 °C, probably by dye leakage or extrusion.

Fluorescence ratio images were obtained and calibrated as described elsewhere (10, 14–16). The images were taken every 5–10 s. The Ca^{2+} values were averages over spots of more than 6.25 μm diameter centered over the cells.

Intracellular Location of Fura-2—The quality of loading and degree of dye compartmentalization were assessed by comparison of the release of acridine orange, fura-2, lactate dehydrogenase (a cytoplasmic marker enzyme), and rhodamine 123 (15, 17, 18), at 5–100 μM digitonin. The total contents of these substances were assumed to be releasable by 0.2% Triton X-100. Isolated hepatocytes were incubated with 10 μM acridine orange, or 10 μg/ml rhodamine 123 for 30 min at 37 °C, or fura-2/AM and Pluronic F127 under the loading conditions described above. The cells were washed twice with Ca^{2+}-free KH buffer containing 1.0 mM EGTA and lysed by a 3-min exposure to Ca^{2+}-free KH buffer containing 1.0 mM EGTA with 5, 10, 20 μM, or 100 μM digitonin or 0.2% Triton X-100. After centrifugation, supernatants were obtained and assayed by fluorescence for content of acridine orange (500 nm excitation, 530 nm emission), rhodamine 123 (535 nm excitation, 580 nm emission), or fura-2 (342 nm excitation, 510 nm emission). Lactate dehydrogenase activity was measured as described previously (15).

Electrophysiological Measurements of Membrane Potential—Resting potentials of isolated hepatocytes were measured by direct microelectrode penetrations roughly following the method of Petzinger and Bigalke (19). Fine-tipped microelectrodes were pulled from aluminosilicate glass on a microprocessor-controlled puller with jet cooling (P-80, Sutter Instruments, San Rafael, CA) and were filled with 4 M potassium acetate, giving tip resistances of 70–100 megohms. The electrodes were advanced with a remote hydraulic micromanipulator (MO-102, Narishige Scientific Instruments, Tokyo). Potentials were recorded with an 8800 electrometer (Dagan Corp., Minneapolis, MN) and chart recorder. Cells were isolated, in some cases loaded with fura-2, adhered to the floor of the chamber, and superfused with normal KH buffer (5.9 mM K^+) as described above.

RESULTS

Quality of Fura-2 Loading—In $[Ca^{2+}]_i$ measurements using fura-2, incomplete hydrolysis of the ester fura-2/AM and compartmentalization of dye fluorescence can be troublesome (15, 17, 20–24). In pilot experiments we found significant variations between different lots of fura-2/AM, all from Molecular Probes. Analysis of four lots of fura-2/AM by reverse-phase high pressure liquid chromatography showed that some were contaminated by two extra components eluting just before and after authentic fura-2/AM but showing a similar absorbance spectrum. Fig. 1*a* compares the chromatograms of the best and worst lots. If the contaminants are assumed to have the same extinction coefficient at 254 nm as real fura-2/AM, the sum of the contaminants ranged from 1 to 20% of

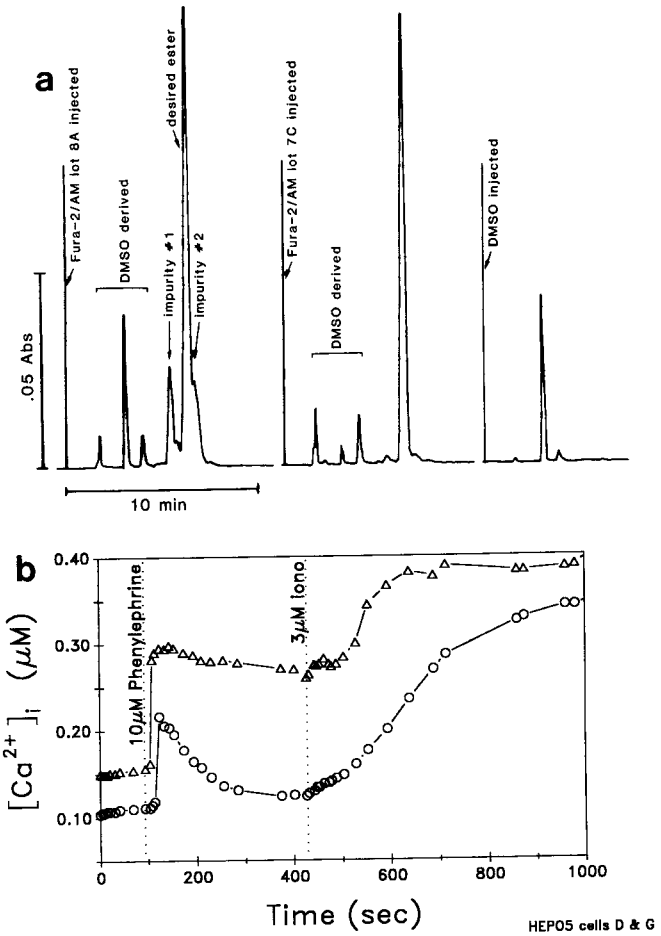

FIG. 1. **Impurities in fura-2/AM and their effect on hepatocyte $[Ca^{2+}]_i$ responses.** *a*, two batches of fura-2/AM (Molecular Probes) were compared by high performance liquid chromatography under isocratic conditions on a reverse phase octadecyl silica column (Supelcosil LC18, 250 × 4.6 mm diameter, Supelco, Inc., Bellefonte, PA), eluting with methanol/water 80:20 (v/v) at 1.0 ml/min. Absorbance was monitored at 254 nm. The first event marker shows the injection of 5 μl of 1 mM fura-2/AM lot 8A in Me_2SO (*DMSO*). After elution of three small peaks probably due to Me_2SO and its aging products, fura-2/AM appears closely flanked by two impurity peaks. This lot of fura-2/AM consistently gave low apparent $[Ca^{2+}]_i$ readings and small non-oscillatory responses to hormones (see *b*). The next injection was of 0.5 μM of 10 mM fura-2/AM lot 7C. The different pattern of Me_2SO-derived peaks is probably due to the lesser amount but greater age of Me_2SO injected. Note the relative absence of impurities immediately surrounding the main fura-2/AM peak. This batch was used for almost all the biological results reported in Fig. 2 and subsequent figures. The final injection was of 5 μl Me_2SO to verify which peaks are attributable to it. (*b*), response to phenylephrine and ionomycin of hepatocytes loaded with impure fura-2/AM. Hepatocytes were loaded by the usual procedure with lot 8A of fura-2/AM, whose chromatogram is shown in the first part of *a*. The cells were successively challenged with 10 μM phenylephrine then 3 μM ionomycin (*iono*) at the times indicated. Note the relatively low basal $[Ca^{2+}]_i$, small amplitude of response to phenylephrine, lack of oscillations, and weak sluggish response to a high dose of the Ca^{2+} ionophore.

the desired material. Batches with higher contents of the contaminants gave low 350:385 nm ratios in resting hepatocytes, which increased little upon hormone or ionophore stimulation and showed no oscillations (Fig. 1*b*), whereas purer lots gave higher, more agonist-responsive 350:385 nm ratios and were used for all the work reported below. Although we have not yet determined the structure of the contaminants or their mechanism of interference, it is worth noting that REF52 fibroblasts seemed much less sensitive than hepatocytes to the presence of the contaminants. Therefore a batch of dye that is satisfactory for one cell type may not be pure enough for another.

Completeness of fura-2/AM hydrolysis was checked in two ways. Addition of several micromolar ionomycin to loaded hepatocytes in KH medium gave 350:385 nm ratios more than 8 times that of fura-2 in EGTA even if the autofluorescence of the cells was not subtracted. If the autofluorescence was measured (by addition of Mn^{2+} to quench the fura-2) and deducted for each cell, the ratios exceeded 12 times that of fura-2 in EGTA. The quality of the fura-2 formed in hepatocytes was more quantitatively assessed by lysing them with 0.2% Triton X-100 in EGTA medium, removing cell debris by centrifugation, and titrating the supernatant with $K_2CaEGTA$ solution to known buffered levels of $[Ca^{2+}]$. The family of fluorescence spectra obtained (Fig. 2) were quite similar to those of reference solutions of fura-2 (25), not only at very low and very high $[Ca^{2+}]$, but also at intermediate values. The correspondence not only of spectral parameters but also of apparent Ca^{2+} dissociation constants between hepatocyte lysate and genuine fura-2 suggests that hydrolysis of fura-2/AM was functionally nearly complete; a small component of the fluorescence, perhaps 5–10%, did not respond until $[Ca^{2+}]$ exceeded several micromolar and may represent a bit of incompletely hydrolyzed ester.

Compartmentalization of fura-2 was assessed (15, 17) by comparison with those of acridine orange, rhodamine 123, and lactate dehydrogenase (Fig. 3). Digitonin at 20 μM released 87% of the intracellular fura-2, more than 95% of the total lactate dehydrogenase, but only 4% of the total rhodamine 123, which is accumulated selectively by intact mitochondria (18). These results suggest that this digitonin concentration permeabilized the plasma membrane but left mitochondria largely intact, and that fura-2 accumulation in mitochondria was very small. On the other hand, digitonin at 20 and 100 μM released 24 and 54% of the total acridine orange, which accumulates in acidic organelles. However, there is no difference between the amounts of fura-2 released by digitonin at 20 and 100 μM. Therefore, the 10–15% of fura-2 that seemed to be in organelles did not appear to be mainly in compartments that accumulated acridine orange.

Resting $[Ca^{2+}]_i$ and Transients Induced by Phenylephrine, Vasopressin, and ATP—Cytosolic $[Ca^{2+}]$ levels in individual resting hepatocytes formed a unimodal distribution (Fig. 4) with a mean of 247 nM and standard deviation of 53 nM (n = 255). This mean value is in reasonable agreement with or

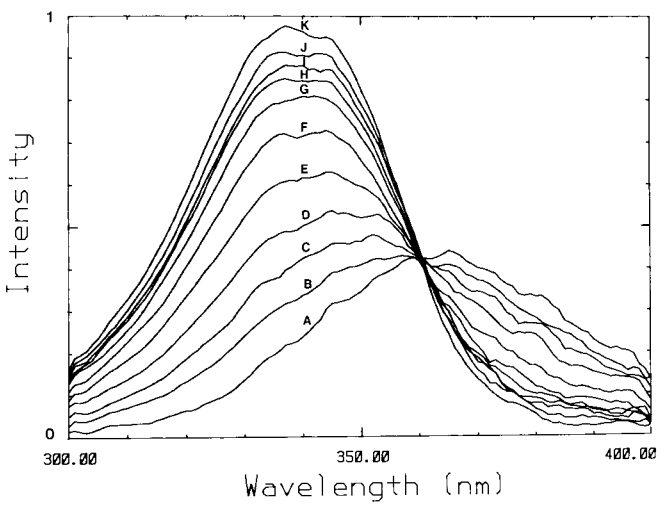

FIG. 2. **Ca^{2+} titration of fura-2 released from hepatocytes.** Cells were loaded by the standard protocol, lysed with 0.2% Triton X-100 in Ca^{2+}-free KH medium containing 1 mM EGTA at pH 7.05. Cell debris was removed by centrifugation, and the excitation spectrum of the supernatant was recorded at 11 free $[Ca^{2+}]$ levels attained by successive additions of 1.00 M $K_2CaEGTA$ (*traces A–J*), concluded by addition of 2 mM $CaCl_2$ for *trace K*. The free $[Ca^{2+}]$ values, calculated as in Ref. 25, were as follows: *trace A*, 0 nM; *trace B*, 19 nM; *trace C*, 38 nM; *trace D*, 76 nM; *trace E*, 151 nM; *trace F*, 303 nM; *trace G*, 630 nM; *trace H*, 1.26 μM; *trace I*, 1.87 μM; *trace J*, 22 μM; *trace K*, 1 mM. Emission was collected at 510 nm; bandwidths of excitation and emission were 9 nm. The autofluorescence spectrum of the lysate (measured by quenching the fura-2 in a separate aliquot of cell supernatant using excess $MnCl_2$) has been digitally subtracted from all the traces shown, which were then smoothed with the 9-point Savitsky-Golay algorithm supplied with the Spex Datamate fluorometer.

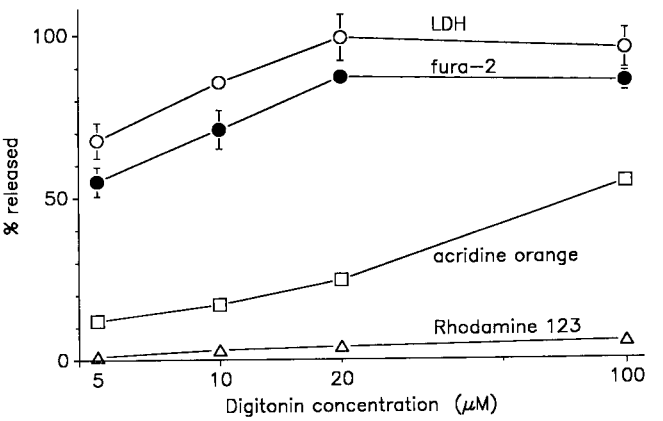

FIG. 3. **Subcellular location of fura-2 assessed by digitonin lysis.** Parallel batches of hepatocytes were lysed with increasing concentrations of digitonin in EGTA medium, centrifuged, and the supernatants assayed for lactate dehydrogenase (*LDH; open circles*), fura-2 (*solid circles*), acridine orange (*squares*), and rhodamine 123 (*triangles*), all expressed as percentages of that releasable by 0.2% Triton X-100. Note the logarithmic scale on digitonin concentrations. *Error bars* denote the standard errors of replicate measurements; where they are not visible, they are within the size of the symbol.

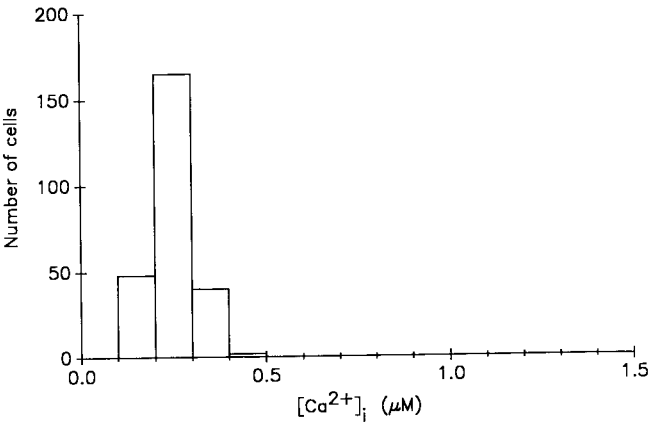

FIG. 4. **Population distribution of $[Ca^{2+}]_i$ values in resting hepatocytes.** The mean and standard deviation of the 255 cells analyzed were 0.247 and 0.053 μM, respectively.

slightly higher than previous measurements of hepatocytes (4, 26–29). Hormones elicited transient elevations, which showed considerable heterogeneity between neighboring cells. Some examples are shown in Fig. 5, *A* and *B*, in which a group of hepatocytes were exposed to 0.1 nM vasopressin. In cells A, B, and E, clear repetitive Ca^{2+} spikes were observed after a lag, though in cell A the timing was irregular. In 47 cells from five runs using cells from five rats, 0.1 nM vasopressin produced repetitive Ca^{2+} transients in 57% of the cells, a single Ca^{2+} spike (similar to cells C and D in Fig. 1) in 37%, and no response in 6% (*e.g.* cell F). In the oscillating cells, the average frequency was about one spike every 3 min. Fig. 6 is a histogram of the distribution of peak responses to 0.1 nM vasopressin, with the population mean near 700 nM $[Ca^{2+}]_i$.

Fig. 7 shows typical responses to 0.5 μM phenylephrine. In 84% of 198 cells from 27 runs derived from eight animals, repetitive Ca^{2+} transients began within 5 min after the addition of phenylephrine, as in cell A. However, in 15% a single Ca^{2+} spike or sustained Ca^{2+} rise, as in cell B, was observed. The oscillation frequency averaged just over one spike/min

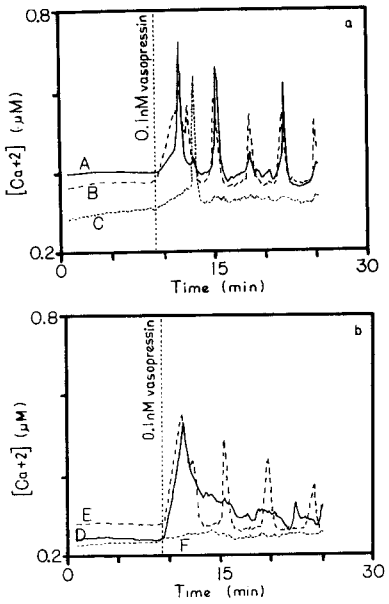

FIG. 5. **Various patterns of $[Ca^{2+}]_i$ response in individual vasopressin-stimulated hepatocytes.** The $[Ca^{2+}]_i$ transients for six different hepatocytes (*A–F*) simultaneously stimulated with 0.1 nM vasopressin are shown in two panels, (*a* and *b*), separated only for clarity.

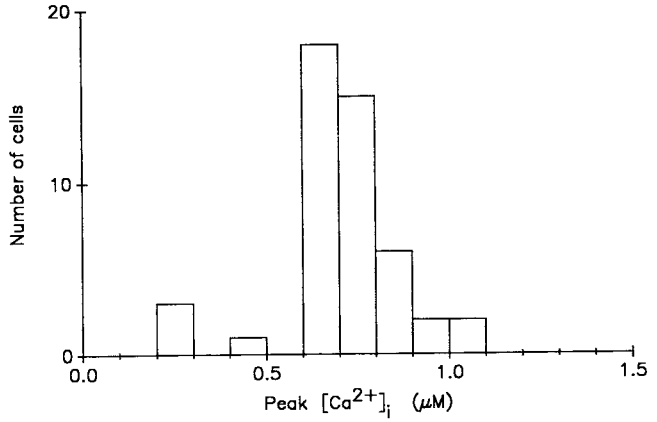

FIG. 6. **Population distribution of peak $[Ca^{2+}]_i$ attained in response to 0.1 nM vasopressin.** The mean and standard deviation of the 47 cells were 0.71 and 0.15 μM, respectively.

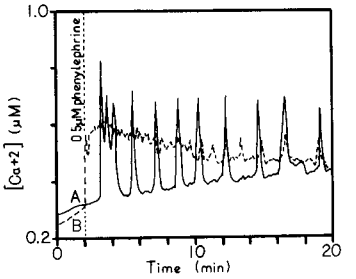

FIG. 7. **Various patterns of $[Ca^{2+}]_i$ responses in individual phenylephrine-stimulated hepatocytes.** Typical transients resulting from 0.5 μM phenylephrine are shown. In cell A (*solid line*), repetitive spikes began promptly, whereas in cell B (*dashed line*), a step-like rise to a sustained plateau was observed, although small oscillations began about 8 min after agonist addition.

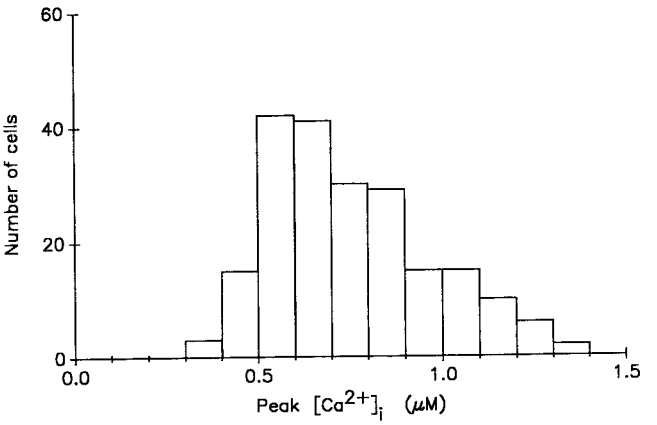

FIG. 8. **Population distribution of peak $[Ca^{2+}]_i$ attained in response to 0.5 μM phenylephrine.** The mean and standard deviation of the 208 cells were 0.75 and 0.17 μM, respectively.

TABLE I

Average parameters of agonist-induced Ca^{2+} oscillations

The highest oscillation frequency and amplitude elicited by 0.1 nM vasopressin and 0.5 μM phenylephrine were determined for each individual cell and the means and standard deviations calculated. A cell was counted as responsive if it produced at least one transient of at least 80 nM amplitude. It was deemed oscillatory if it produced at least two separate spikes of that amplitude within a single period of agonist exposure. The amplitude was defined as the change in $[Ca^{2+}]_i$ from the trough to the peak of the oscillation. The frequency was the number of full oscillations per minute.

Agonist	Cells treated	Responsive cells	Oscillating cells	Oscillation amplitude	Oscillation frequency
		%		*nM*	min^{-1}
Vasopressin	47	94	57	307 ± 112	0.36 ± 0.16
Phenylephrine	198	99	81	288 ± 126	1.11 ± 0.63

at 0.5 μM phenylephrine and increased with agonist dose. In some cells an initial rise in free Ca^{2+} lasting 1–2 min was followed by transients as reported by Woods *et al.* (4, 5) whereas in other cells only a sustained rise was observed (data not shown). Histograms of peak $[Ca^{2+}]_i$ attained in response to vasopressin and phenylephrine (Fig. 8) show population means of around 700 nM, similar to that for vasopressin (Fig. 6). The incidence and properties of the oscillations are summarized in Table I. We also found repetitive Ca^{2+} transients in response to ATP (Fig. 9), though the oscillations were irregular.

Effect of K^+ Depolarization and Gramicidin on Ca^{2+} Transients—Direct microelectrode measurements of the resting

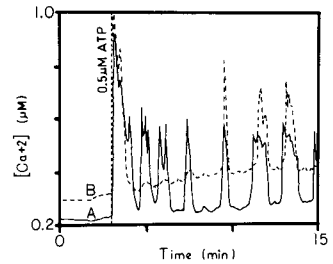

FIG. 9. **Typical patterns of $[Ca^{2+}]_i$ responses to 0.5 μM ATP.** Repetitive spikes were frequently observed, but they were relatively irregular in waveform and timing.

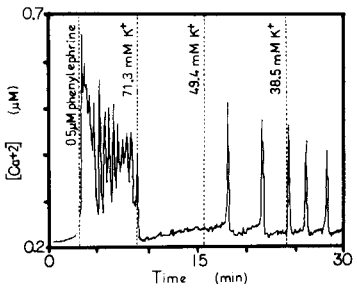

FIG. 10. **Effect of K^+ depolarization on $[Ca^{2+}]_i$ oscillations induced by phenylephrine.** Addition of 0.5 μM phenylephrine to hepatocytes in KH buffer containing 131 mM Na^+ and 5.9 mM K^+ caused $[Ca^{2+}]_i$ oscillations as usual. Then 50% of the 3.0-ml KH buffer in the chamber was removed and replaced with KCl-substituted KH buffer also containing 0.5 μM phenylephrine, to give a net K^+ concentration of 71 mM. Then, one third of the medium (1 ml) was exchanged with normal KH buffer containing agonist to give a net K^+ concentration of 49 mM. 1 ml of normal KH buffer was then added to give a final K^+ concentration of 38.5 mM, all while maintaining the phenylephrine concentration. Two typical response patterns are shown.

potential of hepatocytes isolated under our conditions were made to verify their viability and conformance to previous detailed electrophysiological investigations (19). When fine-tipped microelectrodes filled with 4 M potassium acetate were used, as advocated by Petzinger and Bigalke (19), resting potentials of −69 ± 9 mV (mean ± S.D., $n = 6$) were obtained within a few minutes after impalement, in good agreement with the previous results (−74 ± 8 mV) at the same extracellular K^+ concentration, 5.9 mM (19). Two of the six cells in the present experiments had been loaded with fura-2; their potentials, −65 and −85 mV, respectively, gave no sign that fura-2 had affected the resting potential significantly.

Isotonic substitutions of KCl for NaCl were used to see the effect of membrane depolarizations on Ca^{2+} oscillations. Fig. 10 shows an experiment in which Ca^{2+} oscillations were started with phenylephrine, and then 50% of the NaCl in KH was replaced by KCl ($[K^+]$ = 71.3 mM) in the continued presence of the agonist. This much K^+ should have depolarized the cells to −19 mV (19). $[Ca^{2+}]_i$ fell immediately, and the Ca^{2+} oscillations were slowed or stopped (Fig. 10). The effect of K^+ depolarization was reversible, since partial removal of the KCl increased the frequency or caused them to resume. In other experiments (not shown), the 137 mM extracellular Na^+ was first completely replaced by K^+, which should have depolarized the cells to −8 mV (19). This substitution had no effect on $[Ca^{2+}]_i$, but subsequent addition of 0.5 μM phenylephrine induced Ca^{2+} oscillations in a reduced percentage of cells and at a lower than normal frequency (0.1–0.4/min). The effects of partial substitution of KCl for NaCl are summarized in Table II. A few experiments with other substitutes for NaCl were equivocal: partial replacement of NaCl by choline somewhat slowed oscillations, whereas partial replacement by sucrose had little effect. We also examined the effect of gramicidin, which equilibrates intra- and extracellular Na^+ and K^+ and should also induce membrane depolarization. 270 nM gramicidin seemed to decrease the frequency of the Ca^{2+} oscillations in some cells (5 cells out of 12 stimulated with phenylephrine, and 2 cells out of 8 treated with vasopressin). Gramicidin concentrations of 1 μM or greater stopped the oscillations in almost all of the 61 cells examined, as in Fig. 11.

Some pharmacological antagonists and agonists of L-type voltage-operated Ca^{2+} channels were tested, but their effects were ambiguous. Nifedipine at 10 μM decreased oscillation amplitude but not frequency of phenylephrine-induced oscillations, whereas a reputedly more potent channel blocker, (−)202-791 (30, 31) at 1–5 μM decreased the frequency with little effect on amplitude. The (+) enantiomer of 202-791, which is reported to promote channel opening (30, 31), had a similar effect to the (−) enantiomer, so that the mechanism by which these drugs affect hepatocyte oscillations seems complex or nonspecific.

Effect of Extracellular Ca^{2+} Concentrations on Ca^{2+} Transients—Fig. 12 shows the effect on Ca^{2+} oscillations of changing from KH buffer to Ca^{2+}-free KH buffer containing 1 mM EGTA but still containing phenylephrine. In 7 of the 12 cells from two separate runs from two rats, Ca^{2+} oscillations produced by 0.5 μM agonist were stopped. In the other five cells, Ca^{2+} oscillations persisted even in Ca^{2+}-free medium though at a much reduced frequency (*e.g.* Fig. 12). Restoration of normal Ca^{2+} started the $[Ca^{2+}]_i$ oscillations again at normal frequency in most cells, although a few failed to resume. Interestingly, in all the cells that resumed spiking, the first such transient occurred 25–40 s after the readdition of extracellular Ca^{2+}, even though the period between subsequent spikes was much greater. It would seem that the protocol of Ca^{2+} deprivation then restoration resets the phase of the oscillation mechanism to a stage of incipient spike generation. When Ca^{2+} deprivation with 1 mM EGTA in Ca^{2+}-free KH was begun 1 min before addition of 1 μM phenylephrine, a small number of Ca^{2+} transients were produced for a short period (Fig. 13*A*), and the first peak heights were almost the same as those in KH buffer (Fig. 13*B*). In seven out of nine cells (*e.g.* cell A in Fig. 13*A*), only one spike or cluster of spikes could be observed after Ca^{2+} removal, whereas in the remaining two (*e.g.* cell B in Fig. 13*A*), further spikes could be observed as late as 10 min after Ca^{2+} withdrawal.

Fig. 14 shows the dependency of frequency of Ca^{2+} oscillations on intermediate extracellular Ca^{2+} concentrations. Ca^{2+} oscillations were stopped after the exchange of extracellular solution to Ca^{2+}-free KH buffer. Upon readdition of 0.5–1.0 mM Ca^{2+} the oscillations began again and the frequency increased depending on the concentrations of extracellular Ca^{2+}. Surprisingly, once the spikes restarted, their amplitude did not increase with increasing extracellular Ca^{2+}.

DISCUSSION

In our experiments clear Ca^{2+} oscillations were detected by fura-2 ratio imaging in more than 50% of single rat liver cells in response to phenylephrine, vasopressin, and ATP. In our hands, phenylephrine was the most effective inducer of Ca^{2+} oscillations. The high percentage of oscillating cells and their temporal waveform is in general agreement with the results of Woods *et al.* (4, 5), who used microinjected aequorin as Ca^{2+} detector, but contrasts with the paucity of sustained spiking reported by Monck *et al.* (12) using fura-2 imaging. Monck *et al.* (12) hypothesized that fura-2 somehow might

TABLE II

Effects of varying K^+ depolarizations on average parameters of Ca^{2+} oscillations induced by 0.5 μM phenylephrine

The percentate of oscillating cells and the mean and standard deviation of their amplitude and frequency were defined as in Table I. Membrane potential estimates are from Ref. 19 except for the value in 5.9 mM, the normal extracellular K^+ concentration in KH buffer, determined in this study.

	Membrane potential	Cells treated	Oscillating cells	Oscillation amplitude	Oscillation frequency
	mV		%	*nM*	min^{-1}
Extracellular K^+ (mM)					
5.9	−69	15	100	324 ± 149	1.43 ± 1.71
49.4	−30	15	80	271 ± 60	0.65 ± 0.31
71.3	−19	15	60	300 ± 141	0.54 ± 0.14
136.8	≥−8	6	50	327 ± 70	0.39 ± 0.14

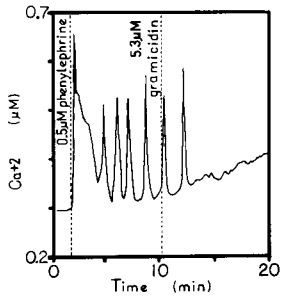

FIG. 11. **Effect of gramicidin on $[Ca^{2+}]_i$ oscillations induced by phenylephrine.** Oscillations were started with 0.5 μM phenylephrine, and then 5.3 μM of gramicidin D, a pore-forming antibiotic, was added where indicated.

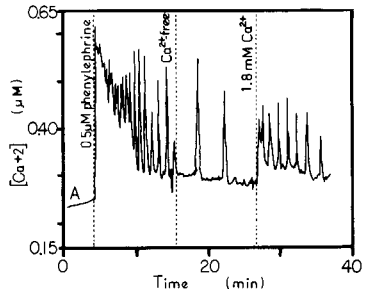

FIG. 12. **Effect of removing extracellular Ca^{2+} on $[Ca^{2+}]_i$ oscillations.** After oscillations were begun with 0.5 μM phenylephrine in normal KH buffer, the medium was changed to Ca^{2+}-free KH buffer containing 1 mM EGTA and 0.5 μM phenylephrine. Finally normal KH buffer with the same drug concentration was restored.

interfere with the feedback mechanisms generating oscillations or that aequorin was seeing localized $[Ca^{2+}]_i$ changes. However, our fura-2 loadings were higher (average 250–350 μM) than those of Monck *et al.* (12) (25–100 μM), a difference inconsistent with fura-2 interference with oscillations. Though considerable variations in dye brightness were seen from cell to cell, there was no obvious tendency for the brighter cells (presumably containing more dye) to oscillate differently from the dimmer cells. No dramatic spatial gradients of $[Ca^{2+}]_i$ were observed, though one cannot rule out the possibility that future technologies with yet better temporal and spatial resolution may detect such gradients. Gray (32) has noted that carbachol-stimulated parotid acinar cells tend to give $[Ca^{2+}]_i$ oscillations only below 30 °C. However, such temperature effects do not explain the difference in hepatocyte behavior, since Monck *et al.* (12) saw little difference in results between 28 and 37 °C, whereas our cells were at 32 °C. One difference in protocol is that we monitored $[Ca^{2+}]_i$ for tens of minutes, because in many cells the initial Ca^{2+} transient lasted several minutes, and spiking developed only later. Monck *et al.* (12) did not report observations lasting

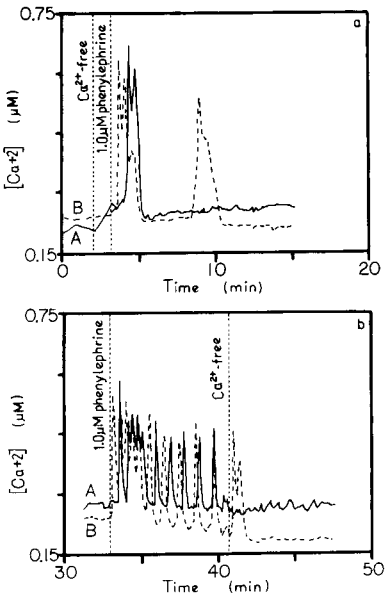

FIG. 13. **Comparison of removal of extracellular Ca^{2+} before *versus* after phenylephrine stimulation.** *a*, the medium was first changed to Ca^{2+}-free KH buffer containing 1 mM EGTA. 1 min later, 1 μM phenylephrine was added. After the $[Ca^{2+}]_i$ transient due to release from internal stores, the medium was changed to normal KH buffer not containing phenylephrine. After a 15-min recovery period, 1 μM phenylephrine was added again (*b*). Finally, the medium was replaced with Ca^{2+}-free KH buffer with 1 mM EGTA and 1 μM phenylephrine.

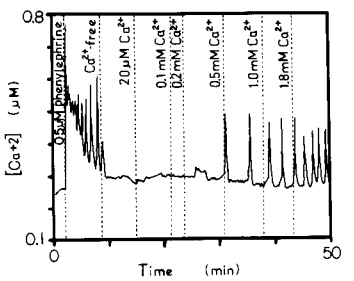

FIG. 14. **Effect of graded readmission of extracellular Ca^{2+} on repetitive spiking.** 0.5 μM phenylephrine was added and maintained at that level during the replacement of normal KH buffer by Ca^{2+}-free medium containing 1 mM EGTA, replacement by KH medium with 20 μM added Ca^{2+}, and then readdition of increasing concentrations of Ca^{2+}.

more than 4 min and may have missed subsequent oscillations. It should also be noted that in many experiments we also obtained small non-oscillatory responses (*e.g.* Fig. 1*b*) somewhat similar to those in Fig. 4, *A* and *C*, of their work, but in our hands such responses were correlated with batches of fura-2/AM containing impurities detectable by high per-

formance liquid chromatography (Fig. 1*a*). Scanlon *et al.* (20) have also noted that difficulties with fura-2/AM loading can be batch-dependent. In a preliminary report, Thomas *et al.* (33) have also observed rhythmic $[Ca^{2+}]_i$ oscillations in fura-2-loaded hepatocytes, with indications of spatial propagation.

Recently, we found (10) that in REF52 fibroblasts, depolarization-induced Ca^{2+} influx synergized with mitogenic hormones to generate Ca^{2+} oscillations. Neither mitogenic hormones nor depolarizations elicited rhythmic spikes in the absence of the other. Gramicidin, ouabain, and high extracellular K^+ all worked with mitogenic hormone to induce $[Ca^{2+}]_i$ oscillations, though K^+ was somewhat less consistently effective. In the present study, however, we find that K^+ depolarization of hepatocytes decreases the frequency of Ca^{2+} oscillations or stops them. The effects of gramicidin are less consistent, although high doses of gramicidin do stop the Ca^{2+} oscillations. Therefore, the effect of depolarizing agents and their relative efficacy on Ca^{2+} oscillations appear to be opposite in hepatocytes from that in REF52 cells. In addition, gramicidin and K^+ depolarization had no effect on the resting level of intracellular Ca^{2+} concentration in hepatocytes (data not shown). On the other hand, in REF52 cells (10) gramicidin or K^+ depolarization by themselves produced one or occasionally two Ca^{2+} spikes followed by a plateau elevation. We have considered the possibility that gramicidin seems to have little effect on hepatocyte $[Ca^{2+}]_i$ merely because the plasma membrane of isolated hepatocytes just after isolation may already be injured, perhaps due to the collagenase required to disperse the cells. If the membrane potential were decreased in injured cells and intracellular Na^+ concentration were already high, the addition of gramicidin would have no apparent effect. However, this explanation is unlikely for the following reasons: (*a*) Direct microelectrode measurements of our hepatocyte membrane potentials give readings averaging −69 mV, in good agreement with the most recent literature values (19). (*b*) High external K^+ is effective but inhibits $[Ca^{2+}]_i$ oscillations. (*c*) Measurements of intracellular free Na^+ with SBFI, a new fluorescent indicator for Na^+ (34) show values of 10–20 mM in these hepatocytes; these values do indeed rise as expected in response to gramicidin or ouabain. For these reasons, it is unlikely that $[Ca^{2+}]_i$ oscillations in hepatocytes are an artifact of membrane damage or depolarization.

At present there is much evidence that Ca^{2+}-dependent hormones initially raise cytosolic Ca^{2+} mainly by releasing Ca^{2+} from nonmitochondrial stores, which may be either components of endoplasmic reticulum (1–3, 35, 36) or newly described organelles called calciosomes (37). In previous studies on large populations of liver cells, either the perfused organ or cuvet suspensions of isolated hepatocytes, such mobilization was associated with an initial large efflux and loss of total cell calcium over about 5 min (38, 39). However, in low Ca^{2+} media (30 μM), the cytosolic Ca^{2+} rise declined rapidly. Therefore, the influx of Ca^{2+} was considered necessary to maintain an elevated Ca^{2+} concentration (39). Our results show how this population response is composed of the more complex and often oscillatory behavior of individual cells. The continued occurrence of bursts of high $[Ca^{2+}]_i$ may help explain the previously puzzling observation (41) that phosphorylase activation can persist at a high level when average $[Ca^{2+}]_i$ in the population is only slightly elevated above resting. Individual cells are now seen to spike to at least many hundreds of nanomolar $[Ca^{2+}]_i$, and the true peaks might be yet higher in unloaded cells, since dye buffering of $[Ca^{2+}]_i$, partial AM ester hydrolysis, compartmentalization into nonresponsive organelles, and imperfect spatial and temporal resolution would all tend to underestimate the peaks. Since Ca^{2+} activation of phosphorylase *b* kinase is a nonlinear function showing strong positive cooperativity (42), occasional spikes that contribute little to the mean of the asynchronized population may be highly effective in stimulating the enzyme.

In Ca^{2+}-free solution only a small number of Ca^{2+} spikes were observed and for a short time (Figs. 12 and 13). The frequency of Ca^{2+} spikes was strongly dependent on the concentration of extracellular Ca^{2+} (Fig. 14). This frequency dependence is similar to that of REF52 fibroblasts (10) but much different from that of BC3H-1 cells derived from smooth muscle (43). Because the amplitude and waveform of the $[Ca^{2+}]_i$ spikes are little affected by extracellular Ca^{2+}, each individual spike is probably governed not by influx of extracellular Ca^{2+} but by the release from intracellular Ca^{2+} stores. Probably Ca^{2+} influx affects the frequency of the oscillations via the rate of Ca^{2+} resupply to the intracellular store. Putney has suggested (44) that such resupply might occur by a mysterious pathway that lets Ca^{2+} bypass the main part of the cytosol. This hypothesis would explain why we generally do not observe a pacemaker ramp of $[Ca^{2+}]_i$ preceding each spike. If replenishment of internal Ca^{2+} stores were controlled by or drawn from cytosolic Ca^{2+}, one might expect to observe such ramps, and indeed they have been reported in endothelial cells (45).

Gelfand *et al.* (46) reported that in lymphocytes the mitogen-induced uptake of Ca^{2+} is depressed by K^+ depolarization. They suggested that the receptor-operated Ca^{2+} permeability is an electrogenic pathway, though K^+ depolarization only slightly reduces the huge electrochemical driving force favoring Ca^{2+} entry. Roughly similar inhibition of $[Ca^{2+}]_i$ elevations by depolarization have been seen in many nonexcitable cell types (Ref. 47 and references therein). Ishida *et al.* (48) observed that even ionomycin-induced $[Ca^{2+}]_i$ rises are diminished by depolarization. Because ionomycin is believed to be an electroneutral ionophore, they concluded that enhancement of Ca^{2+} pump activity by depolarization was the major contributor. We now observe that K^+ depolarization, like removal of external Ca^{2+}, mainly slows the frequency of $[Ca^{2+}]_i$ oscillations with little effect on their amplitude. This finding explains and extends previous reports that K^+ depolarization reduces hormone-stimulated phosphorylase activation (49, 50) and net Ca^{2+} uptake by isolated hepatocytes or perfused livers in which non-parenchymal cells have been inhibited. However, our evidence does not yet say whether inhibition of entry or stimulation of pumping is predominant.

In conclusion, the present data show that in a large percentage of fura-2-loaded isolated hepatocytes, hormones stimulate rhythmic Ca^{2+} spikes under reasonably physiological conditions. The frequency but not the amplitude of the oscillation increases with extracellular Ca^{2+} concentration, suggesting that the amount of Ca^{2+} influx is closely related to the frequency. Depolarization causes no Ca^{2+} rise of its own and slows or inhibits the oscillations in a manner similar to removal of extracellular Ca^{2+}. These results suggest that voltage-operated Ca^{2+} channels are unimportant and that depolarization may inhibit receptor-mediated influx or stimulate efflux. At present we do not know the reason why Ca^{2+} oscillations are observed in some cells but not in other adjacent cells. However, it is possible that Ca^{2+} oscillations do not occur in those hepatocytes where Ca^{2+} influx is very large or very small: if Ca^{2+} influx is large, the first transient will be followed by a sustained phase. If Ca^{2+} influx is very low, $[Ca^{2+}]_i$ may fail to rise high enough to engage positive feedback mechanisms such as Ca^{2+}-induced Ca^{2+} release (8) or Ca^{2+} stimulation of phospholipase C (11).

Acknowledgments—We thank Drs. Donato DiMonte and Martha Sandy for their kind help in making isolated hepatocytes, Dr. S. R. Adams for help with the high performance liquid chromatography, Dr. Joseph P. Y. Kao for help in measuring the fluorescence spectra of fura-2 in lysates, and Prof. J. Exton for helpful discussion on the effects of hepatocyte depolarization.

REFERENCES

1. Campbell, A. K. (1983) *Intracellular Calcium*, John Wiley & Sons, New York
2. Carafoli, E. (1987) *Annu. Rev. Biochem.* **56,** 395–433
3. Exton, J. H. (1985) *Am. J. Physiol.* **248,** E633–E647
4. Woods, N. M., Cuthbertson, K. S. R., and Cobbold, P. H. (1986) *Nature* **319,** 600–602
5. Woods, N. M., Cuthbertson, K. S. R., and Cobbold, P. H. (1987) *Cell Calcium* **8,** 79–100
6. Graf, P., vom Dahl, S., and Sies, H. (1987) *Biochem. J.* **241,** 933–936
7. Capiod, T., Field, A. C., Ogden, D. C., and Sandford, C. A. (1987) *FEBS Lett.* **217,** 247–252
8. Berridge, M. J., Cobbold, P. H., and Cuthbertson, K. S. R. (1988) *Phil. Trans. R. Soc. Lond. B* **320,** 325–343
9. Rink, T. J., and Jacob, R. (1989) *Trends Neurosci.* **12,** 43–46
10. Harootunian, A. T., Kao, J. P. Y., and Tsien, R. Y. (1988) *Cold Spring Harbor Symp. Quant. Biol.* **53,** 945–953
11. Meyer, T., and Stryer, L. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 5051–5055
12. Monck, J. R., Reynolds, E. E., Thomas, A. P., and Williamson, J. R. (1988) *J. Biol. Chem.* **263,** 4569–4575
13. Moldeus, P., Hogberg, J., and Orrenius, S. (1978) *Methods Enzymol.* **52,** 60–71
14. Poenie, M., Alderton, J., Steinhardt, R., and Tsien, R. Y. (1986) *Science* **233,** 886–889
15. Poenie, M., Tsien, R. Y., and Schmitt-Verhulst, A. M. (1987) *EMBO J.* **6,** 2223–2232
16. Tsien, R. Y. (1989) *Cell Calcium*, in press
17. Malgaroli, A., Milani, D., Meldolesi, J., and Pozzan, T. (1987) *J. Cell Biol.* **105,** 2145–2155
18. Johnson, L. V., Walsh, M. L., and Chen, L. B. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 990–994
19. Petzinger, E., and Bigalke, H. (1986) *Biochim. Biophys. Acta* **863,** 318–324
20. Scanlon, M., Williams, D. A., and Fay, F. S. (1986) *J. Biol. Chem.* **262,** 6308–6312
21. Oakes, S. G., Martin, W. J., II, Lisek, C. A., and Powis, G. (1988) *Anal. Biochem.* **169,** 159–166
22. Gunter, T. E., Restrepo, D., and Gunter, K. K. (1988) *Am. J. Physiol.* **255,** C304–C310
23. Davis, M. H., Altschuld, R. A., Jung, D. W., and Brierley, G. P. (1987) *Biochem. Biophys. Res. Commun.* **149,** 40–45
24. Tsien, R. Y. (1989) *Annu. Rev. Neurobiol.* **12,** 227–253
25. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450
26. Charest, R., Blackmore, P. F., Berthon, B., and Exton, J. H. (1983) *J. Biol. Chem.* **258,** 8769–8773
27. Thomas, A. P., Alexander, J., and Williamson, J. R. (1984) *J. Biol. Chem.* **259,** 5574–5584
28. Binet, A., Berthon, B., and Claret, M. (1985) *Biochem. J.* **228,** 565–574
29. Lemasters, J. J., DiGiuseppi, J., Nieminen, A.-L., and Herman, B. (1987) *Nature* **325,** 78–81
30. Kongsamut, S., Kamp, T. J., Miller, R. J., and Sanguinetti, M. C. (1985) *Biochem. Biophys. Res. Commun.* **130,** 141–148
31. Kokubun, S., Prod'hom, B., Becker, C., Porzig, H., and Reuter, H. (1986) *Mol. Pharmacol.* **30,** 571–584
32. Gray, P. T. A. (1988) *J. Physiol.* (Lond.) **406,** 35–53
33. Thomas, A. P., Rooney, T. A., and Sass, E. J. (1988) *J. Cell Biol.* **107,** 445 (abstr.)
34. Minta, A., Harootunian, A. T., Kao, J. P. Y., and Tsien, R. Y. (1987) *J. Cell Biol.* **105,** 89a
35. Burgess, G. M., Godfrey, P. P., McKinney, J. S., Berridge, M. J., Irvine, R. F., and Putney, J. W., Jr. (1984) *Nature* **309,** 63–66
36. Joseph, S. K., Thomas, A. P., Williams, R. J., Irvine, R. F., and Williamson, J. R. (1984) *J. Biol. Chem.* **259,** 3077–3081
37. Volpe, P., Krause, K.-H., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J., and Lew, D. P. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 1091–1095
38. Exton, J. H. (1984) in *Calcium Regulation in Biological Systems* (Ebashi, S., Endo, M., Imahori, K., Kakiuchi, A., and Nishizuka, Y., eds) pp. 141–156, Academic Press, Tokyo
39. Charest, R., Prpić, V., Exton, J. H., and Blackmore, P. F. (1985) *Biochem. J.* **227,** 79–90
40. Reinhart, P. H., Taylor, W. M., and Bygrave, F. L. (1984) *Biochem. J.* **223,** 1–13
41. Joseph, S. K., Coll, K. E., Thomas, A. P., Rubin, R., and Williamson, J. R. (1985) *J. Biol. Chem.* **260,** 12508–12515
42. Burger, D., Stein, E. A., and Cox, J. A. (1983) *J. Biol. Chem.* **258,** 14733–14739
43. Ambler, S. K., Poenie, M., Tsien, R. Y., and Taylor, P. (1988) *J. Biol. Chem.* **263,** 1952–1959
44. Putney, J. W., Jr. (1986) *Cell Calcium* **7,** 1–12
45. Jacob, R., Merritt, J. E., Hallam, T. J., and Rink, T. J. (1988) *Nature* **335,** 40–45
46. Gelfand, E. W., Cheung, R. K., and Grinstein, S. (1984) *J. Cell Physiol.* **121,** 533–539
47. Penner, R., Matthews, G., and Neher, E. (1988) *Nature* **334,** 499–504
48. Ishida, Y., and Chused, T. M. (1988) *J. Exp. Med.* **168,** 839–852
49. Van der Werve, G., Hue, L., and Hers, H.-G. (1977) *Biochem. J.* **162,** 135–142
50. Altin, J. G., Biden, T. J., Karjalainen, A., and Bygrave, F. L. (1988) *Biochem. Biophys. Res. Commun.* **153,** 1282–1289