Hormonal Effects on Calcium Homeostasis in Isolated Hepatocytes*

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A new method is described to determine the cytosolic free Ca"-concentration in isolated hepatocytes. Calcium concentrations in the medium were monitored spectrophotometrically using arsenazo III. Digitonin (5 μg/mg dry weight of cells) was added to make the plasma membrane permeable to Ca", and the magnitude of the Ca" change was measured for different initial external Ca" concentrations. The cytosolic free Ca" concentration was estimated by determining the Ca" null point when no net Ca" changes were observed upon addition of digitonin. Values for the Ca" null point between 100 and 200 nm were obtained for normal hepatocytes from fed or fasted rats, depending upon the pH and Mg" concentration of the incubation medium. Rapid cell fractionation of calcium-loaded cells permitted determination of the intracellular calcium distribution under these conditions. In normal cells, the mitochondrial calcium content accounted for 60 to 70% of the total cell calcium content, with the remainder located mainly in the microsomes. This proportion was approximately constant over a 10-fold range of total cell calcium.

Glucagon had no effect on the cytosolic free Ca". On the other hand, a-adrenergic stimulation increased the cytosolic free Ca" concentration 2- to 3-fold; phenylephrine (10^-4 M) increased mean values from 0.19 ± 0.01 to 0.46 ± 0.04 μM within 2 min. The time course and dose-response relationship of cytosolic free Ca" changes closely followed the increase of phosphorylase a. Rapid cell-fractionation studies showed that the calcium content of the mitochondrial fraction was decreased after norepinephrine addition to the intact cells, while that of the microsomal fraction was increased. The above effects were abolished by β-adrenergic antagonists but were little affected by a-adrenergic antagonists. These data indicate that the mitochondrial calcium pool is highly labile and is influenced by an as yet unknown transducing signal which is regulated by interaction of α-adrenergic hormones with the plasma membrane receptor. Increased efflux of calcium from the mitochondria causes a rise of cytosolic free Ca" and regulates enzymes of carbohydrate metabolism possibly by enhanced binding of calcium to calmodulin.

In recent years, it has become increasingly apparent that changes of intracellular free Ca" exert a vital role in the mediation of chemical events linking hormonal or other external stimuli to alterations of intracellular enzyme activities (1-3). In cardiac muscle and nerve, many studies have described the role of the action potential in the regulation of Ca" entry through the plasma membrane by a gating mechanism (4). With other excitable tissues such as skeletal muscle, as well as nonexcitable or secretory tissues having a lower resting membrane potential, flux of Ca" across the plasma membrane appears to be less important for the transduction of the biochemical signal. Alternatively, it has been suggested that membrane depolarization or hormone-receptor interactions might elicit a release of Ca" from intracellular storage sites and a flux of Ca" between intracellular organelles and Ca"-binding target proteins. The relative roles of the plasma membrane, microsomes, and mitochondria in this process are presently under active investigation (3, 5-8). Of particular interest is the growing body of evidence suggesting that cyclic AMP and Ca" are parallel rather than sequential second messengers for the elicitation of biochemical events (9, 10). Thus, α-adrenergic activity in liver is thought to be mediated by cyclic AMP-independent processes and to involve a direct increase of phosphorylase b kinase activity by a rise of intracellular Ca" concentration to between 10^-7 and 10^-6 M free Ca" (11-13).

Although there is a considerable body of data concerning the effects of catecholamines, glucagon, and other hormones on "Ca" flux in liver (6, 7, 9, 10, 13-16), a more detailed understanding of the sequence and molecular mechanisms of the events involved has been severely hampered by a lack of knowledge of the intracellular Ca" concentrations as well as by a poor understanding of the detailed mechanisms of cellular calcium homeostasis. As a first step toward permitting a greater understanding of the mechanisms of hormone actions and stimulus-response activity of target cells, we have developed methods for the measurement of the concentrations of free Ca" and Mg" in the cytosol of isolated hepatocytes and in the mitochondrial matrix of isolated mitochondria (17). In this paper, we describe in detail a spectrophotometric method using arsenazo III as a calcium indicator for measurement of the free cytosolic Ca" concentration in isolated hepatocytes. In addition, a modification of the rapid cell-disruption technique of Tischler et al. (18) has been used to determine the calcium content of the mitochondria and microsomes after calcium loading of the hepatocytes and after hormonal stimulation. Data are presented describing the effects of the α-adrenergic hormones norepinephrine and phenylephrine on the intracellular distribution of calcium and on the kinetics and dose-response relationship of changes of the free cytosolic Ca" concentration.

**EXPERIMENTAL PROCEDURES**

Hepatocyte Preparation—Rat liver cells were obtained from fed

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1 Portions of this paper (including the experimental procedures for the arsenazo method of Ca" determination and Figs. 1 to 3) are presented in miniprint at the end of this paper. Miniprint is easily
or overnight-fasted rat by previously described (19) modifications of the collagenase liver perfusion method originally introduced by Berry and Friend (20). The hepatocytes were washed twice and subsequently incubated in modified Ca"+- and Mg"+-free Hanks' medium containing 137 mM NaCl, 5.4 mM KCl, 0.44 mM KH2PO4, 4.2 mM NaHCO3, 0.33 mM Na2HPO4, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.4, which was equilibrated with 100% O2. Calcium loading of liver cells was achieved by incubation of the cells (approximately 5 mg dry weight/ml) in ice-cold modified Hanks' medium containing up to 10 mM CaCl2, followed by washing in Ca"+-free medium.

Measurement of Total Calcium Contents in Cellular Subfractions—The total calcium content of isolated hepatocytes was determined by atomic absorption spectroscopy after rapid centrifugation of the cells through silicone oil into 14% (w/v) perchloric acid (28). Corrections for carrydown of medium Ca2+ were made from measurements of the sucrose space by inclusion of [U-14C]glucose and HzO in the incubation medium. The calcium content in the heavy particulate fraction (mainly mitochondria, but also containing peroxisomes, lysosomes, and nuclei) and the light particulate fraction (microsomes) were determined using a modification of the rapid cell-disruption technique of Tischler et al. (18). Cells were mixed with 3 mM EGTA, 15 μM ruthenium red, and 50 μg of digitonin/mg dry weight of cells, and 3 s later passed under controlled pressure (60 psi) through a 25-gauge needle, followed by centrifugation through silicone oil into perchloric acid. The combination of the brief exposure time to digitonin and the shearing forces generated during passage of the cell suspension through the needle caused disruption of the plasma membrane with release of 85 to 95% of lactate dehydrogenase, α-glycerophosphate dehydrogenase, and glucose-6-phosphatase, and 5% release of glutamate dehydrogenase. After the above centrifugation to remove the heavy particulate fraction, the supernatant was recentrifuged at high speed (120,000 × g) for 15 min in a Beckman bench-top ultracentrifuge to remove the microsomal fraction, which was subsequently analyzed for glucose-6-phosphatase activity and for calcium content after extraction with perchloric acid.

Assay Procedures—Protein was determined by a modification of the biuret procedure (29). ATP and glucose in neutralized perchloric acid extracts of mitochondria and cells were assayed fluorometrically by the hexokinase method (30). Chloride was assayed according to Van Rossum (31). Lactate dehydrogenase and glutamate dehydrogenase activities were assayed as described previously (18), glucose-6-phosphatase was assayed according to De Duve et al. (32), and phosphorylase a was assayed as described by Hudson et al. (33).

Materials—Digitonin (Sigma Chemical Co.) was recrystallized three times from hot ethanol. The ionophore A23187 and the uncoupler FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) were gifts from Dr. Robert Hamill, Eli Lilly Co., Indianapolis, Ind., and Dr. Peter Heytler of the Research Division, DuPont Co., Wilmington, Del., respectively. Norepinephrine, phenylephrine, and propranolol were obtained from Sigma, and phentolamine was from Ciba Pharmaceutical Co. Phenoxynbenzamine was a gift from Smith, Kline and French, Philadelphia, and glucagon was a gift from the Eli Lilly Co.

RESULTS

Cytosolic Free Ca2+ Concentration in Hepatocytes—The effects of digitonin addition on changes of free Ca2+ in the medium of the hepatocyte suspensions are illustrated in Fig. 4. The Ca2+ changes were measured spectrophotometrically with arsenazo III using the wavelength pair 675-685 nm. Either EGTA or Ca2+ was added to adjust the free Ca2+ to desired levels prior to addition of digitonin. Calcium was removed from the medium by the digitonin-treated cells, but the final equilibrium free Ca2+ concentration was independent of the initial Ca2+ concentration. This equilibrium value provides a convenient rough estimate of the cytosolic free Ca2+, which reads with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9550 Rockville Pike, Bethesda, Md. 20014. Request document No. 79M-1714, cite authors, and include a check or money order for $1.00 per set of photocopies.

The abbreviations used are: EGTA, ethylene glycol bis(α-aminoethyl ether)N,N,N',N"-tetraacetic acid, FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

![Fig. 4. Effects of digitonin (D) on calcium uptake by hepatocytes. Rat liver cells (4 mg dry weight/ml) were incubated in modified Hanks' medium, pH 7.4, containing 0.5 mM Mg2+ and 40 μM arsenazo III. The free Ca2+ concentration was calculated as described under "Experimental Procedures."](image-url)
after addition of A23187 (10 nmol/mg dry weight of cells which, at this high concentration, results in equilibration of Ca²⁺ across the plasma membrane (34). This latter experiment suggests that release of ions, ATP, proteins, and other cellular constituents by digitonin addition is not interfering with the spectrophotometric measurement of Ca²⁺.

It is readily apparent from the data in Figs. 4 and 5 that the size of the Ca²⁺ change after digitonin addition to the cells is much greater than that expected from a simple mixing of the medium and cytosol volumes. This is illustrated by the following calculation: assuming a cytosolic volume of 2 ml/g dry weight of liver cells (18), the total cytosolic volume in the cell incubation medium (2.5 ml) with 4 mg/ml of cells is only 20 µl. With an initial medium free Ca²⁺ concentration of 1 µM, equilibration of the spaces would only result in a change of the medium free Ca²⁺ of 0.01 µM. Since the measured change at 1.15 µM external free Ca²⁺ is about 0.09 µM, it is clear that calcium is being removed by intracellular organelles once the permeability restriction of the plasma membrane to Ca²⁺ movement is removed.

Both mitochondria and microsomes contribute to net uptake of calcium by the digitonin-treated cells, as illustrated below. Fig. 6 shows the effects of digitonin on cells incubated in the absence and presence of 15 µM ruthenium red. Ruthenium red at this concentration completely inhibits Ca²⁺ uptake by the mitochondria. Digitonin addition caused a 3-fold greater removal of calcium from the medium in the absence than in the presence of ruthenium red. The calcium taken up was released by the subsequent addition of FCCP. The calcium uptake or binding in the presence of ruthenium red can be attributed to nonmitochondrial intracellular components, while that released by FCCP can be attributed to mitochondria. Null point titrations with hepatocytes incubated in the absence and presence of 15 µM ruthenium red prior to addition of digitonin are given in Fig. 7. The results show that although ruthenium red decreased the extent of the Ca²⁺ uptake more than 2-fold, it did not affect the null point. This indicates that the Ca²⁺ null point determination is independent of the activity of the mitochondrial Ca²⁺ uptake system.

Since the intracellular ionic composition is very different from that of the normal cell incubation medium, the effect of digitonin addition to the hepatocytes will be to cause a redistribution of ions across the plasma membrane. The effects of different Mg²⁺ and H⁺ ion concentrations in the medium prior to digitonin treatment were investigated in detail, because it was felt that these ions may be particularly important in altering the binding of Ca²⁺ to intracellular sites, thereby changing the measured Ca²⁺ null point. Table I shows the influence of varying pH over the range from 6.8 to 7.8, and of Mg²⁺ ion concentration from 0 to 1 mM, on the measured Ca²⁺ null points. The Ca²⁺-arsenazo dissociation constants shown in Fig. 2 for the different media were used for calculation of the free Ca²⁺ concentrations. As seen from Table I, the Ca²⁺ null point was not appreciably affected by pH, but tended to increase with increasing Mg²⁺ concentrations. The cytosolic free Mg²⁺ concentration is thought to be between 0.5 and 1.0 mM (17, 35), indicating that the cytosolic free Ca²⁺ concentration should be about 0.2 µM (cf. Fig. 4).

**FIG. 5.** Calcium null point titrations to determine the cytosolic free Ca²⁺ concentration. Rat liver cells were incubated as described in the legend to Fig. 4 except for omission of Mg²⁺. A, the relationship with the total Ca²⁺ change (ΔCa²⁺); B, the relationship with the rate of Ca²⁺ uptake. The curves were obtained by linear regression analysis of the experimental data points.

**FIG. 6.** Effect of ruthenium red on uncoupler-releasable calcium in hepatocytes treated with digitonin. Rat liver cells were incubated in Mg²⁺-free modified Hanks' medium, pH 7.4, containing no substrate. The calibration on the left side of the figure refers to total calcium additions. —, conditions in the absence of ruthenium red; ---, conditions in the presence of 15 µM ruthenium red. The downward slope of the latter line 1 min after digitonin addition is caused by the gradual release of Ca²⁺ from the mitochondria due to Ca²⁺/H⁺ exchange seen when mitochondrial Ca²⁺ uptake is inhibited by ruthenium red.

**FIG. 7.** Effect of ruthenium red on the Ca²⁺ null point titration for determining the cytosolic free Ca²⁺ concentration.
proton electrochemical gradient across the mitochondrial membrane by addition of uncoupling agents causes a release of Ca\(^{2+}\) from the mitochondria (for review, see Ref. 37). Calcium release from intracellular storage sites of isolated hepatocytes has been observed following addition of these agents (7, 38, 39), which results in efflux of Ca\(^{2+}\) across the plasma membrane where it is detected by arsenazo III present in the medium. The maximal amount of Ca\(^{2+}\) released by A23187 (30 \(\mu\)M) was greater than that released by uncoupler (15 \(\mu\)M FCCP), suggesting that while uncoupler may be releasing Ca\(^{2+}\) from the mitochondrial pool alone, A23187 releases Ca\(^{2+}\) from both the mitochondria and other storage sites, such as microsomes (data not shown). Fig. 8 shows a comparison of the quantitative determination of Ca\(^{2+}\) released from hepatocytes by FCCP, measured by the absorbance change of arsenazo III and by the difference of the total calcium content of the cells before and after addition of FCCP.

The Ca\(^{2+}\) calibration produced by seven successive additions of 5 \(\mu\)M total Ca\(^{2+}\) to the suspension of hepatocytes already having about 10 \(\mu\)M contaminant Ca\(^{2+}\) illustrates the nonlinearity of the calibration curve at the higher Ca\(^{2+}\) concentrations. The washed cells contained 3.06 \(\pm\) 0.13 \(\mu\)mol of calcium/g dry weight of cells initially, and 1.00 \(\pm\) 0.03 \(\mu\)mol/g dry weight of cells after FCCP addition. The average difference of 2.06 \(\pm\) 0.12 \(\mu\)mol/g dry weight of cells agreed very well with the average amount of 2.14 \(\pm\) 0.07 \(\mu\)mol/g dry weight of cells for the increase of extracellular Ca\(^{2+}\) obtained in four separate experiments using arsenazo III as a Ca\(^{2+}\) indicator.

Null point titrations for determination of the cytosolic free Ca\(^{2+}\) concentration in cells incubated with 4 \(\mu\)M A23187 and 2 \(\mu\)M FCCP for 90 s prior to digitonin addition are shown in Fig. 9. Both agents are seen to cause about a 4-fold increase of the cytosolic free Ca\(^{2+}\) concentration. In this series of experiments, Mg\(^{2+}\) was absent and the pH was 7.4. The Ca\(^{2+}\) null point for the controls was 0.12 \(\pm\) 0.01 \(\mu\)M (5) compared with means of 0.45 \(\pm\) 0.10 \(\mu\)M (3) for FCCP and 0.44 \(\pm\) 0.12 \(\mu\)M (3) for A23187. Separate experiments showed that FCCP caused a similar increase of the Ca\(^{2+}\) null point when cells were incubated with 3 \(\mu\)M ruthenium red prior to addition of digitonin.

In order to investigate the distribution of calcium between the mitochondrial and microsomal fractions of the cell as a function of total cellular calcium, hepatocytes were loaded with calcium by incubation in the cold (8) in the presence of 3 \(\mu\)M ruthenium red for different times up to 90 min with 5 or 10 mM extracellular Ca\(^{2+}\). After incubation, the cells were centrifuged at 300 \(\times\) \(g\), and washed twice with Ca\(^{2+}\)-free medium. The calcium content of the different subcellular organelles was then estimated by a number of different methods, as shown in Table II. The total calcium content of the cell is shown in Column A and ranged from 3 to 30 nmol/mg of cell protein (0.85 mg of cell protein is approximately equivalent to 1 mg dry weight of cells). The amount of Ca\(^{2+}\) released from the cells after addition of 5 \(\mu\)M FCCP was measured directly in some experiments by arsenazo III (Column B) or by subtraction of the residual cell calcium from the initial cell calcium (Column C). On the assumption that uncoupler releases Ca\(^{2+}\) only from the mitochondrial pool, Columns B and C provide estimates of the mitochondrial cell calcium. An independent check of the mitochondrial cell calcium is provided by rapid disruption of the cells in the presence of EGTA and ruthenium red and separation of the mitochondria, as described under "Experimental Procedures." These data are

**Table I**

| pH of medium | Ca\(^{2+}\) null point | Mg\(^{2+}\) | Ca\(^{2+}\) null point |
|--------------|-----------------------|-----------|-----------------------|
| [Mg\(^{2+}\)] = 0 | \(\mu\)M | mM | \(\mu\)M |
| 6.8          | 0.12 \(\pm\) 0.02 (3) | 0         | 0.12 \(\pm\) 0.01 (5) |
| 7.4          | 0.11 \(\pm\) 0.01 (4) | 0.3       | 0.13 \(\pm\) 0.01 (3) |
| 7.8          | 0.09 (2)             | 0.5       | 0.19 \(\pm\) 0.01 (5) |
|              |                       | 1.0       | 0.20 (2)              |

**Fig. 9** Effects of FCCP and A23187 on Ca\(^{2+}\) null point titrations to determine the free cytosolic Ca\(^{2+}\) concentration. FCCP (2 \(\mu\)M) and A23187 (4 \(\mu\)M) were added 2 min prior to the addition of digitonin.
shown in Column D of Table II. The agreement between the numbers in Columns B, C, and D may be considered satisfactory, and the data indicate that the mitochondrial cell calcium increases roughly in proportion to the total cell calcium. Thus, 60 to 70% of the total cell calcium is located in the mitochondria for the different calcium-loaded cells. The microsomal calcium content was also estimated directly by further high speed centrifugation of the supernatant fraction from the cell disruption (Column E). These values may be compared with those obtained by subtracting the uncoupler-releaseable Ca\(^{2+}\) from the total cell calcium (Column A - Column C) or by subtracting the mitochondrial calcium obtained by cell disruption from the total cell calcium (Column A - Column D). Values obtained by direct measurement of the microsomal fraction were generally higher than those obtained by the subtraction methods, suggesting that some Ca\(^{2+}\) uptake into the microsomes may have occurred during centrifugation.

**Hormonal Effects on Cytosolic Free Ca\(^{2+}\) and Intracellular Calcium Distribution**—Fig. 10 shows the results of Ca\(^{2+}\) null point titrations using liver cells from fed rats incubated at 37°C with Mg\(^{2+}\)-free Hanks’ medium under conditions of control incubation and 90 to 120 s after addition of 10^{-2} M glucagon or 10^{-2} M norepinephrine. These results are interpreted as showing that glucagon had no effect on the cytosolic free Ca\(^{2+}\) concentration while norepinephrine produced a 4-fold increase. For the average of five experiments, norepinephrine increased the Ca\(^{2+}\) null point from 0.10 ± 0.01 to 0.45 ± 0.07 μM, while for the average of three experiments, the Ca\(^{2+}\) null point in the presence of glucagon was 0.12 ± 0.03 μM compared with paired control values of 0.12 ± 0.02 μM.

With further experiments in the presence of 0.5 mM Mg\(^{2+}\) (Table III), the effects of the α-adrenergic agonist phentolamine (10^{-4} M) and the β-agonist propranolol (10^{-4} M) were investigated using phenylephrine (10^{-7} M) as the α-adrenergic agonist. In these experiments, phenylephrine increased the cytosolic free Ca\(^{2+}\) 2.4-fold from a control value of 0.19 μM and increased phosphorylase a 2.2-fold. These effects were completely blocked by phentolamine, but were only slightly attenuated by propranolol, confirming the predominant α-agonist activity of phenylephrine.

The time courses of the effects of 10^{-5} M phenylephrine on the changes of cytosolic free Ca\(^{2+}\) and phosphorylase a activity are illustrated in Fig. 11. Peak phosphorylase a activity was obtained after about 2 min and was slightly preceded by the rise of cytosolic free Ca\(^{2+}\), while the biphasic response for both parameters was complete in about 5 min. Other experiments (not shown) indicated that the duration and the time for the peak of the phosphorylase a response was shifted from 2 min at 10^{-5} M phenylephrine to 30 s at 10^{-3} M phenylephrine. Increased glucose production as a result of enhanced glycogenolysis, however, continued for at least 30 min at the higher hormone concentrations. The dose-response curve for the stimulation of phosphorylase a activity by phenylephrine was also remarkably similar to that for the increase of cytosolic free Ca\(^{2+}\) (Fig. 12). Half-maximal effects were obtained at about 5 × 10^{-6} M phenylephrine.

The effect of norepinephrine on the intracellular distribution of calcium between the "mitochondrial" and "microsomal" fractions after rapid cell disruption is shown in Table IV. The microsomal fraction was calculated from the differences between the total cell calcium and the heavy particulate (mitochondrial) calcium which was obtained by the rapid cell disruption technique. In these experiments, the mitochondrial calcium content represented 65 to 83% of the total cell calcium under control conditions. Of particular interest is the fact that...
norepinephrine decreased the calcium content of the mitochondrial fraction by 40% without appreciably affecting the total cell calcium, with the result that the calculated microsomal calcium content increased almost 3-fold. Glucose production was measured in parallel experiments 15 min after norepinephrine addition. The control rate of glucose output was 29 nmol/mg of protein/15 min, and was increased by 76% after norepinephrine addition. These effects of norepinephrine were blocked by the α-adrenergic inhibitors phenolamine and phenoxybenzamine, which by themselves had little effect.

**DISCUSSION**

Validity of the Cytosolic Free Ca²⁺ Measurements—Very few other measurements of the cytosolic free Ca²⁺ concentration in cells have been reported in the literature, and no others have been reported for hepatocytes. Three general methods have been used for estimation of intracellular free Ca²⁺ concentrations in different tissues. These involve use of (a) calcium-selective microelectrodes; (b) calcium-sensitive photo-polymerase such as aequorin, which emit a blue luminescence when exposed to Ca²⁺; and (c) calcium-sensitive metallochromic indicators, which have different absorption properties when Ca²⁺ is bound. Calcium-sensitive microelectrodes with a tip diameter of about 1 μm and a detection limit of 6 × 10⁻⁶ M in Ca²⁺-buffered solutions have been described (40). However, they are less sensitive and reproducible in solutions approximating intracellular fluid, and few measurements of intracellular free Ca²⁺ using microelectrodes have as yet been reported (41). The useful range of Ca²⁺ measurements with aequorin is from 10⁻⁷ to 10⁻⁴ M, and although it has a Ca²⁺ dissociation constant of 16 μM, it can be used at very low concentrations so that only about 2% of total calcium is bound (42). Both aequorin and arsenazo III have been injected into single cells such as barnacle muscle fibers (43) and giant axons of the squid (44–46), and have provided values of 100 nM or below for the intracellular free Ca²⁺ concentration. Both methods when applied to in situ measurements are subject to large potential absolute errors in this range as discussed by various authors (42, 43, 46, 47) and are more suitable for measuring relative changes of intracellular free Ca²⁺ after perturbing the cell. In addition, this approach is strictly limited to cell types where microinjection is technically feasible.

Arsenazo III has a high selectivity for Ca²⁺, but is sensitive to ionic composition and pH (48). A major disadvantage with arsenazo III as an intracellular Ca²⁺ probe is that its dissociation constant for calcium is about 2 orders of magnitude greater than apparent cytosolic free Ca²⁺ concentrations. Thus, at concentrations of arsenazo III which give a linear Ca²⁺ titration, the ratio of calcium bound to free Ca²⁺ is 3 or greater. This necessitates careful measurement of the Ca²⁺-arsenazo dissociation constants under the experimental conditions and correction for the amount of bound calcium. However, an advantage of arsenazo III, especially when absorbance changes are measured in a highly sensitive dual wavelength spectrophotometer, is that its high extinction coefficient allows measurements to be made at two narrowly spaced wavelengths which minimize light-scattering artifacts. Since the above factors have been taken into account in the present study, it may reasonably be concluded that the free Ca²⁺ concentration at the extrapolated null point with digitonin-treated hepatocytes can be measured with precision.

The relationship between the Ca²⁺ null point as determined by the extracellularly located arsenazo III and the true intracellular free Ca²⁺ concentration requires comment. A number of factors allow us to conclude that the values are very similar, particularly when the extracellular and intracellular Mg²⁺ concentrations are equilized prior to digitonin addition. These are (a) the rapid exchange of Ca²⁺ between cells and medium after digitonin addition; (b) the retention of basic structural integrity of the digitonized hepatocyte (49) and small loss of total protein, which suggests that there is an insignificant loss.
of Ca\(^{2+}\)-binding sites; and (c) the virtual collapse of the plasma membrane potential after digitonin addition, which indicates a negligible ionized equilibrium distribution of Ca\(^{2+}\). The general validity and usefulness of the technique is also evidenced by the fact that FCCP, A23187, and \(\alpha\)-adrenergic hormones as perturbing agents increase the Ca\(^{2+}\) null point 2- to 4-fold. A distinct advantage of this method is that it is applicable in principle to any cell type in suspension and to cells in tissue culture attached to suitable cover slips that will fit inside a spectrophotometer cuvette. The data obtained, however, represent a statistical average for the cell population, and may be misleading if there is appreciable heterogeneity of the cell population.

**Intracellular Calcium Distribution**—Between 65 and 80% of the total cell calcium is mitochondrial (see also Refs. 6 and 7), with most of the remainder being associated with a small particulate (microsomal) fraction that can be sedimented by centrifugation at 120,000 \(\times g\) for 15 min. This relative proportion is essentially independent of total cellular calcium content over a 10-fold range. Studies which will be reported in detail elsewhere (56) have shown that only about 0.6% of the mitochondrial calcium is free, and that the intramitochondrial free Ca\(^{2+}\) rises in proportion to the total calcium in the mitochondria. With liver mitochondria as normally isolated, which have about 10 nmol of calcium/mg of protein, the matrix free Ca\(^{2+}\) concentration is about 60 \(\mu\)M. A similar value can be predicted for mitochondria in situ, since for normal cells with a total calcium content of 4.56 nmol/mg of cell protein, a mitochondrial calcium content of 2.74 nmol/mg of cell protein (Table II), and a mitochondrial content of 28% of total protein, the calculated mitochondrial calcium content is 9.8 nmol/mg of mitochondrial protein. For a cytosolic free calcium concentration of 180 nm (0.004% of the total cell calcium), the gradient of free Ca\(^{2+}\) across the mitochondrial membrane in the hepatocyte is about 350 (see Ref. 51).

**Hormonal Effects on Intracellular Calcium Distribution**—The most important finding reported in this paper relates to the effect of the \(\alpha\)-agonists norepinephrine and phenylephrine on calcium homeostasis in isolated hepatocytes. The present studies show that \(\alpha\)-agonists cause a 2- to 3-fold stimulation of cytosolic free calcium that is closely related to the increase of phosphorylase a activity both kinetically and by a dose-response relationship. Maximum changes were observed within 2 min, the peak effects were almost over within 5 min, and the half-maximal effective concentration of phenylephrine was about 5 \(\times 10^{-6}\) M. Increases of both the cytosolic free calcium and the phosphorylase a activity induced by phenylephrine were completely inhibited by the \(\alpha\)-receptor antagonist phentolamine, but were little affected by the \(\beta\)-receptor antagonist propranolol. It may be concluded, therefore, that there is a cause and effect relationship between binding of phenylephrine to the \(\alpha\)-receptor (52) and an increase of cytosolic free calcium and activation of phosphorylase \(b\) to phosphorylase \(a\) (15, 16). In contrast, glucagon failed to induce a change of cytosolic free calcium, which is in accordance with the generally accepted belief that the stimulatory effects of glucagon on carbohydrate metabolism in liver are mediated by cAMP-dependent rather than calcium-dependent mechanisms.

It has been ascertained that \(\alpha\)-adrenergic agents inhibit glycogen synthase and activate glycogen phosphorylase and gluconeogenesis in hepatocytes (33, 53, 54) and adipocytes (55) without changes in cAMP levels. Calcium is known to increase the activity of phosphorylase kinase and also to enhance the activity of cAMP-independent glycogen synthase kinases (56). Indirect evidence (e.g. Ref. 15) has indicated that \(\alpha\)-adrenergic activation of phosphorylase may be caused by an increase of the intracellular calcium concentration. The present data confirm this hypothesis, and indicate that the maximum increase of the intracellular free calcium (from 0.19 to 0.46 \(\mu\)M) is the correct range to activate hepatic phosphorylase \(b\) kinase, which has a reported \(K_{m}\) for calcium of 0.8 \(\mu\)M (11).

The present results also show that \(\alpha\)-adrenergic stimulation of hepatocytes results in a net efflux of calcium from the mitochondria. After this work was completed, reports from two other laboratories have been published which reached the same conclusion (57, 58). However, our results differ from the results in these and earlier studies from the same laboratories (7, 16) in that we observed little or no net loss of calcium from the liver under the influence of \(\alpha\)-adrenergic stimulation, in agreement with the data of Foden and Randle (6). We conclude, therefore, that flux of calcium across the liver plasma membrane cannot be an intrinsic factor in the mode of action of \(\alpha\)-adrenergic agents. In our studies, the calcium released from the mitochondria appeared to be largely taken up by the endoplasmic reticulum. These effects on a redistribution of intracellular calcium can be attributed directly to the \(\alpha\)-adrenergic hormone, since they were either abolished or greatly attenuated by the presence of \(\alpha\)-adrenergic antagonists.

Calcium transport across the mitochondrial membrane is known to occur by two separate mechanisms (51, 59, 60). Energy-dependent calcium influx occurs by the electrophoretic transport of uncompensated calcium ions, and derives its energy from the outwardly directed proton pump of the electron transport chain (51, 59). The second mechanism of calcium transport in liver mitochondria is by a reversible electro-neutral exchange of calcium with two H\(^+\) ions (51, 60-62). This process has about 2 orders of magnitude lower capacity than the energy-dependent calcium uptake mechanism, while the apparent free Ca\(^{2+}\) \(K_{m}\) for influx is more than 1 order of magnitude greater than that for influx (50). Operation of these two independent influx and efflux pathways permits the mitochondria to buffer the intracellular free calcium concentration at a low value (51). An increase of cytosolic free calcium would thus be expected to result in an increased mitochondrial calcium content. Clearly, a depletion of mitochondrial calcium under the influence of \(\alpha\)-adrenergic stimulation of hepatocytes cannot be secondary to the increased cytosolic free calcium concentration. A specific effect on mitochondria must be postulated; there is either an inhibition of the electrophoretic influx pathway or an increase of the electro-neutral efflux pathway. An increase of the cytosolic free Mg\(^{2+}\) concentration, for instance, would have the effect of inhibiting the Ca\(^{2+}\) influx pathway (51, 60). Alternatively, an oxidation of the mitochondrial nicotinamide nucleotides may stimulate the Ca\(^{2+}\) efflux pathway (61). At present, the nature and mechanism of the transducing signal from the \(\alpha\)-adrenergic receptor on the plasma membrane to the mitochondria remains unknown. On the other hand, the increased calcium uptake by the endoplasmic reticulum after norepinephrine addition to hepatocytes is probably secondary to the rise of the intracellular free Ca\(^{2+}\). Rat liver microsomes are known to have an active MgATP-dependent Ca\(^{2+}\) sequestration system which has a high affinity for Ca\(^{2+}\) (62, 63).

In conclusion, it is evident that the mitochondrial calcium pool in the intact cell is labile. Furthermore, it may be suggested that hormone binding to the plasma membrane \(\alpha\) receptor, by release of a chemical mediator, regulates the kinetics of Ca\(^{2+}\) transport across the mitochondrial membrane, which in turn directly influences the cytosolic free Ca\(^{2+}\) concentration. With \(\alpha\)-adrenergic stimulation under the present experimental conditions, the endoplasmic reticulum Ca\(^{2+}\) ATPase must compete favorably with the plasma membrane.
Ca\textsuperscript{2+}-ATPase for sequestration of the calcium released from the mitochondria in order to account for the low Ca\textsuperscript{2+} efflux from the cell.

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Calcium Homeostasis in Liver Cells

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SUPPLEMENTARY MATERIAL TO THE NORMAL EFFECTS OF CALCULUS HOMESTASIS IN ISOLATED DEPOLARIZED CELLS

BY ELIZABETH MORRIS, KENNETH CRAW, VIOREL L. MICH, AND JOHN R. WILLIAMSON

EXPERIMENTAL PROCEDURES

Calculation of Ca\textsuperscript{2+}-Aromatase Disassociation Constant - Calcium was measured spec-

ically using the Calibrator 

Aromatase Kit (Amersham, Arlington Heights, III). Calcium was determined in a luminometric assay 

using the tissue and/or cell extracts as described. Absorbance at 560 nm was measured in a 

luminometer (SLT, http://www.slt-industrial.com) using a 1:10 dilution of the extract in 

diluent buffer. Values for Ca were calculated from the measured absorbance values using cer-

tain standard curves. The Calibrator Kit was used to determine Ca concentrations in the 

samples. The Ca Calibration Kit is based on the principle of the calcium-specific 

fluorescence resonance energy transfer (FRET) principle. The kit contains a 

fluorescent dye that emits fluorescence when excited by light at a wavelength of 480 nm. 

The emitted fluorescence is then measured at 520 nm. The Ca concentration in the sample is 

determined by the amount of fluorescence emitted. The kit also contains a standard curve 

that is used to determine the Ca concentration in the sample. The Ca concentration in 

the sample is determined by measuring the fluorescence emitted by the sample and comparing it 

to the standard curve. The Ca concentration in the sample is then calculated using the standard curve.

The Ca concentration in the sample is calculated using the following equation:

\[ \text{Ca} = \frac{\text{Fluorescence}_{\text{sample}}}{\text{Fluorescence}_{\text{standard}}} \times \text{Calibration Factor} \]

where \( \text{Fluorescence}_{\text{sample}} \) is the fluorescence emitted by the sample, \( \text{Fluorescence}_{\text{standard}} \) is the fluorescence emitted by the standard, and the Calibration Factor is determined from the standard curve.

The Ca concentration in the sample is determined by measuring the fluorescence emitted by the sample and comparing it to the standard curve. The Ca concentration in the sample is then calculated using the standard curve.

The Ca concentration in the sample is calculated using the following equation:

\[ \text{Ca} = \frac{\text{Fluorescence}_{\text{sample}}}{\text{Fluorescence}_{\text{standard}}} \times \text{Calibration Factor} \]

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The Ca concentration in the sample is determined by measuring the fluorescence emitted by the sample and comparing it to the standard curve. The Ca concentration in the sample is then calculated using the standard curve.