Suppression of Ca$^{2+}$ Oscillations in Cultured Rat Hepatocytes by Chemical Hypoxia

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The model of “chemical hypoxia” with KCN plus iodoacetic acid mimics the ATP depletion and reductive stress of hypoxia. Here, we examined the effects of chemical hypoxia on cytosolic free Na$^+$ and Ca$^{2+}$ in single cultured rat hepatocytes by multiparameter digitized video microscopy and ratio imaging of sodium-binding furan indicator (SBFI) and Fura-2. Intracellular Na$^+$ increased from about 10 mM to more than 100 mM after 20 min of chemical hypoxia, whereas cytosolic free Ca$^{2+}$ remained virtually unchanged. In normoxic hepatocytes, phenylephrine (50 μM) and Arg-vasopressin (20–40 nM) induced Ca$^{2+}$ oscillations in 70 and 40% of cells, respectively. These Ca$^{2+}$ oscillations were suppressed after one spike following the onset of chemical hypoxia. Phenylephrine and vasopressin also increased intracellular phosphate formation by 22 and 147%, respectively. This effect was suppressed by KCN plus iodoacetate. Intracellular acidosis is characteristic of chemical hypoxia. Intracellular acidosis induced by 40 mM Na-acetate suppressed Ca$^{2+}$ oscillations but did not inhibit hormone-induced inositol phosphate formation. Cytosolic alkalinization also suppressed Ca$^{2+}$ oscillations. However, prevention of intracellular acidosis with monensin (10 μM) did not prevent suppression of Ca$^{2+}$ oscillations during chemical hypoxia. Mitochondrial depolarization with uncoupler did not change free Ca$^{2+}$ levels during chemical hypoxia, indicating that mitochondria do not regulate free Ca$^{2+}$ during chemical hypoxia. From these results, we conclude: 1) chemical hypoxia does not block Na$^+$ influx across the plasma membrane; 2) Chemical hypoxia inhibits hormone-stimulated Ca$^{2+}$ flux pathways across cellular membranes by two different mechanisms: (a) by ATP depletion, which disrupts hormone-myoinositol 1,4,5-triphosphate coupling, and (b) by intracellular acidosis, which inhibits myo-inositol 1,4,5-triphosphate-stimulated Ca$^{2+}$ release from intracellular stores; 3) during ATP depletion by chemical hypoxia, mitochondria do not take up Ca$^{2+}$ to maintain cytosolic free Ca$^{2+}$ at low concentrations.

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The model of “chemical hypoxia” with inhibitors of respiration and glycolysis mimics the ATP depletion and reductive stress of hypoxia (1–4). In cultured hepatocytes, cytosolic free Ca$^{2+}$ did not increase after chemical hypoxia for more than 40 min. During this time ATP was more than 95% depleted and cell surface blebbing was well developed (1–3). Several hypotheses might explain why cytosolic free Ca$^{2+}$ does not increase after ATP depletion. First, a generalized blockade of ion movements through the plasma membrane and other membranes may occur, as has been described for mitochondrial membranes during hypoxic stress to hepatocytes (5, 6). Second, specific calcium flux pathways across cellular membranes may become inhibited due to ATP depletion and intracellular acidosis (7) during hypoxic injury. Third, the mitochondrial membrane potential persists after ATP depletion in hypoxic and anoxic hepatocytes (1, 5). Thus, mitochondrial uptake of Ca$^{2+}$ may maintain cytosolic free Ca$^{2+}$ at a constant level during hypoxia.

Mammalian cells typically are bathed in extracellular fluid containing upwards of 120 mM Na$^+$. By contrast, cytosolic Na$^+$ concentration is about 10 mM. This Na$^+$ gradient is important for many biological processes, including nutrient uptake, action potentials, regulation of other intracellular ions, and so on. The concentration gradient of Na$^+$ is maintained by the Na$^+$,K$^+$-ATPase of the plasma membrane, which drives ATP-dependent Na$^+$ efflux from the cell. Therefore, ATP depletion should inhibit the Na$^+$ pump and lead to an increase of intracellular Na$^+$, unless Na$^+$ influx is blocked. Recently, a new fluorescent indicator, SBFI, has been described which is suitable for digital imaging of intracellular Na$^+$ (8). Thus, to determine whether a generalized blockade of ion movements was produced after ATP depletion, we used this new fluorescent indicator to measure cytosolic Na$^+$ during chemical hypoxia.

In recent years, experiments using probes of intracellular Ca$^{2+}$ have revealed that cytosolic free Ca$^{2+}$ is a “second messenger” for many hormones. These hormone agonists increase cytosolic free Ca$^{2+}$ concentration by releasing Ca$^{2+}$ from intracellular stores and promoting Ca$^{2+}$ entry across the plasma membrane (9). In studies with suspensions of hepatocytes, agonist-induced Ca$^{2+}$ transients are typically biphasic (9, 10). However, studies of single hepatocytes have revealed that the Ca$^{2+}$ response to these hormones can be oscillatory.
Suppression of Ca" Oscillations

(11-13). Ca" oscillations presumably are caused by movement of free Ca" across cellular membranes into and out of the cytosol (14-16). Ca" oscillations represent highly regulated and coordinated cellular events. Thus, to test this hypothesis that chemical hypoxia was causing inhibition of specific Ca" flux pathways, we studied the effects of hypoxic stress on hormone-induced Ca" oscillations.

ATP-dependent Ca" pumps in the plasma membrane and endoplasmic reticulum maintain cytosolic free Ca" in the range of 100 to 200 nM. Although isolated mitochondria accumulate Ca" avidly, the Km for this process is too large for mitochondria to regulate free Ca" in situ (17). In pathological states, however, mitochondria may take up large amounts of Ca" (18). Thus, a role for mitochondria in Ca" homeostasis during cell injury cannot be excluded. Accordingly, as mitochondrial Ca" uptake is driven by the mitochondrial membrane potential, a final aim of this study was to determine the effect of mitochondrial depolarization on Ca" homeostasis during chemical hypoxia.

Here, we show that chemical hypoxia inhibits oscillations of free Ca". Chemical hypoxia also inhibits the increase of inositol phosphate formation induced by the calcium-mobilizing hormone agonists phenylephrine and vasopressin. The findings indicate that ATP depletion and acidosis during hypoxia inhibit Ca" release from intracellular stores by separate mechanisms. Na" movement into cells is not prevented, and mitochondria do not play a role in maintaining Ca" homeostasis during hypoxic insult.

MATERIALS AND METHODS

Hepatocyte Isolation and Culture—Hepatocytes from Sprague-Dawley rats (200-300 g) were isolated by collagenase digestion as described previously (2). Isolated hepatocytes were diluted to 0.5 × 10^6 cells/ml in Waymouth's medium MB752/1, containing 5% fetal calf serum, 2 mM glutamine, and 100 mM insulin. In experiments in which inositol phosphate formation was measured, hepatocytes were diluted to 2 × 10^5 cells/ml. Aliquots of 1 ml were cultured on collagen-coated 22-mm square glass coverslips inside 10-mm Petri dishes, as described (7). Hepatocytes were used after 21-30 h of culture in humidified 5% CO2, 95% air at 37°C.

Measurement of Cytosolic Free Na", Ca", and pH by Multiparameter Confocal Microscopy—Hepatocyte cultures were incubated for 60 min with SBFI acetoxymethyl ester (5 μM) for measurement of cytosolic free Na" concentration, and for 30 min with Fura-2 acetoxymethyl ester (3 μM) for measurement of cytosolic free Ca", or for 30 min with BICECF acetoxymethyl ester (5 μM) for measurement of pH. Subsequently, coverslips were washed three times with KRH buffer and mounted in a chamber on the stage of a Zeiss LSM5 inverted fluorescence microscope (Thornwood, NY). Cytosolic free Na" was measured by ratio imaging of SBFI fluorescence excited at 340 and 380 nm. In some experiments, SBFI fluorescence was also imaged at an excitation wavelength of 365 nm. Intracellular free Ca" concentration was measured by ratio imaging of Fura-2 fluorescence excited at 340 and 365 nm. SBFI and Fura-2 fluorescence was imaged through a 395-nm dichroic reflector and a 420-nm long pass filter. Cytosolic pH was measured by ratio imaging of BICECF fluorescence excited at 440 and 490 nm. BICECF fluorescence was imaged through a 510-nm dichroic reflector and a 530-nm long pass filter. Excitation light was provided by a 75-watt Xenon lamp for Fura-2 and SBFI, and a 100 watt mercury lamp for BICECF. Excitation light was passed through an interference and neutral density filter wheel assembly to select wavelength and intensity under computer control. A shutter, also under computer control, automatically blocked the excitation light when measurements were made. A low-light intensified silicon intensified target video camera (model 66, MTVI-Dage, Michigan City, IN) collected fluorescent images, which were fed to a Sun 3/10 workstation (Mountain View, CA) for frame averaging, background subtraction, and ratioing. Ratio images during Ca" oscillations were collected every 10 s, unless otherwise noted.

Calibration of SBFI, Fura-2, and BICECF Signals—Attempts to calibrate cellular SBFI signals by imaging SBFI fluorescence in standard solutions through the microscope optics yielded erroneous results. This procedure often produced estimates of cellular Na" that were less than zero. This problem may result from changes of the properties of SBFI brought about by the intracellular environment (e.g., changes of viscosity) (19). Therefore, intracellular Na" concentration was calibrated in situ by the method of Harootunian et al. (20). Cells were incubated with SBFI for 30 min with 4 μM gramicidin D, 10 μM monensin, and 10 μM nigericin to equilibrate Na" between the cytoplasm and the extracellular solution (Fig. 9, Miniprint). The bathing solution was then changed to a Na"-free glucuronate solution (120 mM K-glucuronate, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 2 mM CaCl₂, and 25 mM HEPES, pH 7.4) containing ionophores. By substitution of Na-gluconate for K-gluconate, Na+ concentration was increased in increments from 0 to 120 mM. A standard relating the 340/380 fluorescence ratio to Na" was thus established. In other experiments, excitation and emission spectra for SBFI were determined with a Perkin-Elmer Cetus 850-40 fluorescence spectrophotometer (Norwalk, CT). Spectra were uncorrected for lamp artifacts. Fura-2 and BICECF signals were calibrated as reported previously (3, 7).

Measurement of Inositol Phosphate Production—Inositol phosphate production was measured as previously described (20) with minor modifications. After 4 h of culture, 5 μCi of myo-[3H]inositol/ml were added to the culture medium. After another 16-20 h, the culture medium was changed to KRH containing 20 mM LiCl. The hepatocytes were incubated for 7 min under air at 37°C, and various chemicals and hormones were then added. After another 15 min, the medium was removed by aspiration and replaced with 0.5 ml of cold 5% trichloroacetic acid. The acid-soluble radioactivity was washed with 0.5 ml of H₂O, which was combined with the perchloric acid fraction. Subsequently, 20 μl of 100 mM EDTA and 2 ml of octylamine/finol (1:1, v/v) were added, vortexed, and allowed to settle at room temperature. [3H]inositol phosphates in the upper phase were separated by anion-exchange chromatography on 1-ml Dowex AG1-X8 columns in the formate form. The samples were applied, and the columns were washed with 10 ml of H₂O and 8 ml of 50 mM ammonium formate. Inositol mono-, bis-, tri-, and tetrrophosphates were eluted with 8 ml of 1.2 mM ammonium formate and 0.1 mM formic acid. The eluate was added to 20 ml of scintillation mixture (15 g of diphenyloxazole and 0.575 g of p-bis[(p-phenylidy)benzenesulfonic acid in 2 liters of Triton X-100 and 2 liters of toluene). Radioactivity was measured with a Beckman LS5000 CE scintillation counter (Palo Alto, CA).

Materials—SBFI (lot 8A), SBFI acetoxymethyl ester (lot 8A), Fura-2 (lot 8D), Fura-2 acetoxymethyl ester (lots 8G, 8H, and 9C), and BICECF acetoxymethyl ester (lot 7J) were purchased from Molecular Probes (Eugene, OR); myo-[3H]inositol from American Radiolabeled Chemicals (St. Louis, MO); bovine serum albumin (Fraction V), digitonin, gramicidin D, mopsin, nigericin, ouabain, phenylephrine, and Arg-vasopressin from Sigma; collagenase A and KB210 from Boehringer-Mannheim; Waymouth's medium MB752/1 from Gibco; inositolomycin from Calbiochem (San Diego, CA); dexamethasone sodium phosphate from American Reagent Laboratories (Shirley, NY); and insulin from Squibb (Princeton, NY). Other reagents were of analytical grade obtained from the usual commercial sources.

RESULTS

Effect of Chemical Hypoxia on Ca" Oscillations Induced by Phenylephrine and Vasopressin—The rapid rise of Na" during chemical hypoxia, together with the absence of a change of free Ca" as reported previously (1, 3), suggested a relatively specific regulation of ion Ca" in ATP-depleted hepatocytes. Accordingly, using ratio imaging of Fura-2 fluorescence, we investigated the response of control and ATP-depleted hepatocytes to the Ca"-mobilizing hormone agonists, phenylephrine and arg-vasopressin. Phenylephrine (50 μM) elicited transient elevations of cytosolic Ca", which showed considerable heterogeneity. In about 30% of hepatocytes, repetitive Ca" spikes were observed (Fig. 1A). These spikes were sustained

2 Portions of this paper (including "Results," Figs. 9-14, Table IV, and Footnote 3) are presented in miniprint at the end of this paper. Full-size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Suppression of Ca\(^{2+}\) Oscillations

**FIG. 1.** Responses of cytosolic free Ca\(^{2+}\) to phenylephrine in single cultured hepatocytes. Cytosolic free Ca\(^{2+}\) in single cultured hepatocytes was measured by ratio imaging of Fura-2 fluorescence, as described under “Materials and Methods.” Illustrated in panels A–C are three typical response patterns to the addition of 50 \(\mu\)M phenylephrine (Phe). Three experiments typical of 32.

**FIG. 2.** Ca\(^{2+}\) oscillations induced by Arg-vasopressin. Cytosolic free Ca\(^{2+}\) was measured in a single cultured hepatocyte, as described in Fig. 1, after addition of 20 nM Arg-vasopressin (Vas). This figure shows the longest period of sustained oscillations observed in 10 cells from 4 rats.

For 5 min or more. The amplitude of oscillations was 30–250 nM, and frequency was 0.5–2 per minute. In about 40% of hepatocytes, damped oscillations of Ca\(^{2+}\) were observed which ceased after 1–3 beats (Fig. 1B). In the remaining 30% of hepatocytes, the increase of Ca\(^{2+}\) after phenylephrine was small (<20 nM) (Fig. 1C). Ca\(^{2+}\) oscillations were also observed in about 40% of hepatocytes after addition of 20 nM Arg-vasopressin, but the oscillations typically were damped within 8 min (Fig. 2).

After addition of KCN plus iodoacetate, oscillations induced by phenylephrine ceased after one spike (Fig. 3A). When phenylephrine was added 1 min after KCN plus iodoacetate to hepatocytes previously shown to oscillate, a single Ca\(^{2+}\) spike, often of diminished magnitude, was observed (Fig. 3B). When phenylephrine was added after 3.3 min, a brief small increase of Ca\(^{2+}\) occurred (Fig. 3C). After 5 min, no increase of Ca\(^{2+}\) was observed (Fig. 3D). Chemical hypoxia also stopped oscillations induced by vasopressin (Fig. 4). Thus, chemical hypoxia led rapidly to suppression of Ca\(^{2+}\) mobilization by both phenylephrine and vasopressin.

**Effect of Changes of pH\(_i\) on Calcium Oscillations**—Chemical hypoxia causes a rapid decrease of pH\(_i\), due largely to proton release during ATP hydrolysis (7, see Fig. 11). Since this
decrease of pHi might account for suppression of Ca\(^{2+}\) mobilization in ATP-depleted cells, the effect of intracellular acidosis on phenylephrine-induced Ca\(^{2+}\) oscillations was examined. Na-acetate (40 mM) was added 3 min after 50 \(\mu\)M phenylephrine to decrease pHi. After the addition, one Ca\(^{2+}\) spike was observed, and subsequently Ca\(^{2+}\) oscillations by phenylephrine were suppressed (Fig. 5A). In parallel measurements we measured pHi by ratio imaging of BCECF fluorescence. Na-acetate produced a 0.3–0.5 unit decrease of pHi within 30 s. pHi gradually returned toward baseline over the next several min (Fig. 5B).

We also examined the effect of intracellular alkalization. Alkalization with 20 mM NH\(_4\)Cl inhibited Ca\(^{2+}\) oscillations sharply (Fig. 6A). Parallel measurements of pHi showed that addition of 20 mM NH\(_4\)Cl increased pHi by 0.6–0.7 units (Fig. 6B).

**Effect of Chemical Hypoxia on Ca\(^{2+}\) Oscillations during pHi Clamping**—To further investigate the hypothesis that inhibition of Ca\(^{2+}\) oscillations during chemical hypoxia is due to cytosolic acidosis, we measured Ca\(^{2+}\) oscillations in cultured hepatocytes treated with 10 \(\mu\)M monensin in modified KRH buffer containing 105 mM choline and 10 mM Na\(^{+}\). Under these conditions, pHi is clamped to pH\(_{\text{N}}\), even during chemical hypoxia, because of monensin-catalyzed Na\(^{+}/\)H\(^{+}\) exchange (7). Phenylephrine produced Ca\(^{2+}\) oscillations in monensin-treated cells (Fig. 7). However, contrary to expectation, these Ca\(^{2+}\) oscillations were fully and rapidly inhibited by KCN plus iodoacetate.

**Fig. 5. Effect of Na-acetate on Ca\(^{2+}\) oscillations and intracellular pHi.** Cytosolic free Ca\(^{2+}\) (panel A) and pHi (panel B) in single hepatocytes were measured by ratio imaging of Fura-2 and BCECF, respectively, as described “Materials and Methods.” 50 \(\mu\)M phenylephrine (Phe) and 40 mM Na-acetate (NaAc) were added where indicated. Panel A is one experiment typical of five. Panel B is one experiment typical of four.

**Fig. 6. Effect of NH\(_4\)Cl on Ca\(^{2+}\) oscillations and intracellular pHi.** Cytosolic free Ca\(^{2+}\) (panel A) and pHi (panel B) were measured as described in Fig. 5. 50 \(\mu\)M phenylephrine (Phe) and 20 mM NH\(_4\)Cl were added where indicated. Panel A is one experiment typical of three. Panel B is one experiment typical of five.

**Fig. 7. Effect of clamping pHi to pH\(_{\text{N}}\) on Ca\(^{2+}\) oscillations.** A cultured hepatocyte was incubated in KRH buffer containing 105 mM choline and 10 mM Na\(^{+}\), and cytosolic free Ca\(^{2+}\) was measured by ratio imaging of Fura-2 fluorescence, as described under “Materials and Methods.” 10 \(\mu\)M monensin (Mon), 50 \(\mu\)M phenylephrine (Phe), and 2.5 mM KCN plus 0.5 mM iodoacetate (IAA) were added where indicated. One experiment typical of four.

**Effect of Chemical Hypoxia and Intracellular Acidosis on Inositol Phosphate Formation**—To investigate whether ATP depletion was suppressing hormone-induced inositol phosphate formation and thereby inhibiting Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores, inositol phosphate formation was measured during metabolic inhibition with cyanide plus iodoacetate (Table I). Phenylephrine (50 \(\mu\)M) and vasopressin (40 nM) increased inositol phosphate formation by 21.5 and 141%, respectively, after 10 min. KCN plus iodoacetate did not change base-line levels of inositol phosphate synthesis.
**TABLE I**

Effect of chemical hypoxia on inositol phosphate formation in cultured hepatocytes

Cultured hepatocytes were labeled with myo-[3H]inositol as described under "Materials and Methods." Subsequently, cells were incubated with (chemical hypoxia) or without (normoxia) 2.5 mM KCN and 0.5 mM iodoacetate. After 5 min, 50 μM phenylephrine, 40 mM vasopressin, or no hormone agonist was added. Samples were then taken after another 10 min for measurement of inositol phosphate formation. Inositol phosphate formation is expressed as percent ± S.E. of the average control value (2282 dpm) from three to six experiments/group.

| Hormone agonist | Inositol phosphate formation | Normoxia | Chemical hypoxia | % |
|----------------|-----------------------------|----------|------------------|---|
| None           | 100 ± 5.5                   | 102 ± 1.7|                  |   |
| Phenylephrine  | 121 ± 3.9                   | 105 ± 3.0|                  |   |
| Vasopressin    | 241 ± 19.8                  | 110 ± 4.2|                  |   |

**TABLE II**

Individual effects of KCN and iodoacetate on inositol phosphate formation

Cultured hepatocytes labeled with myo-[3H]inositol were incubated with 2.5 mM KCN, 0.5 mM iodoacetate (IAA), KCN plus iodoacetate, or no inhibitor and subsequently exposed to 40 mM vasopressin or no hormone agonist as described in Table I. Samples were then taken for inositol phosphate determination. Inositol phosphate formation is expressed as percent ± S.E. of the average control value (2120 dpm) from three experiments per group.

| Inhibitor | Hormone agonist | None | Vasopressin |
|-----------|-----------------|------|-------------|
| None      | 100 ± 10.3      | 236 ± 15.6 |   |
| KCN       | 107 ± 3.5       | 128 ± 3.3 |   |
| IAA       | 137 ± 1.7       | 202 ± 14.5 |   |
| KCN + IAA | 104 ± 3.3       | 101 ± 0.7 |   |

However, when KCN plus iodoacetate were added 5 min before the hormone agonists, the increase of inositol phosphate formation was completely suppressed (Table I).

Bellomo and co-workers (21) showed that pretreatment of hepatocytes with sulphydryl reagents such as p-chloromercuribenzoic acid, diamine, and N-ethylmaleimide inhibited the increase of inositol phosphates induced by hormone agonists. Therefore, we investigated the possibility that the inhibitory effect of chemical hypoxia was due to thiol alkylation by iodoacetate. Iodoacetate in the absence of KCN only slightly inhibited the vasopressin-induced increase of inositol phosphate formation, whereas KCN alone produced inhibition almost as great as KCN and iodoacetate together (Table II).

To investigate whether intracellular acidosis inhibited hormone-induced inositol phosphate formation, the effect of Na-acetate on inositol phosphate formation was determined. Na-acetate (40 mM) at a concentration that caused intracellular acidification and suppression of Ca2+ oscillations had no effect either on basal inositol phosphate formation or on inositol phosphate formation in response to phenylephrine and vasopressin (Table III). Thus, acidic pH did not prevent inositol phosphate formation in response to phenylephrine or vasopressin.

**DISCUSSION**

This study was designed to evaluate mechanisms of ion homeostasis in ATP-depleted cells. Previously, cytosolic free Ca2+ did not increase in cultured hepatocytes after metabolic inhibition with cyanide and iodoacetate, a model of ATP depletion and reductive stress that we call chemical hypoxia (1–3). Accordingly, our specific goals were to determine 1) whether ATP depletion caused a generalized blockade of ion movements across cellular membranes; 2) whether specific hormone-stimulated flux pathways for Ca2+ were inhibited during chemical hypoxia; and 3) whether mitochondrial membrane potential-dependent Ca2+ uptake contributed to free Ca2+ homeostasis in ATP-depleted cells.

Na+ Fluxes after ATP Depletion—After addition of metabolic inhibitors, cytosolic free Na+, as measured with the fluorescent probe SBFI, increased almost immediately and approached extracellular concentrations after 20 min. Hepatocytes acidify during chemical hypoxia (7), and SBFI fluorescence is pH-dependent (19). Therefore, we determined the Kd of SBFI for Na+ as a function of pH, and corrected our intracellular Na+ measurements for actual pH, as measured by BCECF ratio imaging. Ouabain also increased free Na+ measured by SBFI, consistent with the hypothesis that the increase of Na+ after chemical hypoxia was due to inhibition of the Na+,K+-ATPase pump of the plasma membrane.

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**TABLE III**

Effect of intracellular acidosis on inositol phosphate formation

Cultured hepatocytes were labeled with myo-[3H]inositol and incubated without (normal) or with (acidotic) 40 mM Na-acetate for 1 min to lower pH. Subsequently, 50 μM phenylephrine, 40 mM vasopressin, or no hormone agonist was added. After 10 min, samples were taken for inositol phosphate determination as described under "Materials and Methods." Inositol phosphate formation is expressed as percent ± S.E. of the average control value (3347 dpm) from three experiments/group.

| Hormone agonist | Intracellular pH |
|-----------------|------------------|
|                 | Normal           | Acidotic         |
| None            | 100 ± 6.8        | 101 ± 3.1        |
| Phenylephrine   | 121 ± 3.9        | 129 ± 5.9        |
| Vasopressin     | 166 ± 10.2       | 171 ± 5.0        |

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**Ca2+ Oscillations in Cultured Hepatocytes**—In agreement with the recent report by Reddy and co-workers (13), phenylephrine and vasopressin stimulated oscillations of cytosolic free Ca2+ in 40–70% of primary 1-day cultured hepatocytes. However, in some hepatocytes, the Ca2+ response to these hormones was slight, even though high concentrations of phenylephrine (50 μM) and vasopressin (20 μM) were employed. In addition, Ca2+ oscillations were usually damped within 10 min. This contrasts with previous studies that described Ca2+ oscillations lasting more than 30 min in freshly isolated hepatocytes (11, 12). Fura-2 concentration in our cells is estimated to be about 50 μM (23), which is much below the concentration of high affinity Ca2+-binding sites inside cells (24). Thus, Ca2+ buffering by Fura-2 does not explain the absence of hormone sensitivity observed in some cells. A decline of hormone receptors after culturing is a possible explanation (25).

Ca2+-mobilizing hormones increase cytosolic free Ca2+ by releasing Ca2+ from non-mitochondrial stores, either from a portion of the endoplasmic reticulum (17, 26–29) or from newly described organelles called calciosomes (30, 31). During Ca2+ oscillations, each individual spike results from Ca2+ release from these intracellular stores. Data presented by Kawanishi et al. (12) suggest that Ca2+ influx from the extracellular medium regulates the frequency of oscillations by influencing the rate of refilling of the intracellular stores. In the present experiments, KCN and iodoacetate not only stopped Ca2+ oscillations (Fig. 3A), but also suppressed the first spike of Ca2+ (Fig. 3, B–D). Chemical hypoxia did not
deplete intracellular Ca\textsuperscript{2+} stores, as KCN plus iodoacetate, in the absence of added hormone agonists, caused no transient increase of cytosolic free Ca\textsuperscript{2+}. Therefore, it seems most likely that chemical hypoxia suppresses Ca\textsuperscript{2+} oscillations by inhibiting Ca\textsuperscript{2+} release from Ca\textsuperscript{2+} stores.  

**Effect of ATP Depletion and Intracellular Acidosis on Ca\textsuperscript{2+} Oscillations and Inositol Phosphate Formation**—Chemical hypoxia rapidly induces ATP depletion and intracellular acidosis (2, 7). In this study, intracellular acidosis induced by Na-acetate in the absence of ATP depletion suppressed Ca\textsuperscript{2+} oscillations. However, Ca\textsuperscript{2+} oscillations were also suppressed during chemical hypoxia when pH\textsubscript{i} was clamped to pH\textsubscript{i} using monensin. Thus, intracellular acidosis is not the only factor contributing to the suppression of Ca\textsuperscript{2+} oscillations by chemical hypoxia. Notably, clamping of pH\textsubscript{i} to physiologic pH\textsubscript{i} did not influence the amplitude or frequency of the Ca\textsuperscript{2+} oscillations themselves. Thus, oscillations of pH\textsubscript{i} were not providing beat-to-beat initiation of Ca\textsuperscript{2+} oscillations. Nevertheless, since intracellular acidosis and alkalosis both suppressed Ca\textsuperscript{2+} oscillations, pH\textsubscript{i} may play a significant role in regulation of Ca\textsuperscript{2+} oscillations.

The monensin experiments were performed in choline-substituted buffer, in which the ratio of intracellular to extracellular Na\textsuperscript{+} was approximately one to one. In this buffer, cytosolic free Ca\textsuperscript{2+} did not increase after chemical hypoxia. Thus, Ca\textsuperscript{2+} homeostasis in ATP-depleted cells is not linked to the Na\textsuperscript{+} gradient as, for example, by Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange.

We evaluated in detail the effect of changes of pH\textsubscript{i} on free Ca\textsuperscript{2+} measurements by Fura-2 ratio imaging. As pH\textsubscript{i} decreased during chemical hypoxia, the K\textsubscript{D} of Fura-2 for Ca\textsuperscript{2+} increased by about 30\%. Even after correction for this pH\textsubscript{i}-induced change of K\textsubscript{D}, the increase of free Ca\textsuperscript{2+} after ATP depletion was small and much less than that caused by hormones. This interpretation is supported by experiments with monensin. With monensin, pH\textsubscript{i} was clamped to pH\textsubscript{i} and K\textsubscript{D} was constant, but the increase of Ca\textsuperscript{2+} was small and slow after ATP depletion, despite the fact that monensin accelerates the onset of lethal injury (4, 7).

**Inhibition of Inositol Phosphate Formation after Chemical Hypoxia**—IP\textsubscript{3} and IP\textsubscript{4} are second messengers for hormone-induced Ca\textsuperscript{2+} release from intracellular stores (32, 33). In the present study, KCN and iodoacetate strongly suppressed hormone-stimulated inositol phosphate formation. This finding can also explain suppression of Ca\textsuperscript{2+} oscillations by chemical hypoxia. Inhibition of inositol phosphate synthesis was not due to intracellular acidosis, as intracellular acidification with Na-acetate in the absence of ATP depletion did not inhibit inositol phosphate synthesis. Protein thiol alkylation by iodoacetate also could not account for inhibition, as iodoacetate alone did not suppress inositol phosphate formation.

**Lack of Contribution of Mitochondrial Ca\textsuperscript{2+} Uptake to Ca\textsuperscript{2+} Homeostasis**—During anoxia and chemical hypoxia, mitochondria remain polarized for many minutes after ATP depletion, although the mechanism for sustaining this membrane potential remains obscure (1, 5). Perhaps mitochondria assist in cytosolic Ca\textsuperscript{2+} homeostasis by membrane potential-dependent Ca\textsuperscript{2+} accumulation. However, protonophoric uncoupler (carboxyl cyanide m-chlorophenylhydrazide) added after the onset of chemical hypoxia did not increase cytosolic Ca\textsuperscript{2+}. Thus, mitochondrial uptake of Ca\textsuperscript{2+} does not appear important for cytosolic Ca\textsuperscript{2+} homeostasis during chemical hypoxia. Similarly, carbonyl cyanide m-chlorophenylhydrazide did not increase cytosolic free Ca\textsuperscript{2+} in normoxic cells. These findings challenge the assumption that cellular mitochondria normally contain stores of Ca\textsuperscript{2+} that can be released as free Ca\textsuperscript{2+} after mitochondrial depolarization.

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Supplemental Material to “Suppression of Ca\(^{2+}\) Oscillations in Cultured Rat Hepatocytes by Chemical Hypoxia”

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detoxification and noncellular compartments of SBF is single cultured hepatocytes - Fox and 2 and BCCP localise to stress fibres and mitochondria (44). Since SBF compartmentalisation had not previously been determined in cultured hepatocytes, subcellular localization of an unexpected SBF and cellular compartments corresponding to organelles and membrane (e.g., lysosomes and endoplasmic reticulum), respectively (42). Based on the time in SBF fluorescence is a time lag in the intracellular location of SBF (Table 4). 4% of the SBF fluorescence remained after Triton-X-100, which represented autophagic processes and large inclusions and cell components. These studies demonstrate that SBF is located primarily in cytosol, and its use to measure cytosolic Ca\(^{2+}\) in single cultured hepatocyte.

To determine the concentration of SBF in single cultured hepatocytes, were loaded with SBF and examined using MOPS and METHODS). SBF was applied to the cultured hepatocytes, and the concentration of SBF in the individual cells was determined by flow cytometry. The mean fluorescence of the cells was normalized to the fluorescence of Triton-X-100 in KCl buffer containing 100 mM KCl and 4% DMSO (Table 5). The mean fluorescence of SBF was normalized to the fluorescence of the cells was normalized to the fluorescence of Triton-X-100 in KCl buffer containing 100 mM KCl and 4% DMSO (Table 5).

| Treatment | Remaining SBF Fluorescence |
|-----------|---------------------------|
| 0.2% Triton X-100 | 16.3 ± 1.7 |
| 0.5% Triton X-100 | 9.3 ± 1.1 |
| 1% Triton X-100 | 4.1 ± 1.1 |

Effect of chemical hypoxia on intracellular Ca\(^{2+}\) concentration: SBF has been utilized with SBF in other cell types to detect cytoplasmic Ca\(^{2+}\) as described in MATERIALS AND METHODS). SBF, as well as the concentration of SBF in the cytoplasmic space, is determined in MCF-7 cells. SBF was applied to the cultured hepatocytes, and the concentration of SBF in the individual cells was determined by flow cytometry. The mean fluorescence of the cells was normalized to the fluorescence of Triton-X-100 in KCl buffer containing 100 mM KCl and 4% DMSO (Table 5). The mean fluorescence of SBF was normalized to the fluorescence of Triton-X-100 in KCl buffer containing 100 mM KCl and 4% DMSO (Table 5).

Effect of changes in pH on cytosolic Ca\(^{2+}\) concentration: In Figs. 1 and 2, a decrease in pH increases the level of SBF fluorescence (Fig. 1). A decrease in pH increases the level of SBF fluorescence (Fig. 1). A decrease in pH increases the level of SBF fluorescence (Fig. 1).

Effect of changes in pH on cytosolic Ca\(^{2+}\) concentration: In Figs. 1 and 2, a decrease in pH increases the level of SBF fluorescence (Fig. 1). A decrease in pH increases the level of SBF fluorescence (Fig. 1).

Using in the calibration curve, the basal level of Ca\(^{2+}\) in single cultured hepatocytes was about 10 mmol/L (Fig. 1). To determine whether chemical hypoxia was raising a generalized likelihood of intracellular Ca\(^{2+}\) to KCl plus sucrose, 100 mM KCl, 50 mM sucrose, and 0.2% Triton X-100 were used as intracellular concentrations (Table 2). In other cell types and under other loading conditions, Fox and 2 and BCCP localise to stress fibres and mitochondria (44). Since SBF compartmentalisation had not previously been determined in cultured hepatocytes, subcellular localization of an unexpected SBF and cellular compartments corresponding to organelles and membrane (e.g., lysosomes and endoplasmic reticulum), respectively (42). Based on the time in SBF fluorescence is a time lag in the intracellular location of SBF (Table 4). 4% of the SBF fluorescence remained after Triton-X-100, which represented autophagic processes and large inclusions and cell components. These studies demonstrate that SBF is located primarily in cytosol, and its use to measure cytosolic Ca\(^{2+}\) in single cultured hepatocyte.

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Figure 9. In vivo calibration of the fura-2 probe. SHFs in cultured hippocampal neurons were loaded with Fura-2 and incubated in 44 mM glutamate-substituted KRX buffer, as described in MATERIALS AND METHODS. Ratio images at excitation wavelengths of 340 and 380 nm were collected using multiparameter digital image microscopy. Where indicated, 4 mM glutamate, 100 mM monuron and 10 mM nicotinate were added. Subsequently, the buffer medium was changed to 0.5 mM, 2.5 mM, 5.0 mM, 10 mM, 25 mM, 50 mM, and 100 mM NaCl by substituting it glutamic acid for NaCl. The data are presented as mean values ± SEM of four independent experiments. A typical calibration curve is shown. Ratio values are arbitrary units, which are uncorrected for differences in excitation light intensity at the two excitation wavelengths.

Figure 10. Increase of extracellular Na$^+$ after chemical hypnosis. Cultured hippocampal neurons were loaded with Fura-2 and incubated in KRX buffer as described in MATERIALS AND METHODS. After excitation wavelengths (280 nm) were measured to single neurons after addition of 2.5 mM KCl plus 0.5 mM trisodium iodide (TAA) (triangles) or 2.5 mM KCl plus 2.5 mM NaCl (circles). The solid line represents measurements of extracellular Na$^+$, which is based on in vivo calibrations as described in Figure 9. The dashed line represents Na$^+$, following addition of 2.5 mM NaCl at the concentration of 100 mM HEPES as described in the text. One experiment typical of 3.

Figure 11. Increase of extracellular pH after chemical hypnosis. Cultured hippocampal neurons were loaded with BCECF and pH was measured by ratio imaging, as described in MATERIALS AND METHODS. Cells were incubated as described in Figure 9, and KCN and sodium iodide (TAA) were added where indicated. Data points represent means ± SEM of four independent experiments.

Figure 12. Relationship of pH and dissociation constant for extracellular calcium chloride: pH of extracellular calcium concentration. Points for BAPTA, dibromo-BAPTA, and EGTA were calculated by Christensen and Ross (1989) for a temperature of 2°C and an ionic environment of 22°C from the experiments of 25°C from Guter et al. (1982) and unpublished data (Taccetti and Lattanzio, 1984) (triangles and squares). pH for Fura-2 at 2°C may be calculated assuming an ethanol of 10.3 kDa for Fura-2 data are: 300 nm in 2°C may be calculated assuming an ethanol of 10.3 kDa.

Figure 13. Extracellular Ca$^{2+}$ after chemical hypnosis: correction for changes of pH. Cultured hippocampal neurons were loaded with Fura-2 and exposed to 2.5 mM KCN and 2.5 mM NaCl, as above. The solid line represents extracellular calcium concentration after correction for changes of pH as described in Figure 11. Values represent means ± SEM of four independent experiments. One experiment typical of 3.

Figure 14. Effect of CCCP on extracellular Ca$^{2+}$ during chemical hypnosis. Cultured hippocampal neurons were loaded with Fura-2 and exposed to 2.5 mM KCN and 2.5 mM NaCl, as above. After 30 min, 10 mM CCCP, 50 mM CCCP, 50 mM CCCP, and 10 mM bongkrekic acid were added where indicated. Data points represent means ± SEM of four independent experiments.